SPECIES DEFECTS IN DETOXICATION MECHANISMS

by

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Abstract

Conjugation in species with defects in detoxication mechanisms has been studied. ^{14}C-Labelled phenol was administered to cats and pigs (25 mg/kg). The urinary metabolites (mainly phenylsulphate in cat and phenylglucuronide in pig) were identified by paper chromatography and radiochromatogram scanning. Cats appear to form no phenylglucuronide and pigs no phenylsulphate, but detailed examination of radiochromatograms showed that cats excrete small amounts of the glucuronide (less than 2% of dose) and pigs small amounts of sulphate. When phenol was administered intraperitoneally to cats, a new urinary metabolite (5 - 10% of dose) was identified by mass spectroscopy as phenylphosphoric acid, and when ^{32}P-labelled phosphate and phenol were administered, radioactive phenylphosphate was detected in the urine.

Several phenolic compounds were administered to cats and pigs (25 mg/kg). Cats formed only small quantities (less than 5% of dose) of glucuronide when challenged with ^{14}C-labelled 1-naphthol, 2-naphthol or phenacetin, the conjugates being mainly sulphates. Pigs conjugated 2-naphthol and 4-hydroxyamphetamine largely with glucuronic acid excreting only traces of sulphates, but approximately one third of a

dose of 1-naphthol was excreted as sulphate. The rate of conjugation of 1-naphthol and 2-naphthol was compared *in vitro* using pig and rat liver homogenates. The rate of glucuronidation of both compounds was greater in pig than rat (which forms approximately equal amounts of glucuronide and sulphate conjugates). The rates of sulphation of 1- and 2-naphthol were similar in pig, being much less than in rat. ^{14}C-Labelled morphine was excreted in cat urine mainly as a sulphate. Tritiated phenolphthalein was synthesized; when administered to cats, approximately 30% of the dose was recovered in the urine, two-thirds of this as the glucuronide. In conclusion, therefore, although cats have little ability to form the glucuronides and pigs the sulphates of many phenols this is not true for phenolphthalein in cats or 1-naphthol in pigs.

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Ond yr hwn sydd yn gwneuthur gwirionedd, sydd yn dyfod i'r goleuni.

S. Ioan III.21

To Charles Wesley Allen

and Donald Capel

Contents

CHAPTER ONE

GENERAL INTRODUCTION

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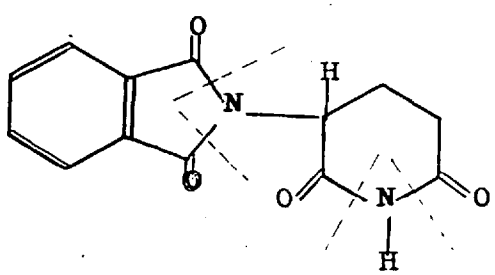
Section A

When foreign compounds, (xenobiotics or anutrients) get into the body they can undergo three possible fates, namely, they can be metabolized by enzymes; they can change spontaneously into other substances without the intervention of enzymes; or they can be excreted unchanged. The majority of xenobiotics undergo the first fate but before considering this the other two possibilities will be outlined.

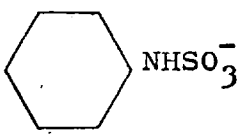
Some xenobiotics when ingested change spontaneously into other compounds because they encounter suitable conditions of pH for spontaneous breakdown, or because they can react chemically with certain compounds or groups in endogenous macromolecules. One of the best examples of a drug which breaks down spontaneously in the body is thalidomide (see Schumacher, et al,, 1965).



Thalidomide
(spontaneous hydrolysis occurs at the substituted amide bonds indicated by the dotted lines)

In aqueous solution at pH 7.4 thalidomide decomposes by spontaneous hydrolysis into some twelve other compounds.

The metabolism of foreign compounds usually tends to make lipid-soluble substances more polar and water soluble and therefore more easily excreted by the kidney. If a xenobiotic is already highly polar then one would not expect it to be readily metabolized and an example of such a compound is the sweetening agent cyclamate (Renwick and Williams, 1969).



cyclamate pKa 1.9

Most, but not all, of the drugs which are not readily metabolized are highly polar.

The enzymes that metabolize xenobiotics are located mainly in the liver, but metabolism by other tissues such as intestine, kidney and lung can also occur to some extent. Although the tissues of the body are the main sites of anutrient metabolism the gut microorganisms can also play a role, especially with compounds that are not readily absorbed from the intestine or that are excreted in the bile in appreciable amounts. The collective term for the metabolic

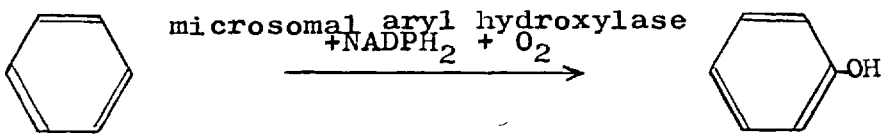
pathways undergone by xenobiotics in the body is detoxication reactions. The majority of these reactions are catalysed by enzymes located mainly in the smooth endoplasmic reticulum of the hepatic cells (Gillette, 1966).

The majority of foreign compounds absorbed are fat-soluble and non-ionized at body pH and their metabolism introduces polar, ionizeable groups, which cause the compound to become more water-soluble and thus more readily excreted. The principal routes of excretion of foreign compounds and their metabolites are via the urine and the bile, but some compounds may also be excreted in the expired air (volatile substances), sweat, milk, saliva and by secretion into the stomach and other parts of the gastrointestinal tract. The extent to which an organic compound is excreted in the bile may be influenced by a number of physiochemical factors, which include molecular-weight, polarity, chemical structure and lipid solubility (Millburn, et al., 1967 a, b; Hirom et al., 1972). Furthermore, with some compounds the extent of biliary excretion varies widely with species and this may result in a marked interspecies variation in the overall pattern of excretion of the compounds. Those compounds and their metabolites that have a relatively low molecular weight

(below about 300) are excreted mainly in the urine rather than in the bile, irrespective of species, but with organic anions of higher molecular weight (300-500) extensive elimination in the bile occurs in the rat and dog but not in the guinea pig, rabbit and rhesus monkey (Abou-El-Makarem, et al., 1967).

It is useful to regard the metabolism of foreign compounds as occurring in two phases. In the first phase (Phase I) the compound undergoes reactions which can be classified as oxidations, reductions and hydrolyses, whilst in the second phase (Phase II) the products of the first phase, having now acquired suitable groups such as -OH, -COOH and $-NH_2$, undergo a synthetic reaction which is usually referred to as a conjugation. During each of these phases, the compound is usually made more polar, and the products of conjugation are mainly fairly strong organic acids. The metabolism of benzene provides an example of this:-

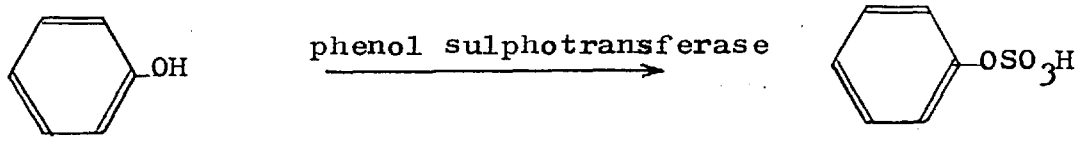
Phase I Reaction (oxidation)



benzene
% ionized at pH 7.4 = 0%

phenol
% ionized at pH 7.4 = 0.25%

Phase II Reaction (conjugation)



phenol
% ionized at pH 7.4 = 0.25%

phenyl sulphate
% ionized at pH 7.4 = 99.9%
(excreted by the kidneys)

Most xenobiotics undergo this biphasic metabolism, although some compounds may be metabolized predominantly by one phase. Thus ethanol is almost entirely metabolized by oxidation (phase I) to carbon dioxide, cyanide is metabolized predominantly by a phase II reaction through which it is converted by synthesis to thiocyanate.

The reactions of the two phases of drug metabolism are catalyzed by enzymes and it is from the study of the qualitative and quantitative variations in these enzymes that the explanations of species differences are likely to be found. Quantitative variations in a metabolic reaction may be due to differences in:-

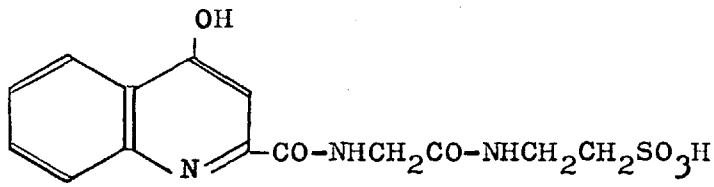
a) the absolute amount of an enzyme,

b) the amount of natural inhibitor of the enzyme,

c) the activity of an enzyme reversing the reaction,

d) the extent of competing reactions using the same substrate.

Some detoxication reactions have a wide phylogenic distribution, e.g. sulphate conjugation, while others are restricted to certain species, e.g. glutamine conjugation in man and some primates (James, 1972). Some metabolic reactions appear to be specific to one species or closely related species and do not occur elsewhere, e.g. cat forms an unusual glycyltaurine conjugate of ~~Kynaurenic~~ Quinaldic acid (Kaihara and Price, 1961).



Glycyltauryl~~kynaurenic~~ Quinaldic acid

When describing a species which is "defective in a detoxication mechanism" this phrase applies to a species in which a common enzymic reaction is not invoked when challenged with a potential substrate. An example of such a species would be the pig which is reported to be unable to form ethereal sulphates readily (Coombs and Hele, 1926); (Stekol, 1936). Knowledge concerning the totality of any defective detoxication mechanism is dependant upon the instrumentation available for the determination of minor

metabolites. Minor metabolites might be formed at very low levels and not be detected because of their dilution in the vehicle of excretion.

If such a species with a defect in detoxication mechanism were challenged with a particular xenobiotic, which could not be metabolized and readily eliminated, then the species would suffer as a result of the defect. Often an alternative detoxication mechanism becomes responsible for the metabolism of the foreign compound but this may not always be adequate. The alternative detoxication mechanism might become "saturated" depending upon the amount of the xenobiotic present and this might result in fatal injury to the species.

By studying species with defects in detoxication mechanisms much useful information concerning these mechanisms might be obtained. Firstly, this information may be of use in estimating safe parameters for the susceptible species at which levels of xenobiotics being ingested must be maintained; whether deliberately ingested foreign compounds such as chemotherapeutic drugs, or unavoidable environmental contaminants. Also by studying the metabolism and elimination of xenobiotics from the species with a defective detoxication mechanism, information concerning the rate

and capacity of the alternate mechanisms may be obtained. Technically one detoxication mechanism may be studied with greater ease when an alternative reaction is absent. By comparing the metabolism of foreign compounds in a species having a defect in a detoxication mechanism with a species that does not have this defect knowledge might be obtained of the genetic basis and metabolic control of detoxication mechanisms. Finally, a laboratory species in which an important detoxication mechanism is absent would be useful for screening new drugs as the species should be more susceptible to the compounds and "exaggerate" any toxic effects likely to arise.

Section B

In this section the metabolic reacions xenobiotics undergo in the body in order to facilitate their excretion will be outlined. The Phase I reactions will be considered first.

Phase I Reactions

The majority of these Phase I detoxication reactions are catalysed by enzymes of the endoplasmic reticulum (microsomal fraction) of the liver. Therefore these reactions may be classified into those which are catalysed by these enzymes (microsomal), and those which are catalysed by enzymes located at other sites (non-microsomal). The microsomal transformations will be considered first.

Microsomal Phase I Reactions

These are classified according to their chemical nature into oxidations, reductions and hydrolyses.

Oxidation Reactions

The endoplasmic reticulum probably consists of an ordered lipoprotein structure with lipid molecules arranged in a bimolecular layer. The substrates for the enzymes in the

reticulum have to be lipid soluble at the pH of the reaction (usually 7 - 8) which probably occurs at the surface. Microsomal oxidation has a specific requirement for $NADPH_2$ and O_2, the overall microsomal oxidative reaction is:

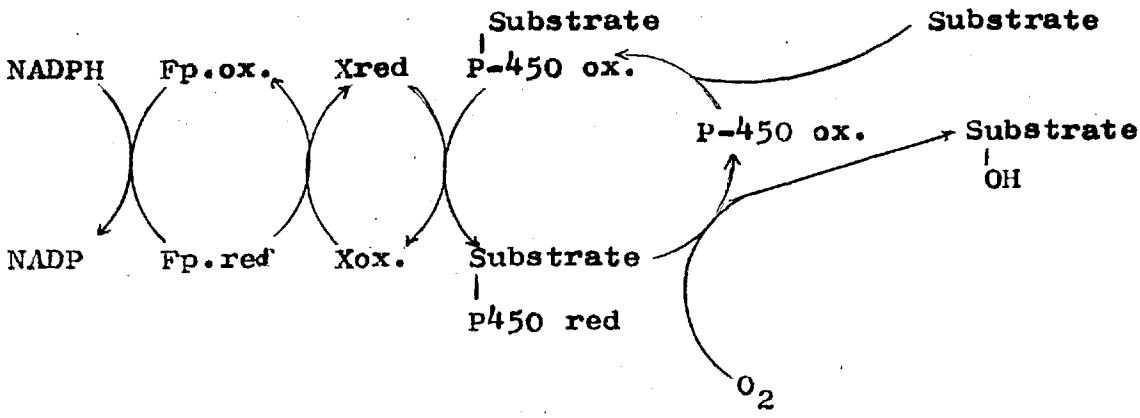
$$NADPH_2 + Sub.H_2 + O_2 \longrightarrow NADP + Sub.HOH + H_2O$$

$Sub.H_2$ = substrate

Sub.HOH = oxidised substrate

The oxidising system contains at least two catalysts, namely the NADPH oxidising flavoprotein, known as NADPH-cytochrome c reductase, and a CO- binding haemoprotein called cytochrome P-450. Much of the speculation regarding the components of the microsomal drug metabolising system exist because attempts to solubilize cytochrome P-450 in an active form failed, although more recently Lu and co-workers have done much toward solving this problem (see review of Mannering, 1971). By their solubilisation techniques they obtained an extract which could be resolved into three components; cytochrome P-450, a fraction containing an NADPH reductase and a fat soluble, heat stable fraction. All three fractions were necessary for the maximal oxidation of compounds.

The interaction of these components in the electron transfer system employed in the microsomal metabolism of compounds is given in the schematic representation below envisaged by Holtzman, et al., (1968):



In this scheme, Fp = flavoprotein, ox. = oxidised, and red. = reduced. In the hepatic microsomal systems the flavoprotein involved is $NADPH_2$ cytochrome c reductase, in adrenal mitochondrial P450 systems adrenodoxin reductase occurs. It has been suggested that a further component, X, is required to bridge electron transport from the flavoprotein reductase to the haemoprotein (Estabrook, et al., 1969). In the adrenal mitochondrial system X is a non-haem iron protein called adrenodoxin. The substrate binds firmly with both the oxidised and reduced forms of cytochrome P450. The substrate-reduced P450 complex reacts with oxygen to give the oxidised substrate and the oxidised form of P-450.

Microsomal oxidations are inhibited by carbon monoxide which readily combines with reduced P-450 and thus prevents the binding of the substrate. Cytochrome P-450 thus binds and hydroxylates the substrate playing an important role in the oxidation of many xenobiotics.

The oxidations carried out by the liver microsomes include the following range of reactions all of which may be ascribed to one common mechanism, namely hydroxylation;

Aromatic hydroxylation

$$C_6H_5X \xrightarrow{[O]} HOC_6H_4X$$

Aliphatic hydroxylation

$$RCH_3 \xrightarrow{[O]} RCH_2OH$$

(R = alkyl chain or alicyclic ring)

O-Dealkylation

$$ROCH_3 \xrightarrow{[O]} \left[ROCH_2OH\right] \longrightarrow ROH + HCHO$$

N-Dealkylation

$$RNHCH_3 \xrightarrow{[O]} \left[RNHCH_2OH\right] \longrightarrow RNH_2 + HCHO$$

Deamination

$$\begin{matrix} R \\ R' \end{matrix}\!\!>\!CHNH_2 \xrightarrow{[O]} \left[\begin{matrix} R \\ R' \end{matrix}\!\!>\!C(OH)NH_2\right] \longrightarrow \begin{matrix} R \\ R' \end{matrix}\!\!>\!CO + NH_3$$

Sulphoxidation

$$RSR' \xrightarrow{[O]} \left[RS^{+}(OH)R'\right] \longrightarrow \underset{\text{(sulphoxide)}}{RSOR'} + H^{+}$$

$$RSOR' \xrightarrow{[O]} \underset{\text{(sulphone)}}{RSO_2R'}$$

Aromatic hydroxylation is one of the most intensely studied of phase I reactions. It has been found to occur in all species examined including fish, insects, birds, reptiles and mammals including man (see Williams, 1967). Although the reaction occurs in most species, there are considerable species variations in the extent to which it occurs with a given compound, in its orientation and also in the extent to which it occurs with different compounds in the same species (see Table 6.1). Thus it appears there is no significant phylogenic development of this Phase I reaction. Some oxidative reactions, for example the 7-hydroxylation of coumarin, are strongly evident in some mammals, absent in others, and yet are found in insects and other lower orders of the animal kingdom (see review of Williams, 1967). In all Phase I reactions the greatest versatility in biotransformations is shown by the simplest of organism, the bacteria.

Table B.1. Aromatic Hydroxylation in Vivo

	Compound	Extent of hydroxylation in Man	Dog	Rabbit	Rat	Mouse	Guinea Pig
+	Amphetamine	L	M	L	M	-	L
o	Ephedrine	-	L	L	M	-	L
o	Imipramine	M	-	H	L	-	-
o	Diethyltryptamine	M	-	-	H	H	L
o	LSD	-	-	-	M	-	L
∓	Aniline	-	H	H	H	H	H

H = 25 – 50% of dose or more M = 5 – 20% L = < 5%

Alternative Phase I metabolic reaction

+ deamination
o dealkylation
∓ none

figure from Williams, 1964

Reductive reactions

Reductions are important Phase I detoxication reactions some of the most important reactions that occur are:

1. Reduction of azo links

$$RN = NR' \xrightarrow{\text{azo reductase}} RNH_2 + R'NH_2$$

2. Reduction of nitro groups

$$RNO_2 \xrightarrow{\text{nitroreductase}} RNH_2$$

3. Reductions of aldehydes or ketones

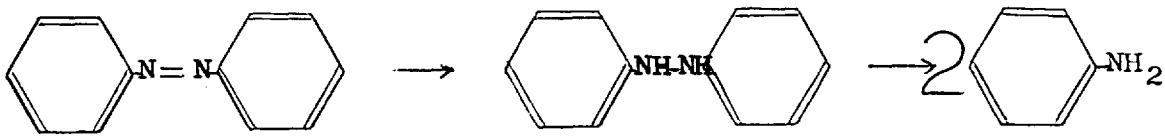
$$RCHO \xrightarrow{\text{aldoreductase}} RCH_2OH$$

e.g. $CCl_3CH(OH)_2$ → $C.Cl_3CH_2OH$

chloral hydrate → trichloroethanol

R = alkyl radicle or alicyclic ring

Reductive reactions have been shown to occur in liver microsomes but the mechanisms of these reactions have not yet been as thoroughly investigated as the oxidative reactions. The reactions which have been investigated in most detail are the reduction of azo and of nitro compounds (Gillette, 1966). Presumably the splitting of the azo groups in these reactions takes place in two steps: first the formation of a hydrazo intermediate and then the reductive cleavage of the N-N bond.



Azobenzene Hydrazobenzene Aniline

Similarly nitroreduction may proceed through various intermediates:

Nitrobenzene Nitrosobenzene Phenylhydroxylamine Aniline

The enzymes responsible for these reactions occur mainly in the liver microsomes. There appears to be at least two pathways for these reactions, one inhibited by carbon monoxide and the other not inhibited by carbon monoxide. The latter pathway does not depend upon cytochrome P-450 and the reduction is carried out by NADPH cytochrome c reductase alone. The first pathway is through cytochrome P-450 since this cytochrome was once described as "CO-binding pigment". Azoreductase activity occurs under both aerobic and anaerobic conditions but nitroreductase activity is inhibited by oxygen and occurs only in anaerobic conditions. Both NADH

and NADPH can act as the electron donors in the reduction of nitro compounds. As with oxidative reactions reductions appear to vary with species and compound; thus rabbit liver reduces p but not m nitrophenol whereas it reduces chloramphenical twice as fast as p-nitrobenzoic or p-nitrotoluene, (see review of Williams, 1964).

Hydrolytic reactions

The main hydrolytic reactions which occur are:

1. The hydrolysis of esters under the influence of esterases

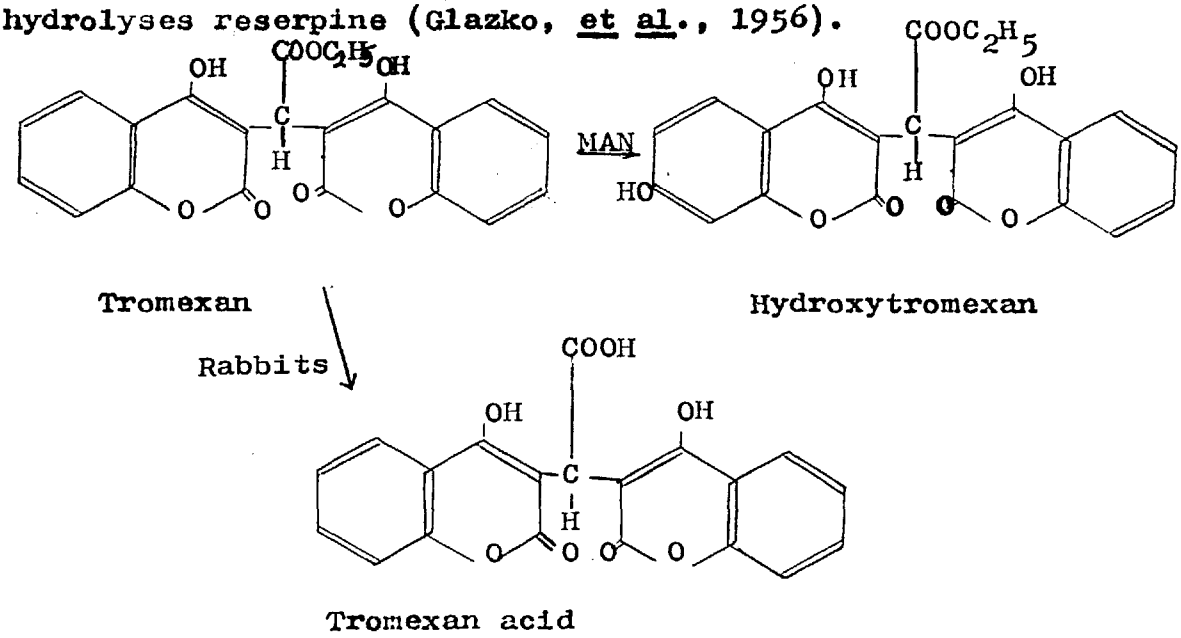
$$ROCOR' \longrightarrow ROH + R'COOH$$

2. The hydrolysis of amides by amidases

$$RCONH_2 \longrightarrow RCOOH + NH_3$$

A large number of drugs are esters of various kinds and many are hydrolysed in the body to yield phenols, alcohols and carboxylic acids. The enzymes which carry out these reactions are widely distributed. Kalow,(1962) has reported that the tissues of rats contain as many as 16 esterases. It was pointed out that esterases are notoriously variable from species to species and in some species variation in pharmacological activity of esters can be explained in terms of the nature and amounts of esterases. The drug tromexan is an ester which

is de-esterified in the rabbit to tromexan acid, while in man this reaction is of little significance, the drug undergoing aromatic hydroxylation to hydroxytromexan (Burns, et al., 1953). Desterification of reserpine does not occur to a large extent in the dog and monkey, but it appears that the intestinal mucosa of rats contains an esterase which hydrolyses reserpine (Glazko, et al., 1956).



Tromexan acid

Non-microsomal Phase I Reactions

Foreign compounds are also metabolised by non-microsomal enzyme systems. In the mitochondria are amine oxidases which convert amines into aldehydes and enzymes which aromatize saturated alicyclic compounds into benzenoid derivatives.

In the soluble fraction are found the enzymes alcohol dehydrogenase, aldehyde oxidase and xanthine oxidase, which oxidise alcohols and aldehydes. Many other enzymes such as amine oxidases and esterases, that metabolise foreign compounds are found in the blood plasma, and enzymes of the intestinal flora are responsible for other metabolic transformations. These numerous reactions by virtue of their diversity are not mechanistically classified as are the microsomal Phase I reactions; they have been reviewed by Parke, (1968).

Having undergone Phase I metabolism a xenobiotic may be excreted, more often however it undergoes a Phase II synthetic (conjugation) reaction and it is these reactions that will be considered next.

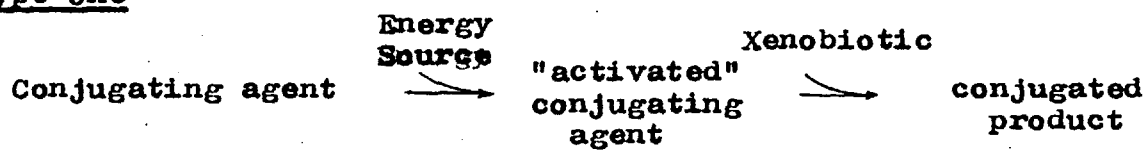
Phase II Reactions

Xenobiotics which possess or are able to acquire a suitable chemical grouping (by one of the Phase I reactions already described) may subsequently undergo one of a number of conjugation reactions. These involve the linking of the xenobiotic with an endogenous conjugating agent and are the Phase II synthetic reactions. The conjugation generally results in the molecule becoming more polar, less lipid-soluble

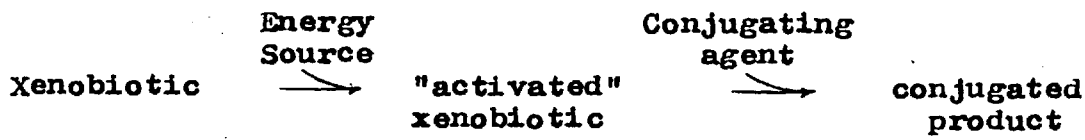
and therefore more readily excreted from the animal body.

These reactions are characterised by the occurence of activated nucleotides as intermediates in the synthetic process and all require energy usually provided by adenosine triphosphate (ATP). The nucleotide intermediate can contain in its molecule either the conjugating agent or the xenobiotic may be activated. Thus mechanistically conjugation reactions may be divided into two types.

Type One



Type Two



Type one reactions occur in many tissues, type two reactions appear to be restricted to liver and/or kidney. Type two reactions are amino acid conjugations and the majority of other conjugations are type one. The conjugating agents are often coenzymes that participate in intermediary metabolism, but the enzymes that catalyse the transfer are specific for the formation of these compounds. The cofactors and the

conjugations with which they are associated are as follows:-

Uridine diphosphate cofactors

Uridine diphosphate glucose (UDPG) - β-glucoside formation

Uridine diphosphate glucuronic acid (UDPGA) - β-glucuronide formation

Adenosine cofactors

3'-Phosphoadenosine-5'-phosphosulphate (PAPS) - formation of sulphate esters

S-Adenosyl methionine O-, N, and S-methylations

Coenzyme A

Acetyl coenzyme A - acetylation

Glutathione

Formation of glutathione conjugates and mercapturic acids

"Sulphur"

Conjugation with sulphur from thiosulphate in the conversion of cyanide to thiocyanate.

Uridine diphosphate cofactors

Glucoside conjugation

Insects and plants apparently do not synthesise glucuronides but form glucosides instead (see Smith, 1964). The mechanism of glucoside formation is probably:-

$$\text{UDP-glucose} + \text{phenol} \xrightarrow{\text{UDP glucose transferase}} \text{phenyl } \beta\text{-glucoside} + \text{UDP}$$

Trivelloni, (1960) demonstrated this reaction in the fat body of locust (Schistocerca cancellata). Miller, (1938) showed that ethylene chlorohydrin was converted into 2-chloroethyl-β-D-glucoside in gladiolus corms. Sorbo, (1957) and others have shown that the glucose donour is UDP-glucose.

Glucuronic Acid Conjugation

Glucuronic acid conjugation is widespread in nature occuring in many species. Glucuronides may be formed in vivo if the xenobiotic contains or acquires during its metabolism any of the groups shown in Table b-2. The mechanism of glucuronic acid formation will be discussed in detail in section D. Most mammals excrete glucuronic acid conjugates of the xenobiotics shown in Table b-2, the most notable exception being the cat. The defect in glucuronic acid conjugation in cat will also be discussed in detail in section D.

Table b.2 Types of Groups Forming Glucuronides

Hydroxyl	
Phenolic	ArOH
Enolic	$-CH=COH$
Primary alcoholic	$-CH_2OH$
Secondary alcoholic	$>CHOH$
Tertiary alcoholic	$\equiv COH$
Hydroxylamine	$>NOH$
Carboxyl	
Aromatic	ArCOOH
Primary aliphatic	$-CH_2COOH$
Secondary aliphatic	$>CHCOOH$
Tertiary aliphatic	$\equiv C.COOH$
Amino and imino	
Aromatic	$ArNH_2$
Carbamate	$-O.CONH_2$
Sulfhydryl	
Sulfhydryl	$-SH$
Sulfonimide	$-SO_2NH-$
Heterocyclic	$>NH$
Dithioic	$-CSSH$

Table taken from Williams, 1967

With birds glucuronide formation has been shown to occur in domestic hen, turkey, goose, duck, domestic pigeon, wood pigeon, dove, carrion crow and parrot (Baldwin, et al., 1960). Although chicken possesses many glucuronyl transferases, it appears a bilirubin-specific transferase is absent or defective since hens do not form bilirubin glucuronide (Dutton, and Burchell, 1969). Glucuronide conjugation has been shown to occur in amphibia and some reptiles. It is not common in fishes and in insects glucoside formation predominates (for general review see Dutton, 1966). In the livers of newborn animals such as man, mouse, guinea-pig and rabbit, glucuronide synthesis is at a low level, neonatal rat is an exception, their livers being more active in this respect than the adult (Dutton, 1964).

Hereditary manifestations of low UDP-glucuronyl transferase are encountered in rat and man. The rat strain described by Gunn, (1938) exhibits acholuric jaundice, having a complete lack of bilirubin conjugation (Axelrod, et al., 1958; Carbone and Gradsky, 1957; Lathe and Walker, 1957). This condition is similar to the Crigler-Najjar syndrome in man (Schmid, 1972; Crigler and Najjar, 1952). Both conditions are inherited as autosomal recessive characters and in the homozygous individual an elevated plasma (unconjugated)

bilirubin is present (17 - 40 mg/100 ml in man normal plasma level is 0.3 - 1 mg/100 ml), and the bile is almost free of the pigment. The heterozygous rat has 55% of enzyme activity of the normal (Strebel and Odell, 1971). The homozygous rat and human can form other glucuronides although some at reduced rates, Fevery et al., (1971) showed that Gunn rat could not form the glucoside or xyloside of bilirubin. The other hereditary defect in bilirubin conjugation is Gilbert's disease which is an idiopathic constitutional hyperbilirubinaemia associated with a plasma bilirubin level of 1 - 3 mg/100 ml. This is inherited as an autosomal dominant character (Powell et al., 1967). Biopsy specimens conjugate 270 $\pm$ 140 (S.D.) μg. bilirubin/g.liver/h. compared with normal 1100 $\pm$ 280 (Black and Billing, 1969). In many, if not all, of these patients plasma bilirubin can be reduced by giving phenobarbitone, the inductive effects of which have been reviewed by Gelboin, (1971).

Adenosine Cofactors

Sulphate conjugation

Another important class of conjugate is the sulphate ester or "ethereal sulphate" the types occuring are:-

Aryl sulphates - esters of phenolic compounds

$ArOH \longrightarrow ArSO_3H$ e.g. Phenyl sulphate

Alkyl sulphates - esters of primary aliphatic alcohols

$ROH \longrightarrow ROSO_3H$ e.g. ethyl sulphate

Sulphamates - esters of amines and so contain a sulphamyl (N-Sulphates) group

$RNH_2 \longrightarrow RNHSO_3H$ e.g. phenyl sulphamate

Steroid Sulphates - esters of alcoholic or phenolic groups of steroids, e.g. androsterone sulphate (alcoholic), oestrone sulphate (phenolic)

Carbohydrate Sulphates - esters of hydroxyl or amino groups of carbohydrates. chondroitin sulphate (alcoholic).

Sulphate conjugation of phenols is a widely distributed process and has been found to occur in most mammalian species Phenol sulphotransferases are a well studied group of enzymes and although little information is available on their properties they have been shown to occur in man, dog, rabbit, rat, cat horse, cow, guinea-pig, goat and species of whale (Schmidt-Nielsen and Hdmson, 1921). Birds, reptiles and amphibia can also form aryl sulphates (Smith, 1964, 1968; Hitchcock and Smith, 1964; Darby, Heenan and Smith, 1966). Sulphates are

also reported to be formed in a variety of invertebrates. It appears this conjugation is even more widely distributed than is glucuronide synthesis and is therefore an important detoxication reaction. The mechanism of ethereal sulphate formation will be discussed in section D.

Methylation

Another conjugation reaction involving adenosine cofactors is methylation. Methylation is the fate of a number of endogenous compounds, e.g. adrenaline, as well as xenobiotics. It is a potential reaction of compounds containing amino or substituted amino groups, hydroxyl or sulphydryl groups. Methylation is not a general reaction of aromatic amines, e.g. aniline is not methylated in the rabbit (Smith, 1958). Methylation of hydroxy compounds is only observed in certain phenols, usually polyhydroxyphenols such as catechols. The methylation of various phenols was studied in rat by Bakke, (1970). None of the monohydric phenols administered were methylated to their corresponding anisoles. An example of S-methylation which is the rarest type, is the methylation of thiouracil to 2-methylthiouracil in small quantities in the rat described by Sarcione and Sekol, (1958).

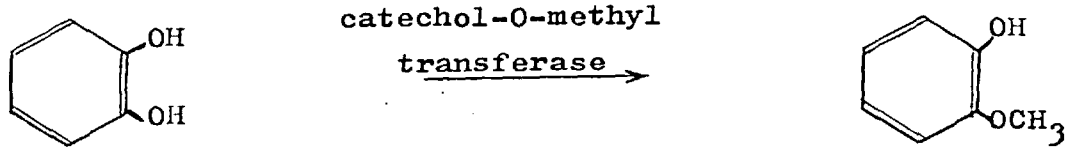
The methyl donor is "activated methionine", i.e. S-adenosyl methionine which transfers its methyl group to a suitable acceptor and is converted into S-adenosylhomocysteine. Specificity in methylation depends on the transferase, several enzymes exist which catalyse a specific O-, N-, or S-methylation.

ATP + methionine ⟶ S-adenosylmethionine + Pi + PPi

S-adenosylmethionine + acceptor —(methyl transferase)→ methylated acceptor + S-adenosylhomocysteine.

O-methylations have been observed in a number of mammals (Axelrod and Tomchick, 1958; Booth et al., 1957). N-methylations are also widely distributed. Both O- and N-methylations have been noted in amphibia, (Marki, et al., 1962); fish and plants, (Towers, 1964).

O-Methylation

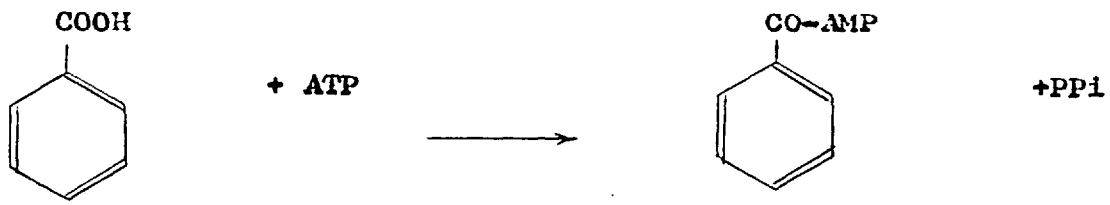


Coenzyme A cofactors

There are two types of conjugative reactions involving coenzyme A intermediates. In the first type to be considered Coenzyme A derivatives of foreign carboxylic acids combine with endogenous amino acids, principally glycine, to form amino acyl conjugates. In the second type of reaction to be considered foreign aromatic amines are acetylated by acetyl CoA.

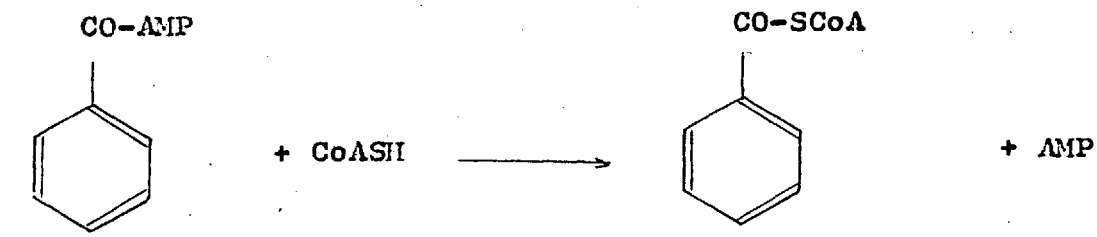
Glycine conjugation

Conjugation with glycine and other amino acids is a characteristic metabolic reaction of aromatic carboxylic acids such as benzoic acid, substituted benzoic acids and heterocyclic carboxylic acids. Table b.3 shows the amino acids used for conjugation by various species. The formation of amino acid conjugates can be illustrated by the following example where a glycine conjugate (i.e. hippuric acid) of benzoic acid is formed:

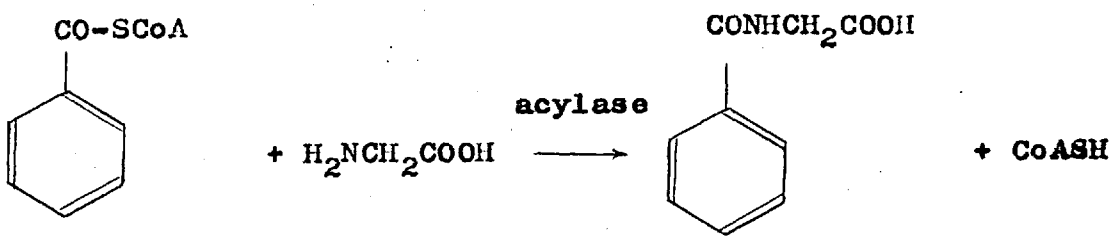


Benzoic Acid

Adenyl benzoate



Adenylbenzoate Benzoyl Coenzyme A



Benzoyl CoenzymeA Glycine Hippuric Acid Coenzyme A

An example of a species defective in amino acid conjugation is the Indian fruit bat (Pteropus giganteus) which is apparently unable to form glycine conjugates of such xenobiotics as benzoic acid (see French, 1970).

Table b.3 Amino Acid Conjugation in Various Species

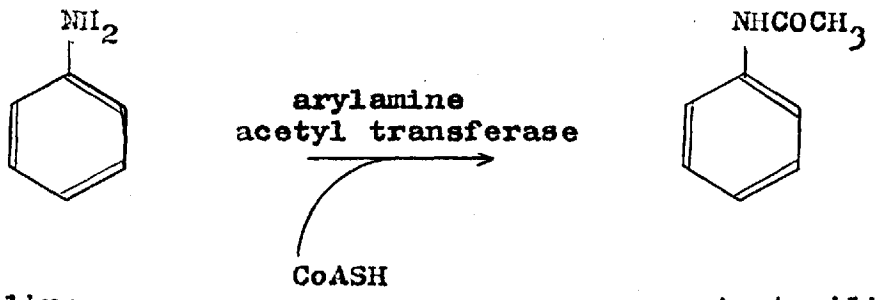
Species	Xenobiotic	Aminoacid conjugated
Mammals[+]	Aromatic acids	Glycine
Birds (columbiformes)	Arylacetic acids	
(Insects)		
Birds (galliformes, anseriformes)	Aromatic acids Arylacetic acids	Ornithine (mainly)
(Reptiles)		
Man	Arylacetic acids	Glutamine
(Old World Monkeys)		
Arachnids	Aromatic acids	Arginine

[+] excluding man and Old World monkeys

Acetylation

Acetylation is a general pathway for detoxication of aromatic amines, sulphonamides, and some foreign aromatic amino acids such as phenylcysteines. With sulphonamides acetylation yields N'- and N^4-acetyl and diacetyl derivatives. The acetyl group arises from endogenous sources, different acetylase enzymes catalyse the acetylation of different substrates. It appears in dog (and fox) some of these enzymes are defective since this species can acetylate some amino groups but not others (Marshall, 1954; Williams, 1967). The dog can acetylate the N'-sulphonamido group of sulphanilamide and acetylate phenylcysteine derivatives into mercapturic acids. Arylamines and the N^4-amino group of sulphonamides are not acetylated in the dog, Leibman and Anaclerio, (1961) reported that dog liver and kidney contains an inhibitor of arylamine acetyl transferase. The dog is also unable to acetylate hydrazines and hydrazides, (McKennis, et al., 1959). The guinea-pig is unable to acetylate arylcysteines. Sulphanilamide for example is readily acetylated by guinea-pig liver slices, but S-(-p-chlorophenyl)-L-cysteine is not. (Bray, et al., 1959).

acetylation of an aromatic amine



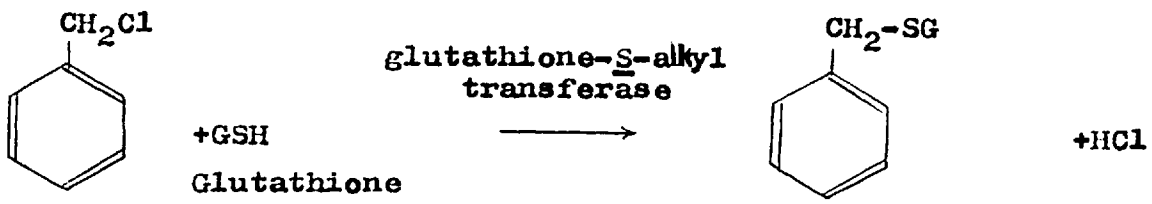
Aniline Acetanilide

No Cofactor known

Glutathione (and Mercapturic acid) formation

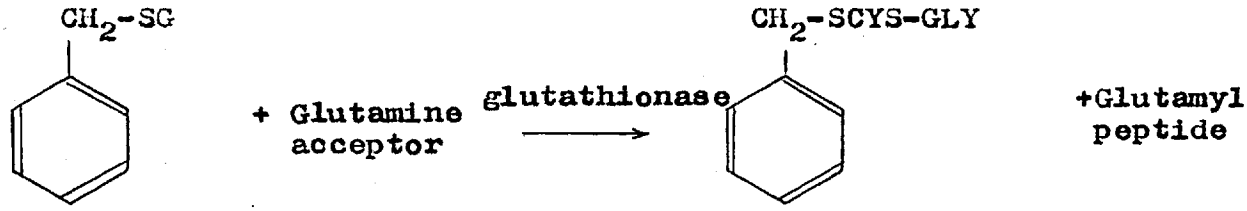
Xenobiotics may be conjugated with glutathione to form S-alkyl or S-aryl glutathione derivatives, cysteines and ultimately mercapturic acids. Benzene, naphthalene, anthracene and the monohalogenated benzenes are metabolised into mercapturic acids, the L-acetyl-cysteine moiety replaces a hydrogen atom. The formation of a mercapturic acid takes place in four steps:-

1) Conjugation of compound with glutathione



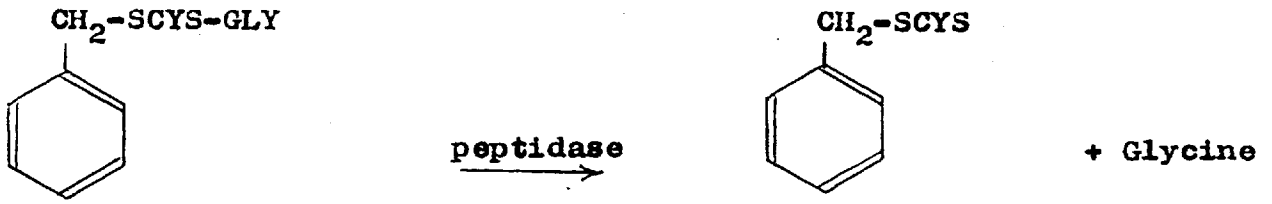
Benzylchloride Benzylglutathione

2) removal of the glutamyl group from the glutathione conjugate by transpeptidation to form S-arylcysteinyl-glycine,



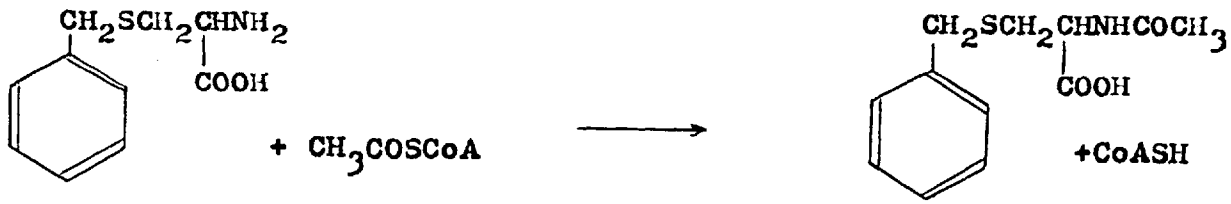
Benzylglutathione Benzylcysteinylglycine

3) hydrolysis of the S-arylcysteinylglycine by peptidases to give an S-arylcysteine



Benzylcysteinylglycine Benzylcysteine

4) acetylation of the S-substituted cysteine to give a mercapturic acid



Benzylcysteine Acetyl Coenzyme A Benzylmercapturic Acid Coenzyme A

Mercapturic acids are excreted by most mammals and also by a number of insects (Gessner and Smith, 1960). In man it has been reported that there is no detectable mercapturic acid in urine following doses of bromobenzene (Wainer and Lorincz, 1963); however, small amounts of mercapturic acid conjugates have been detected chromatographically in human urine following the administration of naphthalene (Boyland and Sims, 1958). In the guinea-pig it is probably that all the reactions of mercapturic acid synthesis except the last can occur, for the guinea-pig is unable to acetylate arylcysteines (Bray, et al., 1959). When the hen is dosed with bromobenzene no mercapturic acid conjugate is detected in the excreta (Baldwin, 1961). Grover and Sims (1964) have shown that liver preparations from hens and ducks contain very low levels of glutathione S-aryltransferase. It is therefore probable that the first step in mercapturic acid synthesis is defective in these species.

Thiocyanate Formation

Hydrogen cyanide and alkali cyanides are highly toxic substances, since these are converted into thiocyanates which

are a hundred times less toxic, this is an example of true detoxication. The enzyme responsible, namely, rhodanese is distributed throughout the body, the highest concentration occuring in the liver. Rhodanese appears to require no coenzyme for its activity, it is a highly specific enzyme catalysing the reaction:

$$CN^- + S_2O_3^{2-} \longrightarrow SCN^- + SO_3^{2-}$$

where sulphur is transferred from thiosulphate (which arises from endogenous sources) to the cyanide ion. This simplest of conjugation mechanisms occurs widely in nature; in man, other vertebrates, non-vertebrates, plants and bacteria.

In this section (B) the general enzymic reactions foreign compounds undergo have been outlined, and some species which apparently have little ability to perform one or more of these reactions mentioned. In the following section (C) other factors which affect these enzymic reactions and thus the overall detoxication of foreign compounds are discussed.

Section C

Factors Influencing the Metabolism of Xenobiotics

The control of the Phase II metabolism of xenobiotics may be at various stages:-

1. at the uptake of the endogenous conjugating agent into the cells;
2. at the conjugation of the xenobiotic with the conjugating agent;
3. at the accessibility of the xenobiotic and conjugating agent to the enzyme catalysing their conjugation;
4. at the level of active enzyme;
5. at the availability of cofactors necessary for the conjugation;
6. at the secretion of the conjugate from the cell;
7. at the possible hydrolysis of the conjugated xenobiotic.

Control at any stage will depend upon the state of the animal; its age, diet, hormonal or genetic complement, intake of toxicants or drugs, its response to external environment and its health.

Age

At birth many of the hepatic enzymes show a marked increase in levels of activity, presumably to enable the

neonate to become adapted to individual existence. However, many microsomal enzymes, especially those involved in detoxication are greatly reduced at birth. Thus deficiency in forming glucuronic acid conjugates in newly born animals is well known, although rat appears to be an exception to this rule (see review of Dutton, 1971). Some compounds are glucuronidated in neonates, however, but when the glucuronide-forming capacity develops to adult levels is not certain. An attractive theory is that the transferase activity is "triggered" by accumulation of natural substrates, such "triggering" by xenobiotics would be far less predictable. It is likely that newly born animals of species in which a major conjugation mechanism is defective (e.g. glucuronic acid conjugation in cats; sulphate conjugation in pigs) would be even more susceptible to the toxic effects of xenobiotics normally detoxicated by this reaction than the adult.

Very little information is available on the ability of aged animals to metabolise xenobiotics. Dutton, (1971) reported that although UDPGA and glucuronidation have not been specifically studied in old animals indirect evidence suggested it appeared lower. Thus it is probably that aged

animals of species with defective conjugation mechanisms would be even less able to metabolise and excrete foreign compounds then mature animals.

Diet

The influence of the diet on detoxication mechanisms has long been known (see Braunstein, et al., 1931). Fasting animals of a number of species produce smaller amounts of glucuronic acids than normal but show no decrease in transferase activity in vitro (Miettinen and Leskinen, 1963). Glucose loading increases liver UDPGA (Wong and Sourkes, 1967) and possibly glucuronidation, (Müller-Oerlinghausen et al., 1967), Since it is the glucuronyl transferase activity that is defective in cat, increasing UDPGA by loading with glucose is unlikely to increase glucuronidation substantially.

It is known that addition of sulphate or sulphate precursors increases sulphate conjugation. Laidlaw and Young, (1948) showed by use of radioactively labelled inorganic sulphate that this was incorporated into sulphate conjugates. Coombs and Hele,(1927) found that addition of sulphate precursors did not stimulate ethereal sulphate formation in pig. Furthermore since the pig is an omnivore it is unlikely

that the defect in sulphate conjugation is due to a dietary deficiency in sulphate.

Sex Differences

Adult male rats metabolise many xenobiotics at higher rates than female rats (Kato and Gillette, 1965). This sex difference in the activity of the microsomal enzymes has not been observed to the same extent in species other than rat. Sex differences in liver UDPG-transferases and glucuronide biosynthesis have been cited and denied in many species. Except for hamsters any greater activity appears in males (see Dutton, 1966). It is unlikely however that a species will be found where a specific detoxication reaction is absent in one sex but not the other sex of the species. No difference has been reported in the ability of male and female; cats to form glucuronic acid conjugates, or pigs to form sulphate conjugates. During late pregnancy the glucuronic acid conjugation of foreign compounds is markedly reduced, (Creaven and Parke, 1965). A similar inhibition of sulphate conjugation has been observed in pregnant guinea-pigs and rats (Pulkkinen, 1963). Obviously a pregnant animal in a species such as cat or pig would be even more susceptible to the toxic effects of xenobiotics.

Hormones

Various hormones are known to affect drug metabolism *in vitro* so that administration of hormones might possibly alleviate the conjugation defect. Most of the information derives from the studies of glucuronic acid conjugation. Cortisone, ACTH, thyroxine and insulin had little effect on glucuronidation or its enzymes, (Dutton, 1966). Many other hormones inhibit glucuronic acid conjugation *in vitro*, e.g. progesterone and pregnanediol, (Hsia *et al.*, 1963). These hormones are possibly responsible for the decrease in glucuronide synthesis associated with pregnancy mentioned above. Adrenaline and hydrocortisone raise liver UDPGA (Wong and Sourkes, 1967) thus administration of these hormones might increase glucuronic acid formation in cat.

Disease

It is well known that human patients with liver damage show an increased sensitivity to a wide variety of drugs, this has been attributed to impairment of the detoxicating function of the liver. Patients with obstructive jaundice, hepatitis, cirrhosis and other liver diseases exhibit impaired formation of glucuronic acid and sulphate conjugates (Muting, 1963). UDP glucuronyltransferase does not fall in

damage or disease as rapidly as some drug metabolising enzymes (Dutton, 1969). Therefore, damage to the liver of species with defect in conjugative mechanisms will only worsen the capacity to detoxicate xenobiotics.

Other Compounds

The metabolism of certain foreign compounds is enhanced by simultaneous administration of other foreign compounds such as drugs, pesticides and polycyclic hydrocarbons. Foreign compounds are believed to elicit their response by increasing the amounts of microsomal enzymes, including cytochrome P-450 and $NADPH_2$-cytochrome-c reductase (Ernster and Orrenius, 1965; Remmer and Merker, 1965). Much of the information available is on stimulation of Phase I reactions in *in vitro* systems. Could administration of a suitable chemical stimulate the formation of glucuronic acid conjugates in cats or sulphates in pigs? Much of the relevant work has been performed on UDP glucuronyl transferase.

UDP glucuronyl transferase activity and glucuronidation were increased in animals given the carcinogens 3,4-benzpyrene, 3-methylcholanthrene, 1,2-,5-,6-dibenzanthracene (Dutton 1966; Goldstein and Taurog, 1968) and diethylnitrosamine

(Stevenson, *et al.*, 1968). Of these carcinogens only diethylnitrosamine was found to stimulate UDPGA transferase activity *in vitro* with rat liver preparations. The stimulation of glucuronide formation by diethylnitrosamine *in vitro* appears to differ from that reported with ATP and UDP-*N*-acetylglucosamine (Stevenson and Dutton, 1962) since diethylnitrosamine, unlike these nucleotides, did not affect the rate of destruction of UDPGA. However, the stimulatory effect of diethylnitrosamine is limited to the liver of rats and the glucuronide acceptors *o*-aminophenol and paracetamol (Stevenson, et al., 1968). Mowatt and Arias, (1970) studied the stimulatory effect of diethylnitrosamine on UDPGA transferase activity in liver preparations of rats, Gunn rats, goats and cats, using *o*-aminophenol, *p*-nitrophenol and bilirubin as substrates. Enhancement of UDPGA transferase was only detected in the rat. In the Gunn rat treatment with diethylnitrosamine did not increase UDPGA transferase activity *in vitro* with bilirubin as substrate, and *in vivo* administration of diethylnitrosamine did not reduce serum bilirubin levels. Therefore it is unlikely that diethylnitrosamine treatment could enhance the ability of cat *in vivo* to form glucuronic acid conjugates.

No stimulatory drug appears an obvious substrate for

for UDPGA transferase; the potential substrate morphine stimulates UDP glucose dehydrogenase instead (Takemori, 1960) A large number of xenobiotics have been examined as possible inducers of the transferase, only some of the successful examples will be cited.

Phenobarbital treatment was used by Yaffe et al., (1966) to enhance the glucuronide conjugating capacity and thereby increase the elimination rate of bilirubin in a hyperbilirubinaemic infant. Jansen and Hendersen, (1972) compared the influence of phenobarbital treatment on the glucuronidation of p-nitrophenol and bilirubin in Wistar rat, Gunn rat and cat. In the Wistar rat both p-nitrophenol and bilirubin conjugation were induced; in the Gunn rat p-nitrophenol but not bilirubin conjugation was increased; conversely in the cat bilirubin glucuronidation was induced. Thus by administration of certain foreign compounds the ability of the cat to form glucuronic acid conjugates might be enhanced, but it must be remembered that many xenobiotics inhibit rather than enhance conjugation. Inhibition of glucuronide conjugation of o-aminophenol and bilirubin has been shown to occur in vitro in the presence of therapeutic concentrations of the anti-inflammatory drugs, phenylbutazone, ibufenac (4-isobutylphenylacetic acid) (Hargreaves, 1965). The

porphyriogenic drug sedormid inhibits the *in vitro* conjugation of p-nitrophenol (de Barriero, 1965), chlorpromazine inhibited p-aminophenol glucuronidation *in vitro* in rat (Dybing, 1972), a variety of inhalation anaesthetics were shown to reduce glucuronidation *in vitro* (Brown, 1972). Administration of Eugenol (4-allyl-2 methoxy phenol) showed competitive inhibition with p-nitrophenol conjugation (Yuasa, 1972) in rats liver preparations.

Thus although it might be possible to enhance a conjugation reaction which does not proceed readily in a particular species owing to the unpredictable effect of varied xenobiotics an inhibitory response with a resulting detrimental effect might occur.

Mode of Administration

When considering a species which is unable to perform a particular detoxication reaction the route of administration of the xenobiotic is particularly important. In the case of cat the hepatic UDPGA transferase has little ability to conjugate various potential substrates (Dutton and Grieg, 1957). It is possible that other tissues might contain UDPGA transferas with higher activity against potential substrates. UDPGA transferase has been reported to occur in many organs of

mammals (see Dutton, 1966). This enzymic activity has been demonstrated in the skin of guinea-pig and mice (Stevenson and Dutton, 1960; Dutton and Stevenson, 1962). Possibly the skin of cat might contribute to the glucuronidation of some xenobiotics. Thus, appropriate administration of the xenobiotic to the cat might cause an increase in the amount of glucuronic acid conjugate excreted. The intestinal mucosa of many species possibly conjugate xenobiotics with sulphate as they are absorbed from the gut, (Roy and Trudinger, 1970). Thus in the case of pig which is reported not to form sulphate conjugates readily a suitable substrate would be more likely to form sulphate conjugates following oral than intraperitoneal administration. Where in the former case the foreign substrate would traverse the gut wall, whereas by intraperitoneal injection it would pass directly to the liver. It is also possible that a xenobiotic could be metabolised by the organisms of the gut flora and such a compound would be metabolised following oral administration but not following administration by another route. Thus the mode of administration can have a profound affect on the metabolism and toxicity of xenobiotics.

In the next section glucuronic acid conjugation in cats and sulphate conjugation in pigs will be considered in more detai

Section D

The Metabolic Defect in the Cat and Pig

The apparent inability of cats to form appreciable quantities of glucuronic acid conjugates and the reported lack of sulphate conjugation in the pig are the metabolic defects in detoxication reactions chosen for further investigation. A large range of compounds are detoxicated by these mechanisms, and in the case of some compounds, e.g. phenols, glucuronic acid conjugation in the cat and sulphate conjugation in the pig may be investigated by administering the same compound to each species. It is also advantageous that cat and pig are more easily obtained than the exotic species in which some defects are manifested. These animals are also suitable for urine collection for metabolic studies (young pigs of limited size being used).

Toxicity of xenobiotics in the cat and pig

A toxic xenobiotic is likely to have a deleterious effect on an animal ingesting it and if a metabolic defect results in a delay in the excretion of this compound, the toxic action will be prolonged. It would be expected, therefore, that animals unable to conjugate and thus excrete xenobiotics, will be more sensitive to compounds normally detoxicated by these

reactions. This is the case in the cat and pig. In Table d.1 it is seen that the cat is far more susceptible to the toxic effects of phenolic, potentially glucuronogenic agents than is the rabbit, which can excrete glucuronic acid conjugates of many xenobiotics. Similarly it is seen that the cat is far more susceptible to phenol poisoning than the other laboratory species cited (Spector, 1956). This is therefore a true example of a "detoxication defect" where the toxicity as measured by the LD_{50} appears to be lowered by the absence of important conjugation mechanisms.

There are a number of examples in the veterinary records of phenols not being readily metabolised in pigs. Coal tar "pitch" poisoning is well known in pigs (Aitken, 1956). It is recommended that care should be made in the choice of tar for pig pens.

If flooring made from lignite pitch and bitumen contains more than 6 mg. per cent of phenolic constituents, it must be considered potentially dangerous to pigs (Rummler, 1962). Tarred roofing materials are also toxic to pigs (Sautter and Ferstermacher, 1957). Unconjugated cresols have been demonstrated in the ingesta and livers of pigs dead from pitch poisoning (Oliver and Ingram, 1957). It seems, therefore, that pigs are susceptible to phenols and cresols.

Table d.1 Toxicity of Some Compounds in Cat and Other Species

Compound	Animal	Route	Dose	Dosage mg/kg
Aniline	Cat	p.o.	LD	100-200
	Rabbit	s.c.	LD	500
m-cresol	Cat	s.c.	LD	180
	Rabbit	s.c.	LD	500-600
Morphine	Cat	s.c.	MLD	40-80
	Rabbit	s.c.	LD_{50}	266
1-Naphthol	Cat	p.o.	LD	100-150
	Rabbit	p.o.	LD	9000
2-Naphthol	Cat	p.o.	LD	100-150
	Rabbit	p.o.	LD	3800
o-nitrophenol	Cat	s.c.	LD	600
	Rabbit	s.c.	LD	1700
p-nitrophenol	Cat	s.c.	MLD	197
	Rabbit	s.c.	MLD	1700
Phenol	Cat	s.c.	MLD	80
	Mouse	s.c.	MLD	420-450
	Guinea-pig	s.c.	MLD	450-550
	Rabbit	s.c.	LD_{50}	620
	Rat	s.c.	LD	500-600

Phenothiazine 0.44 g/kg is extremely toxic to young pigs, although with older pigs 2.2 g/kg can be toleraged (Lopage,1943). However this drug is non-toxic to cattle in three daily consecutive doses of 100g; repeated daily doses of 160 g in sheep; and 1g was fed daily to hens for 45 days without ill effects (cited in Garner, 1967). It appears therefore that the lack of sulphate conjugation in pig renders this animal, as cat, more susceptible to phenol poisoning. It was interesting to note the high susceptibility of young pigs to phenothiazine. As stated in the previous section glucuronic acid conjugation is low in very young animals so that the pig, which is also unable to form sulphate conjugates readily would be particularly vulnerable to such drugs.

The Glucuronide Defect in Cat

Conjugated glucuronic acids are β-glycosides of D-glucuronic acid, the mechanism of formation is shown below the product being fully described as β-D-glucopyranuroniside (see fig. d.1).

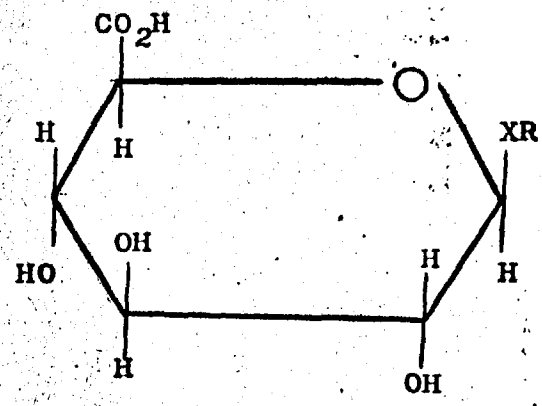
$$\text{Glucose-1-Phosphate} + \text{UTP} \xrightarrow{\text{uridyl transferase}} \text{UDPG} + \text{PPi}$$

$$\text{UDPG} + 2\text{NAD} \xrightarrow{\text{UDPG dehydrogenase}} \text{UDPGA} + 2\text{NADH}_2$$

$$\text{UDPGA} + \text{ROH} \xrightarrow{\text{UDP transglucuronylase}} \text{ROC}_6\text{H}_9\text{O}_6 + \text{UDP}$$

FIG d.1

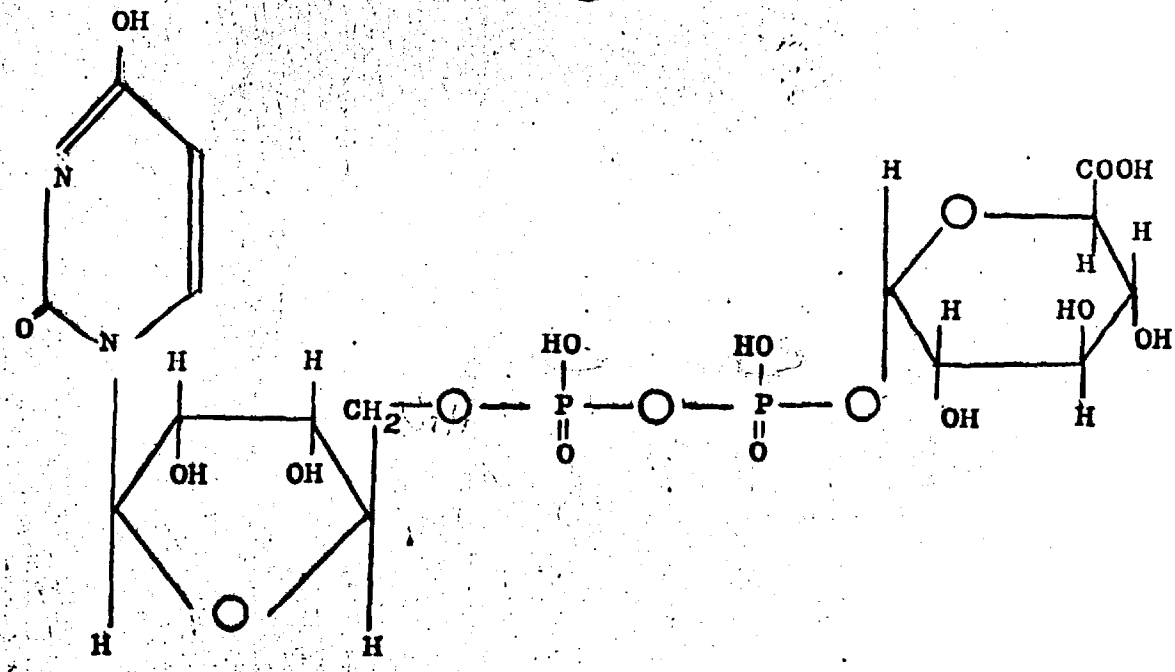
Conjugated Glucuronic Acid



In the majority of glucuronides, X in this formula is O others include

X = NH
X = S
R = Radicle from foreign compound

UDPGA



(UDPG = Uridine diphosphateα-D-glucose)

(UDPGA = Uridine diphosphateα-D-glucosiduronic acid)

($ROC_6H_9O_6$ = β-D-glucopyranosiduronic acid)

Glucuronides of β-D-glucosiduronic acids are formed when a molecule condenses with the C-1 atom of glucuronic acid. Glucuronide formation is one of the commonest conjugation mechanisms, presumably from the ready availability of glucuronic acid in the tissues and the many groups forming glucuronides. Glucuronide formation occurs in most mammals the most notable exception being the cat. It was shown with tissue slices, (Dutton and Grieg, 1957) also Hartiala,(1955) and in vivo (see Robinson and Williams,1958) that if glucuronide formation occurs it is at a very low level.

Cats excrete injected glucuronides so that the conjugation with glucuronic acid not excretion is defective (see Dutton, 1959; Dutton and Montgomery, 1958; Abou-El-Makarem, et al, 1967) UDPGA is plentiful in cat liver so that the enzymes responsible for the formation of UDP glucuronic acid are not deficient. It appears therefore that the defect lies with the transferase enzymes. Cat liver has a very low value when assayed for UDP-glucuronyl transferase activity, this could indicate a defect in the synthesis of the transferase. Attempts to

solubilise the enzyme have proved very difficult as it is intimately associated with the vesicular membranes of the endoplasmic reticulum. The best purification of UDP glucuronyl transferase so far described is that by Mowat and Arias, (1970). The purified enzyme was obtained from guinea-pig liver and was achieved by ultrasonication, sucrose gradient fractionation and sephradex G-200 elution. Electron microscopy studies showed that membranous fragments were always associated with active fractions. The purification increased the capacity to conjugate bilirubin in vitro twenty-fold and o-aminophenol fifty-fold.

This purification procedure was applied to cat and Gunn rat livers. Enzymatic activity was studied in the stages of the purification procedure as devised for guinea-pig liver and failed to show any change in the relative ability to conjugate the various potential substrates tested, nor was any substrate conjugated by the purified preparations if it had not been conjugated by the crude homogenate.

It is not possible at present to suggest whether synthesis, activity or both factors are responsible for the defect in glucuronide formation. There is at present an unresolved controversy concerning the possible multiple nature

of UDP-glucuronyl transferase. One school of thought is that the enzyme consists of a series of isoenzymes with differing, if overlapping, specificities towards various substrates (Dutton, 1971). This could be very important in cat, if there are a number of glucuronyl transferases then they might not all be defective. This would mean that some substrates might be conjugated with glucuronic acid in the cat.

However, as regards the cat which should be a key species for study concerning the multiplicity of the transferase we can only summarise observations on an assortment of foreign and endogenous substrates and exercise caution comparing substrates, tissues and other species. The other evidence derives from four main sources; studies on kinetics, activations, developmental rates and on mutants lacking specific transferase activities. The first three especially are difficult to interpret with an insoluble enzyme, and the mutant strains have been poorly characterised.

One distinguishable transferase forms N-glucuronides, Marsh,(1966) points out the chemical peculiarities of these conjugates. Liver slices from Gunn rats cannot synthesize a large number of O-glucuronides but form aniline N-glucuronide (Arias, 1961) Illing, et al., (1972) stated that although

differences exist between the transferase enzymes forming S- and O-glucuronides these are often no greater than the differences existing between the "O-" enzymes specific for varying phenolic substrates.

UDP glucuronyltransferase activities towards hydroxyl and carboxyl groups are not clearly divisible, although the acyl glucuronide of bilirubin probably requires a different transferase from those synthesizing phenolic glucuronides. Gunn rat appears to be unable to glucuronidate bilirubin whereas cat appears unable to conjugate most compounds other than bilirubin. There are confliciting reports on the ability of the Gunn rat to conjugate carboxyl and hydroxyl groups (Dutton, 1971). Cat forms no appreciable glucuronide of either benzoic acid (Bridges, et al., 1970) or phenol (French, 1970) so that if there is more than one transferase for these groups they both appear to be defective.

The Sulphate Defect in Pig

Probably because it is a less convenient animal to study, even less is known of the defect in sulphate conjugation in pig, than the defect in glucuronic acid conjugation in the cat. In terms of toxicity the cat is at a greater disadvantage than pig. Williams, (1938) stated that with increasing dose of

phenol, sulphate conjugation decreased and glucuronide conjugation increased. Extending this idea it seems likely that the glucuronide conjugation "copes" with greater concentrations of xenobiotics.

As was mentioned earlier the cofactor involved in the formation of sulphate esters contains adenosine. As seen in Fig d.2 the sulphate is transferred from adenosine-3'-phosphate-5'-phosphosulphate (PAPS) to the hydroxyl group of the xenobiotic by a sulphotransferase or sulphokinase. PAPS is formed from ATP. Several sulphokinases are known. Phenol sulphokinases are relatively non-specific but can be distinguished from another sulphokinase which forms oestrone ethereal sulphate (Roy, 1971). Steroid sulphokinases are restricted to the liver. The formation of aryl sulphamates requires a specific sulphokinase and transfers sulphates to such xenobiotics as aniline, 1- and 2-naphthylamine but not to others such as benzylamine or glucosamine (Roy and Trudinger, 1970).

It was mentioned earlier that sulphate was even more widespread than glucuronic acid conjugation. Coombs and Hele,(1927) and Stekol,(1936) however, reported sulphate conjugation to be at a very low level in the pig. It was also reported recently that the hamster could not form a

FIG 4.2

The Formation of Ethereal Sulphates

$$ATP + SO_4 \xrightleftharpoons{\text{ATP-sulphate adenyl transferase}} APS + P_2O_7$$

$$APS + ATP \xrightleftharpoons{\text{ATP-adenylyl sulphate 3-phosphotransferase}} PAPS + ADP$$

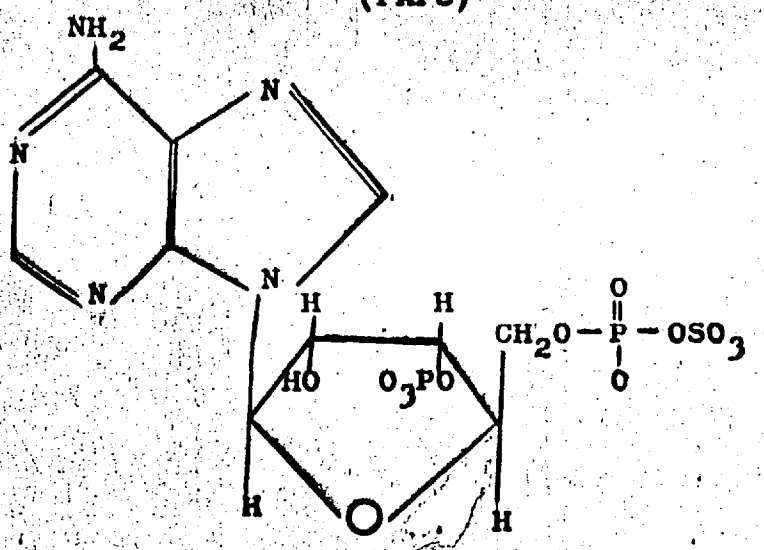
$$PAPS + ROH \xrightleftharpoons{\text{sulphotransferase}} ROSO_3H + ADP$$

APS = Adenosine-5 -phosphosulphate

$ROSO_3H$ = Sulphate ester

3 -phosphoadenosine-5 -phosphosulphate

(PAPS)



a sulphate conjugate of phenol (French, 1970). The Marsupial Opossum (Trichosurus vulpecula) has also been demonstrated to have little ability to form sulphate conjugates (Roy, 1963).

It is likely therefore that the defect in sulphate conjugation in pig, as with glucuronic acid conjugation in cat, is owing to a defect in the transferase enzyme. The sulphate activation systems are widespread in nature having been demonstrated in a variety of mammals, plants and invertebrates. Pig colonic mucosa was used to study the effect of vitamin A deficiency on sulphate activation (Wolf, et al., 1961). It is likely therefore that the pig has the ability to form PAPS. Furthermore, addition of PAPS to pig liver homogenates does not stimulate sulphation (French, 1970).

There are a number of substrate specific sulphotransferase enzymes (Roy, 1971). Enzymes specific for phenol, oestrone, other steroids, choline, arylamines, and mucopolysaccharides are known. In addition there are a number of enzymes which transfer sulphate ions to various endogenous compounds. The question lies with the specificity of these enzymes. As with the defective UDPGA transferase enzyme of cat the specificity is important, if all the transferase enzymes are not defective,

then there might be some compounds which are detoxicated by sulphate conjugation in the pig.

The phenol sulphotransferases are probably among the most studied group of enzymes but remarkably little information is available on the properties of the purified enzymes. Roy and Trudinger, (1970) suggest that, from the evidence available, it appears the transferase is probably ubiquitous but present in greatly differing amounts. How many individual phenol sulphotransferases occur in mammalian tissues, in particular liver, is not known. Banerjee and Roy, (1966) by chromatography on DEAE-Sephradex, separated three fractions showing phenol sulphotransferase activity from extracts of guinea-pig liver. One of these appears a true phenol sulphotransferase in that the enzyme will not catalyse the transfer of the sulphuryl group from PAPS to any other type of acceptor which has been investigated. The other phenol sulphotransferases are closely associated with steroid sulphotransferase and arylamine sulphotransferase respectively. The phenol sulphotransferase from guinea-pig liver was isolated, partly purified and its kinetics investigated by Banerjee and Roy, (1968). Their findings regarding the specificity of the phenol sulphotransferase are given in Table d.1

Table d.2

The values of Km and relative maximum velocities, V, for different substrates for the phenol sulphotransferase of guinea pig liver.

Substrate	Km	V
phenol	2.5	0.30
p-nitrophenol	0.070	1.00
1-naphthol	0.025	1.02
2-naphthol	0.025	0.95
5,6,7,8-tetrahydro-2-naphthol	0.056	0.63
4-nitro-1-naphthol	0.018	0.39
2-phenanthrol	0.017	0.51
15,16,dihydro-3 hydroxy-17-oxo-cyclopentena- a phenanthrene	0.015	0.13
equilin	0.020	0.21
equilenin	0.015	0.10
oestrone	0.015	0.00

figures from Roy and Trudinger, 1970

The conclusion from these data is that phenol sulphotransferase can sulphate a wide variety of phenolics. Tentatively extrapolating the result from guinea-pig to pig tissues, it seems likely that the defect in sulphate conjugation in pig ought to apply to all these compounds.

How the defect in the above transferase enzyme in cat and pig arose is uncertain, or how far back in the evolutionary history of the species it arose. This might be determined by the administration of suitable xenobiotics to related species. Unfortunately the other members of the felidae are unsuitable for metabolic studies. Pig is a member of the artiodactyla which includes deer, cattle and sheep, the close relatives however are more exotic wild pig species again unsuitable for metabolic studies. The in vitro studies performed on the livers of some artiodactyls, especially sheep suggest that the sulphate defect is not common to all artiodactyls applying only to pig.

The foregoing chapter has traced the nature of the conjugative defects of the cat and pig. In the following chapters experiments designed to elucidate and compare these defects are described.

CHAPTER 2

THE METABOLIC FATE OF PHENOL IN VARIOUS SPECIES

Contents

Tables and Figures

The following abbreviations will appear on the figures:-

O = Origin

SF = Solvent Front

cpm = Counts Per Minute

Introduction

Previous work on the metabolism of phenol (see Williams, 1959) has shown that its main metabolites are phenyl sulphate and phenyl glucuronide together with small amounts of conjugated quinol and lesser amounts of conjugated catechol (Parke and Williams, 1953). More recently the metabolism of phenol in 19 species was described by French (1970) - see Table 2.1. In the case of the cat no glucuronide conjugates were detected by radiochromatogram scanning and in the case of the pig no sulphate conjugates were detected. The hamster also did not appear to form any sulphate conjugates. Radioactive $[^{35}S]$ sulphate was administered to the hamster together with non-radioactive phenol and no radioactive phenylsulphate was detected in the urine.

Thus, it appeared that the cat was defective in conjugating phenol with glucuronic acid and the pig and hamster in conjugating phenol with sulphate. The metabolism of phenol in these three species was therefore further investigated in order to establish whether the above conjugation reactions were completely absent or occurred at a low level.

Materials and Methods

Chemicals

Phenol, m.p. 42°, catechol, m.p. 103°, resorcinol, m.p. 109° and quinol, m.p. 170°, were purchased and purified. Phenylglucuronide, m.p. 161-162° $[\alpha]_D^{17}$ - 78.5 (c, 2 in water) was a sample previously prepared in this laboratory (cf. Garton, et al., 1949). Potassium phenyl sulphate and potassium 4-hydroxyphenyl sulphate (quinol monosulphate) were prepared according to Garton,(1949). These sulphates were characterised (see Table 2.2) by colour reactions before and after hydrolysis with 2M -HCl and with an arylsulphatase preparation from Helix pomatia (Sigma Chemical Company, London). Urine (1 ml.) was heated with an equal quantity of 2M HCl for 15 min at 80-90°C in a water-bath. Under these conditions ethereal sulphates, but not glucuronides, are hydrolysed (see Garton et al., 1949). 4-hydroxyphenylglucuronide (quinol monoglucuronide) was obtained as a gum from the urine of rabbits fed with quinol according to Garton and Williams,(1948). The gum was purified by t.l.c. in solvent A (see Table 2.2) in which the glucuronide had an Rf value of 0.10. It was characterised by colour reactions (see Table 2.2) before and after hydrolysis

with a beef liver β-glucuronidase preparation (Ketodase; Warner-Chilcott Laboratories, Eastleigh, Hampshire).

[U-^{14}C] Phenol (266 μCi/mg) was purchased from The Radiochemical Centre, Amersham.

Animals

Details of animals used will be found in Appendix 1, the animals being obtained from dealers in the London area and maintained on appropriate diets. [^{14}C] Phenol, dissolved in water, was administered orally or intraperitoneally. For the collection of urine animals were placed in suitable metabolism cages and their urine was collected daily in receptacles containing a few ml of saturated aqueous $HgCl_2$ solution to prevent bacterial breakdown of conjugates.

Chromatography

The Rf values and colour reactions of phenol and its metabolites on paper and t.l.c. are given in Table 2.2. For radiochromatogram scanning the urine (0.1 - 0.5 ml) was placed as a band on strips (5 cm wide) of Whatman No. 1 paper and developed with the solvents listed in Table 2.2. The paper was then scanned in a Packard radiochromatogram scanner, model 7200.

Thin layer chromatography was carried out with fluorescent silica gel G (silica gel HF_{254}, Merck, Anderman & Co. Ltd., London), 0.1 mm layers being used for detection and 0.8 mm for preparative work.

Radiochemical Techniques

^{14}C radioactivity was measured in a Tri-Carb Scintillation Spectrometer (Model 3214) manufactured by the Packard Instrument Company. Two scintillator systems were used; the "Dioxan" system consists of 60g. naphthalene, 4g. PPO = 2, 5 diphenyloxazole, 200 mg. POPOP = 1, 4-di[2-(5-phenyloxazolyl)] benzene, 100 ml. methanol and 20 ml of ethane -1, 2-diol made up to one litre with dioxan. The "Gel" system is a 5% suspension of Cab-O-Sil, a thixotropic gelling agent supplied by the Packard Instrument Company, in "Dioxan" scintillator.

Samples. Urine samples (0.5 - 1.0 ml) were counted in quadruplicate in the dioxan scintillator fluid (15 ml). In some experiments the paper strip chromatograms were cut into 1 cm pieces and placed in dioxan scintillation fluid and the associated ^{14}C was determined.

Counting Procedure. All samples were counted at $0^{o}C$ and recounted several days later to avoid possible errors due to chemiluminescence. The "Channel Ratio" method (Bridges, et al., 1967) was used to determine the counting efficiencies of samples. Knowing the ratio of the counts given by a sample in the two channels of the scintillation counter, the counting efficiency and hence the absolute activity in disintegrations/minute of the sample, can be determined. A counting time of 10 minutes was usually sufficient to give a suitable number of counts for estimation by the channel ratio method.

Identification and Estimation of Metabolites

The metabolites were identified by radiochromatogram scanning of the urine and the Rf of the ^{14}C peaks compared with those of authentic reference compounds (see Table 2.2).

The intergrator incorporated into the radiochromatogram scanner gave an approximate value of the relative sizes of the peaks of activity (corresponding to the various metabolites of phenol) occurring on the trace. This method was not sufficiently accurate when comparing minor metabolites with with major ones. Furthermore metabolites present in quantities of less than two percent of the activity banded onto the strips

could not be detected amongst the background trace of the radiochromatogram scanner. Cutting the strips into 1 cm pieces and estimating the associated ^{14}C by scintillation counting solved both these problems. Thus accurate percentage values for the metabolites could be calculated. When sufficient material is banded onto the chromatography paper minor metabolites present in quantities as low as 0.1% of the total activity can be detected after cutting up the chromatogram and counting the associated ^{14}C as described above. The Rf values can then be determined by constructing histograms of the distribution of the ^{14}C along the strip. The histograms can be compared with the radiochromatogram scans.

The results shown in the tables are the mean values for three animals in 3 chromatography solvents. Results with the same specimens in different solvents did not normally vary by more than five percent. The chromatograms and histograms are typical photographs of the results obtained.

Results and discussion

From radiochromatogram scanning it appears that in the pig there is only one metabolite, namely, phenylglucuronide, whereas in the cat there is no glucuronide conjugate; phenol

being excreted mainly as phenylsulphate together with smaller quantities of quinol sulphate (see Fig 2.1). This therefore agrees with the work of French (see Table 2.1).

The paper chromatograms were then cut into 1 cm pieces and the ^{14}C on each piece estimated by counting in the dioxan scintillator. The distribution of the ^{14}C along the strip was then plotted as a histogram (see Fig 2.3). In the case of the cat a small amount of radioactivity corresponding to phenylglucuronide and in the case of the pig a small peak corresponding to phenylsulphate was detected. In each case the minor metabolite was separate from the main radioactivity peak and had an Rf corresponding to authentic samples. The minor metabolite was further identified by specific enzyme hydrolysis (see above).

Cat

In the cat the major urinary metabolite of phenol given orally or intraperitoneally at a dose of 5 or 25 mg/Kg is phenylsulphate. There is however a small but definite excretion of phenylglucuronide which is less than about one percent of the ^{14}C in the urine (see Fig 2.2). Quinol glucuronide was detected in the urine of one cat only in very small amounts

(0.1% of the ^{14}C in the urine). The cat also excretes appreciable amounts of quinol sulphate, twice as much being excreted after the intraperitoneal injection of phenol than after the oral administration of the compound. This may be a reflection of the ability of the intestinal tissue of the cat to conjugate phenol given orally. If phenol is partly conjugated during absorption from the intestine, then less free phenol is available for oxidation to quinol.

Pig

In the pig the major metabolite of phenol is phenylglucuronide, with only traces of quinol glucuronide being formed. Table 2.3 shows, however, that sulphate conjugation is not entirely absent, but occurs at a very low level (see also Fig. 2.2). A very low dose (1.5 μg/Kg) of phenol was administered to pigs to determine whether sulphate conjugation was greater with lower doses of phenol. The results in Table 2.3 illustrate that the excretory pattern of phenol in the pig is similar at 1.5 μg and 25 mg/Kg. It was interesting to note that the pig had little ability to oxidise phenol to quinol at these dose levels.

The cat and pig therefore appear to have defective glucuronic acid and sulphate conjugation mechanisms respectively for phenol. However these conjugations are not entirely absent but occur at very low levels with this compound.

Hamster

The hamster had been reported by French,(1970) to be unable to form sulphate conjugates of phenol (see Table 2.1). Phenol was therefore administered to three female hamsters and the metabolites detected by chromatography using the solvents given in Table 2.2. The results are summarised in Table 2.4 and are the mean of values obtained in 3 solvents. Phenylsulphate was detected and further characterised by specific enzymic hydrolysis using a sulphatase preparation and also by acid hydrolysis (see above). Chromatography and radiochromatogram scanning revealed that the hydrolysis had removed the radioactivity peak ascribed to phenyl sulphate but not that associated with phenyl glucuronide. Phenol was administered both orally and intraperitoneally to hamsters at two dose levels and in all cases approximately twenty-five percent of the dose was recovered as phenylsulphate. It was concluded that the hamster was able to conjugate phenol with sulphate, which is in contrast with the findings of French,(1970)

The total urinary recovery of ^{14}C quoted by French (approx. 47% see Table 2.1) was lower than in this study (85%). It is possible that in French's experiments bacterial contamination from faeces of the urine resulted in deconjugation of the phenol metabolites. If this occurred then the major urinary metabolite, i.e. phenyl glucuronide, might appear to be the only conjugate present especially if the urine contained only relatively low levels of ^{14}C. French did not detect any free phenol in the urine of hamsters administered the compound. If, in his experiments, phenyl sulphate was converted to free phenol by the action of bacteria contaminating the urine then the urine would contain free $[^{14}C]$ phenol. This $[^{14}C]$ phenol was not detected by radiochromatogram scanning and this may have been due to the phenol vaporizing when the urine being applied to the paper was dried. French was unable to demonstrate phenyl sulphate formation in hamster using $[^{35}S]$ sulphate. However, the $[^{35}S]$ labelled sulphate and cold phenol were administered simultaneously and it is possible that the phenol was excreted before the $[^{35}S]$ was available for conjugation.

SUMMARY

1) The metabolism of [^{14}C] phenol has been described in the cat, pig and hamster.

2) The cat was found to conjugate phenol mainly with sulphate forming only traces of the glucuronide. The pig conjugated phenol almost exclusively with glucuronide only traces of phenyl sulphate being detected.

3) The hamster was found not to be defective in sulphate conjugation.

Table 2-1 Urinary metabolites of [^{14}C]phenol in various species

[^{14}C]Phenol in water was administered by stomach syringe or tube except in the case of the primates, carnivores and pigs, when it was given incorporated in the food or drink. Urine was collected for 24 h and chromatographed (see text). Radiochromatograms were prepared and the areas corresponding to peaks were cut out and their ^{14}C determined by scintillation counting. Where 3 or more animals were used averages are given with ranges in parentheses. Individual values are given where only one or two animals were used. tr. means < 1% of urinary ^{14}C; — means no ^{14}C peak was detected by radiochromatogram scanning. m male; f female

Species (No., sex and trivial name)	Family or other description	Dose: Phenol (mg/kg)	Dose: ^{14}C (μCi/animal)	^{14}C excreted in 24 h (% dose)	%^{14}C excreted in 24 h found as: Phenyl sulphate	Quinol sulphate	Phenyl glucuronide	Quinol glucuronide	Total sulphate	Total glucuronide	Total quinol
Primates											
Man (3 m)	Homo	0·01	2·7	90(85–98)	77(69–90)	1†	16(4–23)	tr	78	16	1
Rhesus monkey (2 f)	Macaca	50	10	37, 49	60, 70	—	40, 30	—	65	35	—
Squirrel monkey (3 f)	Saimiri	25	8	31(18–52)	7(2–17)	—	68(55–86)	25(11–36)	7	93	25
Capuchin (1 f)	Cebus	25	8	73	14	—	65	21	14	86	21
Carnivores											
Ferret (3 f; mongrel)	Mustelidae	25	5	51(49–54)	28(19–34)	30(21–34)	40(22–52)	—	58	40	30
Cat (3 f; mongrel)	Felidae	25	8	59(52–61)	87(86–89)	13(11–14)	—	—	100	—	13
Dog (1 f, 1 m; mongrel)	Canidae	25	10	53, 62	33, 68	43, 20	24, 12	—	82	18	32
Miscellaneous											
Pig (3 f; large White)	Suidae	21	10	51(18–72)	—	—	100	—	—	100	—
Hedgehog (2 m; European)	Erinaceidae	20	7	34, 43	63, 86	17, 4	20, 10	—	85	15	10
Fruitbat (2 f; Indian)	Pteropidae	25	5	50, 58	9, 11	—	91, 89	—	10	90	—
Rabbit (3 f; New Zealand white)	Leporidae	25	10	48(35–60)	45(41–48)	9(7–13)	46(39–52)	—	54	46	9
Chicken (3 f; Rhode Island red)	Phasianidae	25	10	57(53–60)	78(57–91)	—	22(9–43)	—	78	22	—
Rodents											
Rat (3 f; Wistar albino)	Muridae	25	5	95(91–100)	54(44–65)	1(0–2)	42(35–55)	2(0–5)	55	44	3
Mouse* (3 × 10 f; ICI)	Muridae	25	2·5	66(64–68)	46(40–51)	5(3–8)	35(33–37)	15(12–17)	51	50	20
Jerboa (3 f; Egyptian)	Dipodidae	25	6·5	47(28–65)	61(51–71)	12(9–15)	26(20–33)	1(0–4)	73	27	13
Gerbil (2 f; 1 m)	Cricetidae	25	3	55(45–68)	42(22–53)	19(13–23)	35(30–49)	1(0–4)	61	36	20
Hamster (3 f; golden)	Cricetidae	25	5	47(41–59)	—	—	68(67–70)	32(30–33)	—	100	32
Lemming (3 f; steppe)	Cricetidae	25	5·6	40(15–67)	35(18–51)	10(8–12)	39(28–48)	15(10–23)	45	54	25
Guinea-pig (2 f; English)	Caviidae	25	2·7	64, 64	13, 22	tr, tr	82, 73	5, 5	18	82	5

*The urines of 10 mice were pooled for each determination. †One subject only.

(Figures taken from French, 1970).

Table 2.2 Chromatography of Phenol and its Metabolites

For paper chromatography, Whatman No. 1 paper and the descending technique were used; for t.l.c plates were prepared with fluorescent silica gel (see text). Solvents, paper chromatography: A, propan-1-ol- ammonia soln. (s.g.0.88)(7:3 by vol.); B, butan-1-ol-ammonia soln. (s.g. 0.88)-water (10:1:1); C, butan-1-ol-benzene-pyridine-water (5:1:3:2); t.l.c.: D, benzene-methanol-acetic acid (45:8:4); E, benzene-dioxan-acetic acid (90:25:4). Detecting reagents: freshly diazotized p-nitroaniline followed by 0.5 M-KOH in ethanol. $FeCl_3$ + ferricyanide: chromatograms were dipped in a mixture of equal vol. of $FeCl_3,6H_2O$(0.3% w/v) and $K_3Fe(CN)_6$(0.3% w/v) in water, then in 2 M-HCl and then water. Gibbs reagent is a spray of ethanolic 2,6-dichloroquinonechloroimide (1%) followed by saturated $NaHCO_3$. HCl then Gibbs reagent: the paper was sprayed with 5 M-HCl, dried and then sprayed with Gibbs reagent and $NaHCO_3$. Naphtharesorcinol is a spray of naphtharesorcinol (1% w/v) in aq. trichloroacetic acid (33% w/v) followed by heating the paper at 120° for 5-10 min. Phenols on the t.l.c. plates were detected as dark spots on illumination with u.v. light (254 nm) from a Hanovia Chromatolite lamp, s=streaks; -, not tested.

	Rf values in solvents					Colour reactions on paper				
	Paper		Thin-layer			Diazotized	$FeCl_3$ +	Gibbs	HCl then	Naphth
Compound	A	B	C	D	E	p-nitroaniline	ferri-cyanide	reagent	Gibbs reagent	resor inol
Phenol	0.92	0.94	0.97	0.68	0.82	Yellow red	Blue	Blue	Blue	None
Catechol	s	s	0.90	0.54	0.62	Yellow red	Blue	Blue	Blue	None
Resorcinol	-	-	-	0.45	0.55	-	-	-	-	-
Quinol	s	s	0.89	0.42	0.49	Red‡	Blue	-	-	-
Phenyl sulphate	0.75	0.52	0.60	-	-	None‡	None	None	Blue	None
4-Hydroxyphenyl sulphate	0.55	0.25	0.55	-	-	Yellow red	Blue	Blue	Blue	None
Phenylglucuronide	0.45	0.10	0.25	-	-	None	None	None	-	Blue
4-Hydroxyphenyl-glucuronide	0.20	0.00	0.20	-	-	Yellow red	Blue	Blue	-	Blue

‡Turns yellow red after 5-10 min

Table 2.3 Urinary Metabolites of [^{14}C] Phenol in the Cat and Pig

Four fully grown mongrel cats were used, A, B and D being females and C, a male. Five pigs (large White 11-22 weeks old; 18-40 kg.body wt.) were used. Nos. 1, 2, 3 and 5 being females and No. 4 a male. [^{14}C] Phenol was administered either orally mixed with food or in water by i.p. injection. Urine was collected for 24 h and chromatographed on Whatman No.1 paper using solvent system C (see table 1) for cat urine and A for pig urine. The radiochromatograms were cut into suitable pieces then counted (see text). For cat average values are given with the range in parentheses. In column 5, the ^{14}C output for each cat is given in the order found in column 1. The values for pigs are in the order given in column 1; - means <0.1% of the ^{14}C in the urine.

Animals	Dose Phenol (mg/kg)	Dose ^{14}C (μCi/animal)	Route of administration ‡	^{14}C excreted in 24 h (% dose)	% ^{14}C excreted in 24 h found as Phenyl Sulphate	Quinol Sulphate	Phenyl Glucuronide	Quinol Glucuronide
Cats ≠								
B, D, A	5	10	o	57(35,57,78)	93(89-95)	6.1(4.0-9)	1.4(1.0-2.0)	-
B, C, B	25	10	o	49(23,60,65)	88(81-98)	9.3(1.2-15)	0.2(0.1-0.3)	0.1*
B, A, A	5	10	i.p.	79(66,85,86)	85(81-89)	10(8.2-14)	0.3(0.1-0.5)	-
B, B, A	25	10	i.p.	63(49,69,72)	67(64-69)	21(20-21)	0.2(0.1-0.3)	-
Pigs								
5, 3	5	10	o	60,65	0.4,0.3	-	99,99	-
4, 3	25	10	o	76,95	3.6,1.2	-	96,99	-
1, 2	5	5	i.p.	89,97	3.3,0.2	-	93,96	3.8,3.4
5, 4	25	10	i.p.	79,115	4.3,8.3	-	96,92	-
3, 5, 4	1.5μg/Kg	10	i.p.	68(70,58,77)	6(4-8)	-	87(82-89)	7(5-9)

≠ With some cat urine some unidentified ^{14}C-containing material remains at the origin of the chromatograms. This accounted for 5 and 12% respectively of the ^{14}C excreted after intraperitoneal doses of 5 and 25 mg/kg and 0.2 and 2.5% for the same doses given orally.

‡ o = oral; i.p. = intraperitoneal

* [^{14}C] Quinol glucuronide was found in the urine of the male cat (C) only.

Table 2.4 Metabolism of [^{14}C] Phenol in Hamster

Three female Golden hamsters (140 - 180 g. body wt.) were used. [^{14}C] Phenol was administered orally by stomach tube or injected intraperitoneally. Urine was collected for 24 h. and chromatographed on Whatman No. 1 paper using solvents A, B and C. Average values are given with, in parentheses, the value for each animal.

Phenol mg/kg	^{14}C (μCi/animal)	Route of admin.+	^{14}C excreted in 24 h	% ^{14}C excreted in 24 h as - Phenyl Sulphate	Quinol Sulphate	Phenyl Glucuronide	Quinol Glucuronide
5	5	i.p.	68(64,70,69)	27(36,16,29)	2.7(0.1,0.1,7.8)	42(35,51,39)	28/27,38,28)
25	5	i.p.	97(102,95,93)	27(27,26,29)	0.8(0.1,0.1,2.2)	50(50,48,50)	22(24,24,19)
5	5	p.o.	91(98,93,82)	23(21,26,21)	-	47(45,49,48)	28(34,25,26)
25	5	p.o.	82(78,83,85)	24(24,27,21)	-	50(50,44,56)	26(27,28,22)

+i.p. = intraperitoneal
p.o. = oral

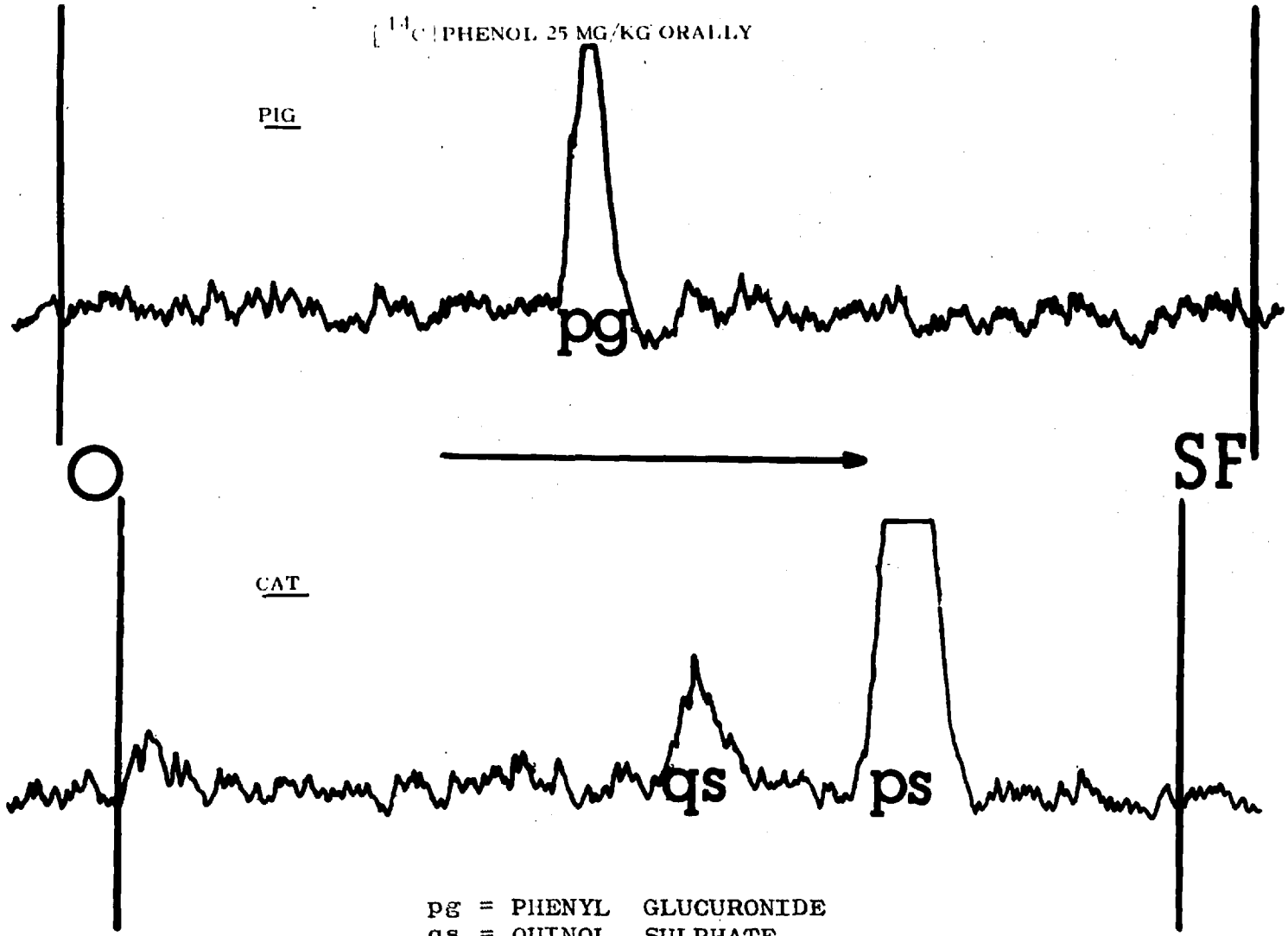
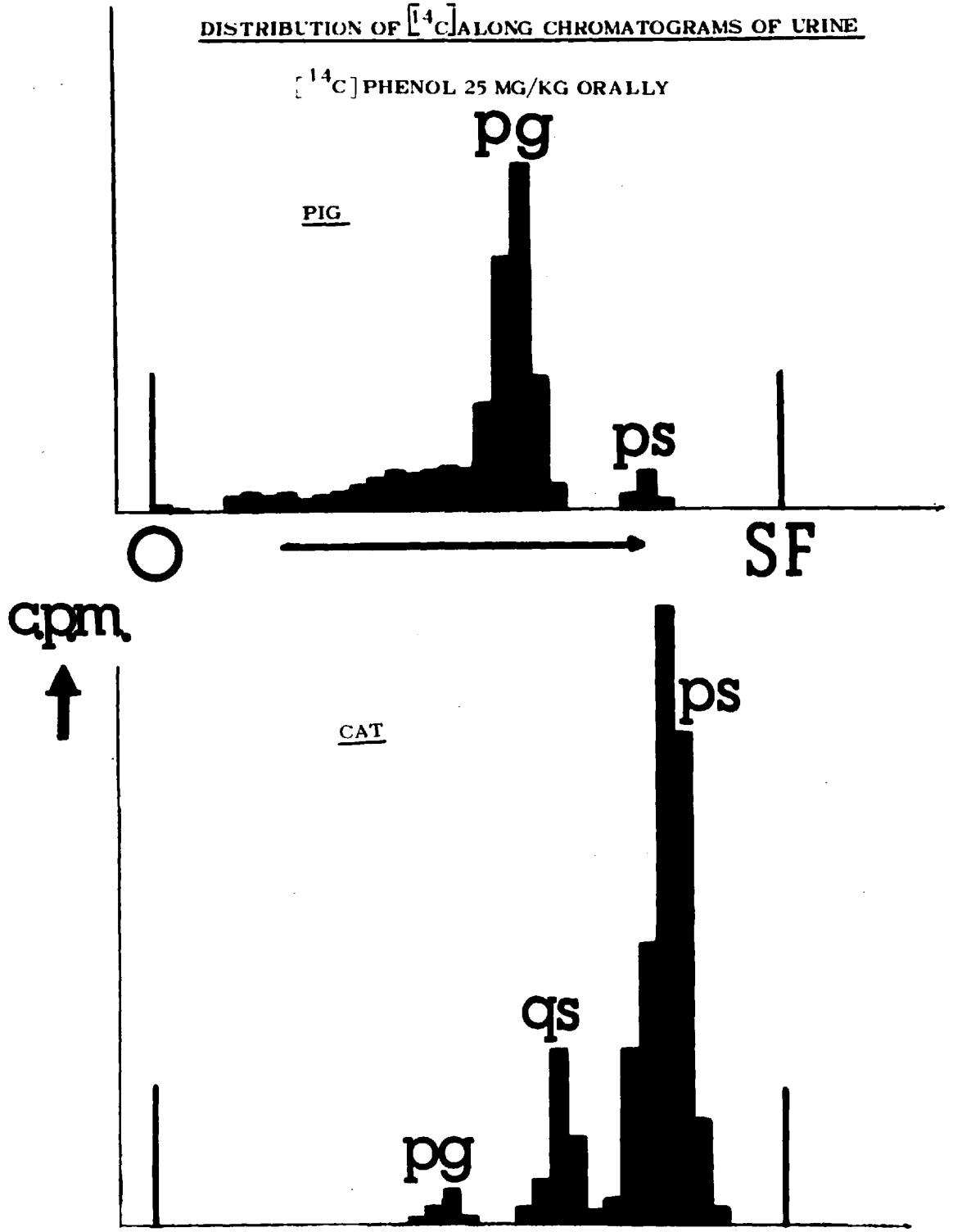

RADIOCHROMATOGRAM SCANS OF URINE
[14C] PHENOL 25 MG/KG ORALLY
PIG
pg
O
SF
CAT
qs
ps
pg = PHENYL GLUCURONIDE
qs = QUINOL SULPHATE
ps = PHENYL SULPHATE

FIG 2-1

FIG 2-2


DISTRIBUTION OF [14C] ALONG CHROMATOGRAMS OF URINE
[14C] PHENOL 25 MG/KG ORALLY
pg
PIG
ps
O
SF
cpm
CAT
ps
qs
pg

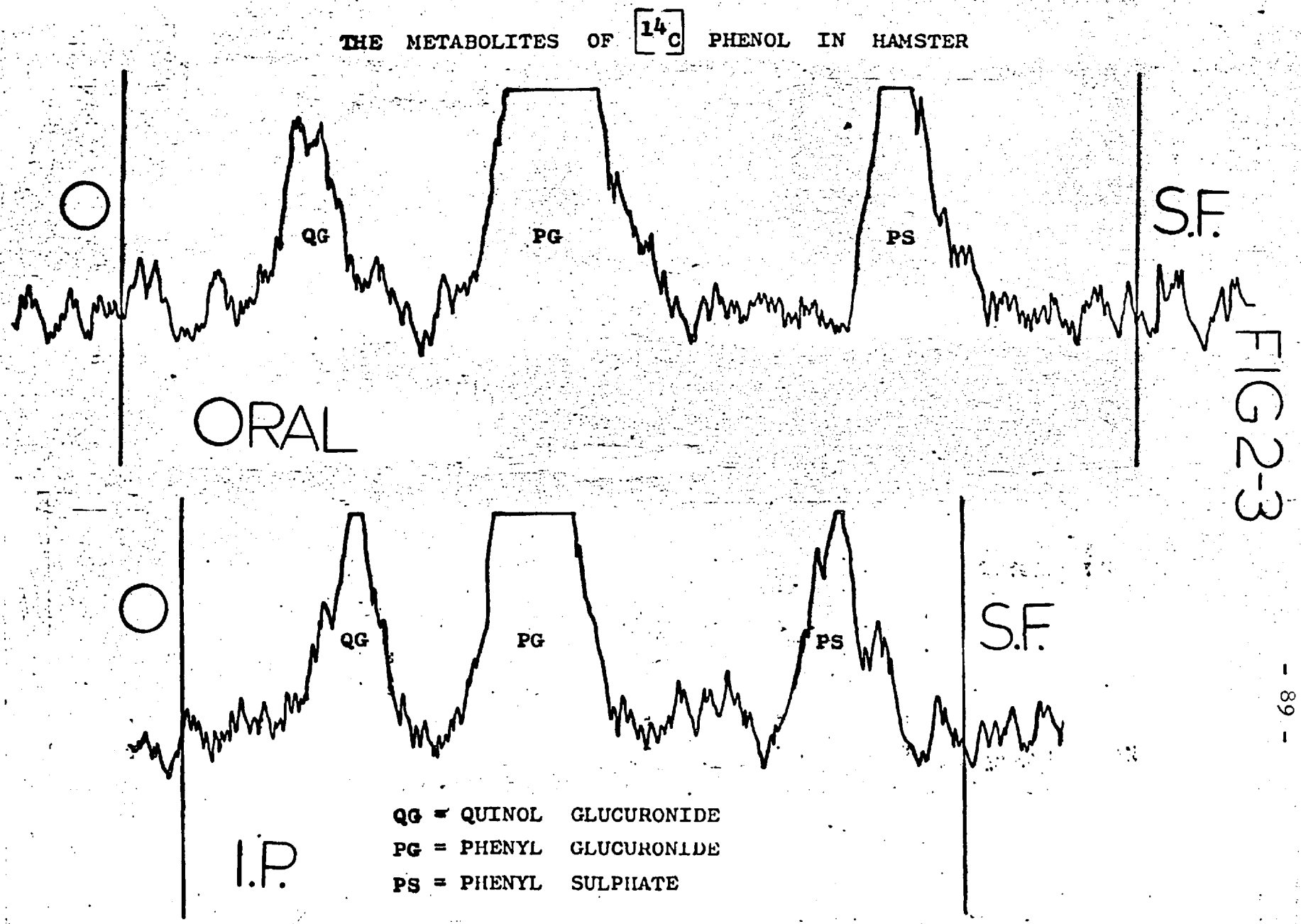

THE METABOLITES OF [14C] PHENOL IN HAMSTER
O
QG
PG
PS
S.F.
ORAL
O
QG
PG
PS
S.F.
I.P.
QG = QUINOL GLUCURONIDE
PG = PHENYL GLUCURONIDE
PS = PHENYL SULPHATE

FIG 23

CHAPTER THREE

THE METABOLISM OF 1- AND 2-NAPHTHOL IN THE CAT AND PIG

Contents

Tables and Figures

Introduction

In order to investigate the substrate specificity in these two species with defects in conjugation mechanisms a number of low molecular weight phenols (whose conjugates have a molecular-weight of less than 300) were administered to cat and pig. As stated in Chapter One there is possibly a family of UDPGA transferases, and also phenol sulphotransferases. By administering structurally-differing potential substrates to these two species some active transferase isoenzymes might be invoked. Among the compounds chosen were 1- and 2-naphthol to determine whether the presence of another aromatic ring might require an alternative enzyme to that used for phenol for conjugation. The metabolism of 1- and 2-naphthol is discussed in this chapter and that of a number of other phenols in the following chapter.

1- and 2-naphthol undergo the typical metabolism of phenols in most species studied and are conjugated with either glucuronic acid or sulphate in mammals. Thus, rats excrete about 50-60% of a subcutaneous dose (0.1g/rat) of 1- or 2-naphth as 1- or 2-naphthylglucuronide and about 20% as 1- or 2-naphthyl sulphate respectively and these conjugates have been isolated from rat urine (Berenbom and Young, 1951). It has been

reported that both naphthols are excreted mainly as the glucuronides by the dog (Lesnik and Nencki, 1886). Rabbits metabolize 1-naphthol to the glucuronide, for this conjugate has been isolated from rabbit urine after dosing with the phenol (Smith & Turbert, 1964).

Materials and Methods

Chemicals

[1-^{14}C] 1-Naphthol (106 μCi/mg.) (Radiochemical Centre, Amersham, Bucks) 1-naphthol, m.p. 94° and 1-naphthyl β-D-glucuronide, sodium salt, (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) were purchased. Potassium 1-naphthyl sulphate was synthesised by sulphating 1-naphthol with chlorosulphonic acid in pyridine (method of Feigenbaum and Neuberg, 1941). This conjugate ran as one spot on chromatography in 3 solvent systems (see Table 3.1) and gave a positive test for a free aromatic -OH group only after acid hydrolysis (see Table 3.1). The Rf value of the sulphate in solvent A was the same as that quoted by Binning *et al.*, (1967).

[8-^{14}C] 2-Naphthol (104 μCi/mg) (Schwartz-Mann, New York, U.S.A.) and 2-Naphthyl glucuronide, sodium salt (Koch-Light Laboratories Ltd., Colnbrook, Bucks) were purchased. 2-Naphthol (May & Baker Ltd., Dagenham, Essex) was purified

by recrystallisation from aqueous ethanol, when it gave a melting point of 121°-123°. Potassium 2-Naphthyl Sulphate was synthesised and characterised in a manner similar to 1-naphthyl sulphate (see above) according to Feigenbaum and Neuberg (1941). The Rf value of the sulphate in solvent D was similar to that quoted by Binning, et a., (1967) 1,3-dihydroxynaphthalene (naphthoresorcinol) m.p. 124° and 2, 7-dihydroxynaphthalene m.p. 192° was obtained from British Drug Houses, Poole, Dorset: 2, 3-Dihydroxynaphthalene m.p. 164° and 2, 6-dihydroxynaphthalene m.p. 220° from Koch-Light Laboratories, Colnbrook, Bucks., U.K. 1, 6-dihydroxynaphthalene m.p. 138° and 1, 7-dihydroxynaphthalene m.p. 179° from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Uridine diphosphate glucuronic acid (UDPGA: Sigma Chemical Company, London) and adenosine triphosphate (ATP: British Drug Houses Ltd. Poole, Dorset) were purchased.

Animals

The cats and pigs (see Appendix 1) were obtained from animal dealers in the London area and were maintained on appropriate diets. [1-^{14}C] 1-Naphthol dissolved in water (10 μCi/ml) was administered (1 ml per animal) intraperitoneally together with sufficient carrier naphthol dissolved in ethanol (0.5 ml/animal) to give a dose level of 1-naphthol of

25 mg/kg. [8-^{14}C] 2-Naphthol was similarly administered. Female Wistar Albino rats (190-210 g. body weight) received a similar dose of 1- and 2-naphthol. The radioactive compound was dissolved in water (10 μCi/ml) and was administered (0.5 ml/rat) intraperitoneally, together with sufficient carrier naphthol dissolved in aqueous ethanol (1/1, v/v 0.5 ml per rat) to give a dose level of 25 mg/kg.

Chromatography

For chromatography, Whatman No. 1 paper (5 cm strip) and the descending technique were employed. The Rf values and colour reactions of 1- and 2-naphthol and their metabolites are given in Tables 3.1 and 3.2.

Radiochemical Techniques

Estimation of ^{14}C in urine samples and radiochromatogram scanning were carried out as described for [^{14}C] phenol in Chapter Two. In some experiments the paper strip chromatograms were cut into 1 cm pieces and placed in dioxan scintillation fluid and the associated ^{14}C determined.

Incubation Techniques for Liver and Kidney Samples In Vitro

Preparation of Homogenates. Liver samples (10g cooled on ice) from several male and female rats (Wistar albino) and

male and female pigs (Large White), removed immediately after killing, were homogenized at 5° in a Waring blender for 30 s. A Potter-Elvjeim homogeniser was then employed to make up a 10% homogenate in a medium consisting of 0.154 M-KCl (80 ml), and 0.1 M-sodium phosphate buffer, pH 6.8 (20 ml). The sub-mitochondrial fraction of the homogenate was prepared by centrifuging in an MSE "high speed"17" refrigerated centrifuge at 10,000 (G av.) for 10 min and part of this 10,000 (G av.) supernatant was used for investigating glucuronide synthesis. The remaining sub-mitochondrial fraction was centrifuged at 100,000 (G av.) for 1 h in an MSE Automatic "Superspeed 40" centrifuge to sediment the microsomal fraction and the supernatant (soluble fraction) was used for investigation of ester sulphate synthesis. De Meio, et al., (1953) have reported that the microsomal fraction inhibits the synthesis of arylsulphates.

Samples (10g.) of kidney were also taken from 3 different pigs and homogenates prepared and incubations conducted in a manner identical with that used for the liver. The conjugating ability of the pig kidney towards 1- and 2-naphthol could thus be assessed and compared with that of the liver.

Incubations

(a) For investigations of glucuronide synthesis, the incubates in duplicate consisted of the 10,000 (G av.) supernatant (2 ml); aqueous [1-^{14}C] 1-naphthol or [8-^{14}C] 2-naphthol solution (0.05 ml 5μCi/incubate); 0.2 ml of carrier 1- or 2-naphthol solutions of sufficient concentration to give a range of incubation mixtures of substrate concentration 30-70 μg/incubate; 0.05 ml of 0.154 M $MgSO_4$ solution; and UDPGA soln. (0.5 ml; 10 mg/ml). Suitable controls in which the UDPGA was omitted were also prepared. The mixtures were then incubated in glass-stoppered tubes for 20 min at 37° in a shaking water-bath (H. Mickle, Gomshall, Surrey). At the end of the incubation UDPGA soln., 0.5 ml as above, was added to the control incubates, and the reaction in all the tubes terminated by adding 10% aqueous sodium tungstate (0.5 ml). The incubates were adjusted to 5 ml with water and then centrifuged at 2,000 rev./min for 10 min in the MSE Minor centrifuge to remove precipitated protein.

(b) For the investigation of sulphate synthesis, the incubates (duplicates) consisted of the 100,000 (G av.) supernatant (2 ml); aqueous [1-^{14}C] 1-naphthol or [8-^{14}C] 2-naphthol

solution (0.05 ml 5μCi/incubate); 0.2 ml of carrier 1- or 2-naphthol solutions of sufficient concentration to give a range of incubation mixtures of substrate concentration 30-70) μg/incubate; 0.05 ml or 0.154 M $MgSO_4$ soln.; and an aq.soln. of ATP (0.2 ml; 31.2 mg/ml). In the controls the ATP was omitted. The mixtures were incubated in glass-stoppered tubes in a shaking water-bath for 1 h at 37°. At the end of the incubation, ATP as above was added to the controls and then all the incubation mixtures were treated with 10% aq. sodium tungstate (0.5 ml), diluted to 5 ml. with water and centrifuged as above to remove protein.

Samples (0.1 ml) of the protein-free incubates were chromatographed as bands on 5 cm paper strips (Whatman No.1) in solvents A and B, (Table 4.1) and scanned for ^{14}C. 1- and 2-Naphthol and their conjugates were detected using reference samples of these compounds which were chromatographed simultaneously. The conversion of 1- and 2- naphthol to the sulphate or glucuronide conjugates was estimated by cutting out the areas of the chromatograms corresponding to the ^{14}C labelled peaks and counting them in dioxan scintillator.

Results and Discussion

The *in vivo* metabolism of both 1- and 2-naphthol was studied in the cat, pig and rat.

Rat

In the case of the rat dosed with 1- and 2-naphthol, almost equal quantities of sulphate and glucuronide conjugates are formed, (see Tables 3.3, 3.4, and the radiochromatogram shown in Fig 3.3). This is comparable with results obtained when phenol is administered to the rat (see Table 2.1). It is interesting to note that very little oxidation to dihydroxynaphthalene(s) occurs, presumably owing to the rapid rate of conjugation of 1- and 2-naphthol in the liver.

Cat

When [1-^{14}C] 1-naphthol was administered to cat only one radioactive peak was clearly detected by radiochromatogram scanning (see Fig 3.2). This was identified as 1-naphthyl sulphate so that unlike phenol (see Chapter Two) 1-naphthol was not appreciably oxidised by enzymes of the liver. The radiochromatograms were cut into 1 cm pieces and the total ^{14}C distribution along the strip estimated as in the case of the phenol studies. A small, but clearly defined, peak of

radioactivity of Rf value corresponding to 1-naphthyl glucuronide was detected (see histogram shown in Fig 3.4). This was not present on chromatograms of cat urine incubated with ketodase (β-glucuronidase) except when the incubation was carried out in the presence of saccharo 1:4 lactone, which specifically inhibits this enzyme.

When [8-^{14}C] 2-naphthol was administered to the cat two ^{14}C labelled peaks were observed in the urine by radiochromatogram scanning (see Fig 3.3) and both were hydrolysed by a sulphatase preparation from Helix pomatia and also by dilute acid in the manner described by Garton, et al., (1949). It was concluded that each peak was a sulphate conjugate. Furthermore, the larger one corresponded in Rf value to 2-naphthyl sulphate. Following the enzymic hydrolysis two ^{14}C peaks were observed on radiochromatogram scanning, one of which was 2-naphthol and the other appeared to be a dihydroxynaphthalene. It was not possible to identify with certainty which isomer of dihydroxynaphthalene was present although 2, 7-dihydroxynaphthalene seems to be the most likely candidate from a comparison of its Rf values with those of the unknown (see Table 3.2). We see, therefore, that cat hydroxylates 2-naphthol to form a dihydroxynaphthalene,

this being excreted as a sulphate conjugate. When the chromatograms were cut up to determine the % of metabolites present a small but distinct amount of ^{14}C which corresponded in Rf value with 2-naphthyl glucuronide was observed, (see Fig 3.5). This could be removed by Ketodase but the hydrolysis was inhibited by Saccharo 1:4, lactone. It was concluded that this metabolite was 2-naphthyl glucuronide. Thus cat metabolises 2-naphthol in a similar fashion to phenol.

Pig

When 1-naphthol was administered to pigs two radioactivity peaks were detected on radiochromatogram scanning of the urine (see Fig 3.2). One of these peaks had an Rf value corresponding to 1-naphthyl glucuronide and the other, which accounted for approximately one-third of the radioactivity on the chromatogram, had an Rf value similar to that of 1-naphthyl sulphate. The identity of these metabolites was confirmed by acid and enzyme hydrolyses; 1-naphthyl sulphate was attacked by sulphatase and not Ketodase, and it was hydrolysed to free 1-naphthol on heating with dilute acid as described by Garton, et al., (1949). The peak ascribed to 1-naphthyl glucuronide was hydrolysed by Ketodase and sulphatase (which contained β-glucuronidase as impurity). This peak was not attacked by

acid under the conditions which removed the sulphate peak completely from radiochromatogram scans. The chromatograms were cut up as before and the percentages of the metabolites present accurately determined (see Table 3.4). It was found that pig excreted approximately one-third of a dose of 1-naphthol as 1-naphthyl sulphate and thus does not have a defective sulphate conjugation mechanism for this substrate.

The pig metabolised 2-naphthol in a manner similar to phenol the compound being excreted almost exclusively as 2-naphthyl glucuronide (see Fig 3.2 and Table 3.4) the identity of which was confirmed by enzymic and acid hydrolyses as for 1-naphthyl glucuronide (see above). 2-Naphthyl sulphate was however detected by cutting up the chromatograms as described for the cat and determination of the associated ^{14}C by liquid scintillation counting (see Fig 3.5). The ^{14}C radioactivity corresponding in Rf value to 2-naphthyl sulphate was not present on chromatograms of urine which had been treated with sulphatase or dilute acid (under which conditions standard 2-naphthyl sulphate was hydrolyzed).

In the case of one pig no 2-naphthyl sulphate was detected even on cutting up the chromatograms (see Table 3.4). This pig was given a dose of 1-naphthol to ensure the results

were due to differences in the isomer administered and not due to differences in the pigs used. This pig excreted 29% of the dose of 1-naphthol as 1-naphthyl sulphate. Thus pigs are not defective in sulphate conjugation with respect to 1-naphthol. Neither 1-naphthol nor 2-naphthol were oxidised to dihydroxynaphthalenes in pig.

Section b

In Vitro Studies

In Section a of this Chapter it was shown that the pig was able to form substantial amounts of 1-naphthyl sulphate although it formed only traces of 2-naphthyl sulphate. In this section the *in vitro* metabolism by liver and kidney of these two naphthols is compared.

De Meio, *et al.*, (1953) found that phenyl sulphate was synthesised from phenol and sulphate ions in the presence of ATP and Mg^{2+} by the "microsome-free" supernatant preparations from rat liver. They further showed that the enzyme system could be separated into two components, one of which produced an activated form of sulphate (PAPS), the other carrying out the transfer of sulphate from this active nucleotide to a phenolic acceptor (De Meio, *et al.*, 1955). The synthesis of glucuronides is also a multi-stage process, the final stage taking place in the microsomal fraction of liver homogenates. UDPGA, the essential cofactor necessary for the synthesis of glucuronides was isolated by Dutton and Storey, (1953). The glucuronyl moiety is transferred to the acceptor under the influence of UDP-glucuronyl transferase to form a β-D-glucuronide. The mechanism of glucuronidation and sulphation are more fully described in section D, Chapter One.

French,(1970) used the technique of De Meio *et al.*, (1953) to compare the rates of sulphation and glucuronidation of phenol in pig and rat liver (see Table 3.5) and found that pig liver preparations glucuronidate phenol at least as readily as rat liver, but in the case of sulphate conjugation the pig liver preparation was only able to conjugate phenol at a rate of 3-8 times slower than the rate found with rat liver homogenates. This agrees with the *in vivo* findings with phenol (see Chapter 2) where sulphate formation is at a very low level but is not entirely absent in the pig.

Results and Discussion

The rate of conjugation of 1- and 2-naphthol at several different concentrations was determined and the average rate of conjugation for each pig liver and 10 g of pooled rat liver is quoted in Table 3.6. No significant difference was seen between the conjugation of 1- and 2-naphthol in the pig. Similarly in the rat, 1- and 2-naphthol are conjugated at the same rate, both in the case of glucuronic acid or sulphate conjugation. In the case of pig this result would not be predicted from the *in vivo* studies (see section a). It would be expected that 1-naphthol would be sulphated at a greater rate than 2-naphthol *in vitro* since 1-naphthol but not 2-naphthol is readily sulphated *in vivo*.

The difference between the sulphation of 1- and 2-naphthol might be explained if more than one phenol sulphotransferase enzyme exists and the various phenol sulphotransferase enzymes have different requirements, (e.g. differences in pH optimum) for activity. Thus it can be hypothesised that one phenol sulphotransferase enzyme accepts phenol, 1- or 2-naphthol as substrates but this enzyme is defective in pig, however, and very little sulphation is observed in this species. Possibly another phenol sulphotransferase enzyme can accept 1-naphthol but not phenol or 2-naphthol as substrates. This enzyme is not defective in pig and is responsible for the appreciable sulphation of 1-naphthol *in vivo*.

In the *in vitro* studies the conditions employed are the optimum for the first enzyme mentioned above which has little ability to conjugate phenol, 1- or 2-naphthol in pig. Under these conditions the other enzyme which conjugates 1-naphthol *in vivo* might be inactive and therefore no sulphation of 1-naphthol would be observed. In order to test this hypothesis further experimentation is necessary; varying the incubation conditions. If this hypothesis is correct incubation conditions will be found where 1-naphthol but not phenol or 2-naphthol will be sulphated *in vitro*. It must be remembered however

that the enzyme which sulphates 1-naphthol might have a different intracellular location and might possibly have been removed in the preparation of the homogenate, e.g. in discarding the microsomes.

Summarising, it is possible that this difference between the sulphation of 1- and 2-naphthol *in vivo* might be evidence for the existence of more than one phenol sulphotransferase enzymes, capable of conjugating 1- or 2-naphthol. In this study there was no apparent difference in ability between pig liver and pig kidney to conjugate either 1- or 2-naphthol with glucuronic acid or sulphate.

Summary

1) The cat excretes ^{14}C labelled 1-naphthol and 2-naphthol almost exclusively as sulphates forming only traces of glucuronides.

2) The pig excretes ^{14}C labelled 2-naphthol almost exclusively as a glucuronide forming only traces of sulphate. However, the pig excretes approximately one third of a dose of 1-naphthol as 1-naphthyl sulphate.

3) From the *in vitro* studies it appeared there was no difference in the rate of sulphation of 1- and 2-naphthol in pig and this was much less than the rate in rat.

Table 3.1 The Chromatography of 1- and 2-Naphthol and Their Principle Metabolites

For paper chromatography, Whatman, No.1. paper and the descending technique were used. Solvents; A, butan-1-ol: ammonia soln. (s.g.0.88): water (4:1:5, by vol.); B, pyridine-pentan-1-ol-water (7:7:6); C, butan-2-one:water (2:1); D, butan-1-ol: acetic acid (glacial):water (4:1:5). The upper organic phase was used for solvents A, C and D; B is miscible. Detecting reagents; the free naphthols gave an orange colour on spraying the chromatograms with freshly diazotized p-nitroaniline followed by 0.5M KOH in ethanol. The naphthyl sulphates were visualised by spraying with 2M HCl and heating at 100° for 2 min followed by spraying with freshly diazotized p-nitroaniline and KOH (see above), when they appeared as orange spots. The naphthyl glucuronides gave a blue colour on spraying with naphtharesorcinol (1% w/v) in aqueous trichloroacetic acid (33% w/v.) followed by heating the paper at 120° for 5-15 min.

Compound	Rf in solvents			
	A	B	C	D
1-Naphthol	0.95	0.95	0.95	-
1-Naphthylglucuronide	0.40	0.50	0.00	-
1-Naphthylsulphate	0.70	0.82	0.50	-
2-Naphthol	0.95	0.95	0.95	0.95
2-Naphthylsulphate	0.70	0.74	0.60	0.66
2-Naphthylglucuronide	0.33	0.45	0	0.75

Table 3.2 The Chromatography of Some Dihydroxynaphthalene Isomers

The dihydroxynaphthalene isomers were purchased (see text). Attempts were made to separate them using the solvents listed below. For paper chromatography 5 cm. Whatman No.1 paper and the descending technique were used. For T.L.C. fluorescent silica Gel G HF_{254} (Merck) was used. Solvents: Paper chromatography; B[+] Pyridine-Pentan-1-ol Water (7:7:6 by vol.), D[+] Butan-1-ol-Acetic acid-Water (4:1:5); T.L.C. solvent E, Ethyl acetate-cyclohexane (3-7); F Benzene-Ethyl Formate-Formic acid (75:24:1). Detecting reagents: freshly diazotized p-nitroaniline followed by 0.5 M-KOH in ethanol.

Compound	Rf Value in Solvents Papers B	D	Thin-layer E	F	Colour with diazotized p-nitroaniline
Unknown	0.89	0.90	0.57	0.52	-
2, 3-dihydroxynaphthalene	0.85	0.87	0.60	0.54	cherry-red
1, 7-dihydroxynaphthalene	0.86	0.87	0.55	0.52	purple
2, 6-dihydroxynaphthalene	0.84	0.90	0.55	0.52	mauve
2, 7-dihydroxynaphthalene	0.88	0.90	0.56	0.52	dark-orange
1, 6-dihydroxynaphthalene	0.91	0.93	0.54	0.50	dark-green
1, 3-dihydroxynaphthalene	0.92	0.88	0.54	0.50	orange

[+] These two solvent systems were also used for the chromatography described in Table 3.1

Table 3.3 The Urinary Metabolites of 1-naphthol in the Cat, Pig and Rat

Three full grown mongrel cats (2.5-3.5 kg body wt) were used. A was a neutered one year old male and B and C were females. Three female pigs (large White: 10-15 weeks old; 12-15 kg body wt) were used. Three female Wistar albino rats (190-210 g body wt) were used. 1-Naphthol [1-^{14}C] was injected intraperitoneally (see text). Urine was collected for 24 h and chromatographed on Whatman No.1 paper using the solvent systems described in Table 3.1. The radiochromatograms were cut into suitable pieces which were then counted (see text). Average values are given with, in parentheses, the value for each animal in the order found in column 1.

Species	Dose 1-Naphthol mg/kg	Dose ^{14}C μCi/animal	^{14}C excreted in 24h (% dose)	% of ^{14}C excreted in 24 h found as:- 1-Naphthyl-glucuronide+	1-Naphthyl-sulphate ‡
Cat (D,B,A)	25	10	91(100,72,101)	1.4(0.8,1.6,1.8)	98(99,98,98)
Pig (7,8,9)	25	10	77(93,61,76)	64(52,79,62)	33(42,22,36)
Rat (A,B,C)	25	5	59(41,63,73)	47(45,44,51)	53(54,56,49)

+ This ^{14}C peak was hydrolysed by both β-glucuronidase (Ketodase) and sulphatase (contains both sulphatase and β-glucuronidase activity) preparations to give a ^{14}C peak with the Rf value of free 1-naphthol.

‡ This ^{14}C was hydrolysed to free 1-naphthol by the sulphatase preparation only.

Table 3.4 The Urinary Metabolites of 2-Naphthol in the Cat, Pig and Rat

Three full grown mongrel cats (body wt 2.5 kg) were used. A was a neutered male, B and C were females. Three male but sexually immature pigs, (large White; 16weeks old 20-22 kg body wt) were used. Three female Wistar albino rats (190-210 g. body wt) were used. 2-Naphthol [8-^{14}C] was administered intraperitoneally as described in the text. 24h urines were chromatographed in the solvent systems of Table 3.1. Radiochromatogram scans were also cut into suitable pieces which were then counted (see text). Average values are given with, in parentheses, the value for each animal in the order found in column 1.

Species	Dose mg/kg	Dose μCi/animal	^{14}C excreted in 24h	% of ^{14}C excreted found as 2-Naphthylglucuronide+	2-Naphthylsulphate±	Other✓
Cat (E,D,F)	25	10	73(73,72,74)	2.6(1.5,1.9,4.5)	78(80,89,65)	19(19,9.3,31)
Pig (4,5,6)	25	10	84(64,98,90)	94(90,100,93)	6(10,0,7)	n.d.
Rat (D,E,F)	25	5	86(87,78,93)	52(49,57,50)	48(51,43,50)	n.d.

+ This ^{14}C peak was hydrolyzed by both β-glucuronidase (Ketodase) and sulphatase (contains both sulphatase and β-glucuronidase activity) preparations to give a ^{14}C peak with the Rf value of free 2-naphthol.

± This ^{14}C peak hydrolysed to free 2-naphthol by the sulphatase preparation only.

✓ This ^{14}C peak was hydrolyzed by sulphatase only, to give a ^{14}C peak which had an Rf value similar to 2,7-dihydroxynaphthalene (see Table 3.2)

n.d. = not detected

Table 3.5

Conjugation of [^{14}C] Phenol with sulphate and Glucuronic acid in Rat and Pig Liver Homogenates

Substrate	Species	Conversion to sulphate ester		Conversion to glucuronide	
		% added substrate	rate (μmol/g liver/h)	% added substrate	rate (μmol/g liver/h)
Phenol	Rat				
	1	80	8.6	-	-
	2	72	7.7	89	9.6
	3	67	7.2	91	9.8
Phenol	Pig				
	1	22	2.5	100	10.8
	2	10	1.1	100	10.8
	3	20	2.2	86	9.2

(Figures from French, 1970)

Table 3.6

The Conjugation of ^{14}C 1 and 2-Naphthol with Sulphate and Glucuronic Acid in Rat and Pig Liver Homogenates

For details of incubates see text.

Substrate	Species	Conversion to Glucuronide % added substrate	Conversion to Glucuronide rate (µmol/g liver/h)	Conversion to Sulphate % added substrate	Conversion to Sulphate rate (µmol/g liver/h)
1-naphthol	Pig 1	56	5.3	11	0.46
	" 2	83	4.5	25	0.60
	" 3	75	5.5	39	0.69
	" 4	93	3.8	16	0.59
	" 5	60	4.9	18	0.55
			MEAN 4.7		MEAN 0.57
	Rat 1	92	3.8	82	2.5
	" 2	91	3.7	74	2.3
	" 3	88	3.0	72	2.8
			MEAN 3.5		MEAN 2.6
2-naphthol	Pig 1	64	5.5	15	0.51
	" 2	49	4.4	32	0.64
	" 3	62	3.9	26	0.63
	" 4	55	4.2	27	0.50
	" 5	85	3.3	18	0.72
			MEAN 4.2		MEAN 0.60
	Rat 1	88	3.9	77	2.8
	" 2	83	3.2	74	2.7
	" 3	92	3.4	72	2.0
			MEAN 3.5		MEAN 2.5

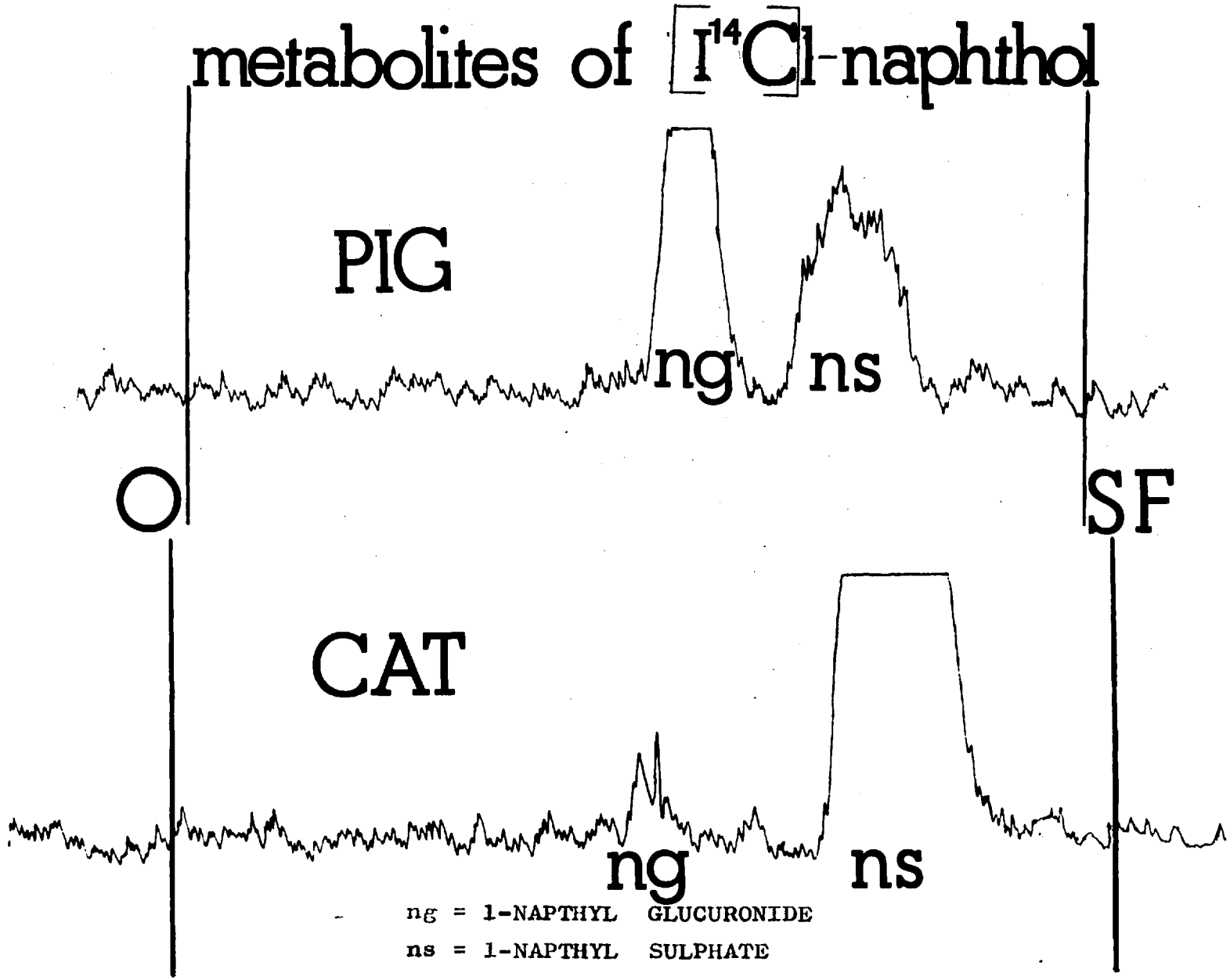
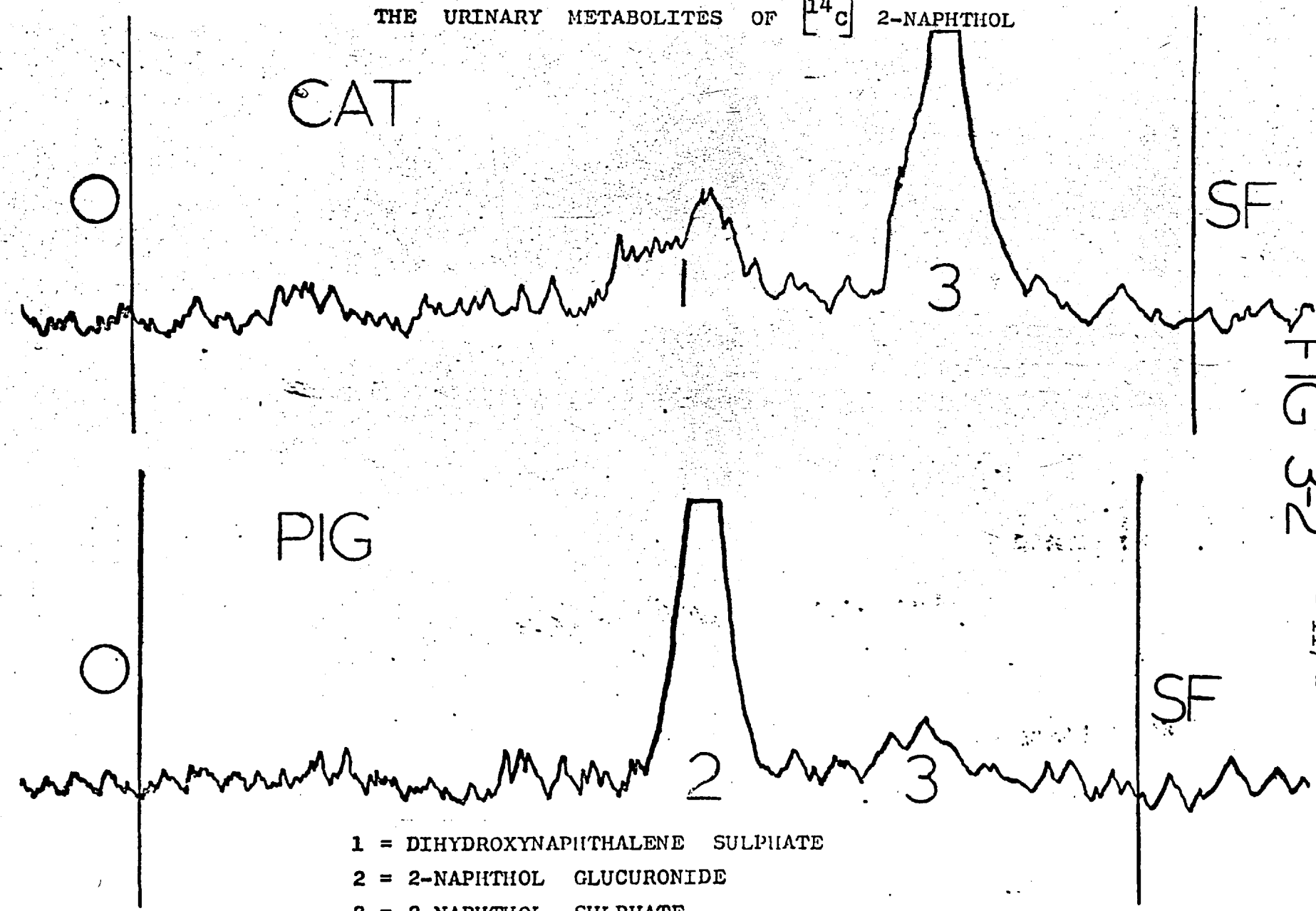

metabolites of [1-14C]-naphthol
PIG
ng
ns
O
SF
CAT
ng
ns
ng = 1-NAPTHYL GLUCURONIDE
ns = 1-NAPTHYL SULPHATE

FIG 3-1

THE URINARY METABOLITES OF [^{14}C] 2-NAPHTHOL



1 = DIHYDROXYNAPHTHALENE SULPHATE

2 = 2-NAPHTHOL GLUCURONIDE

3 = 2-NAPHTHOL SULPHATE

FIG 3-2

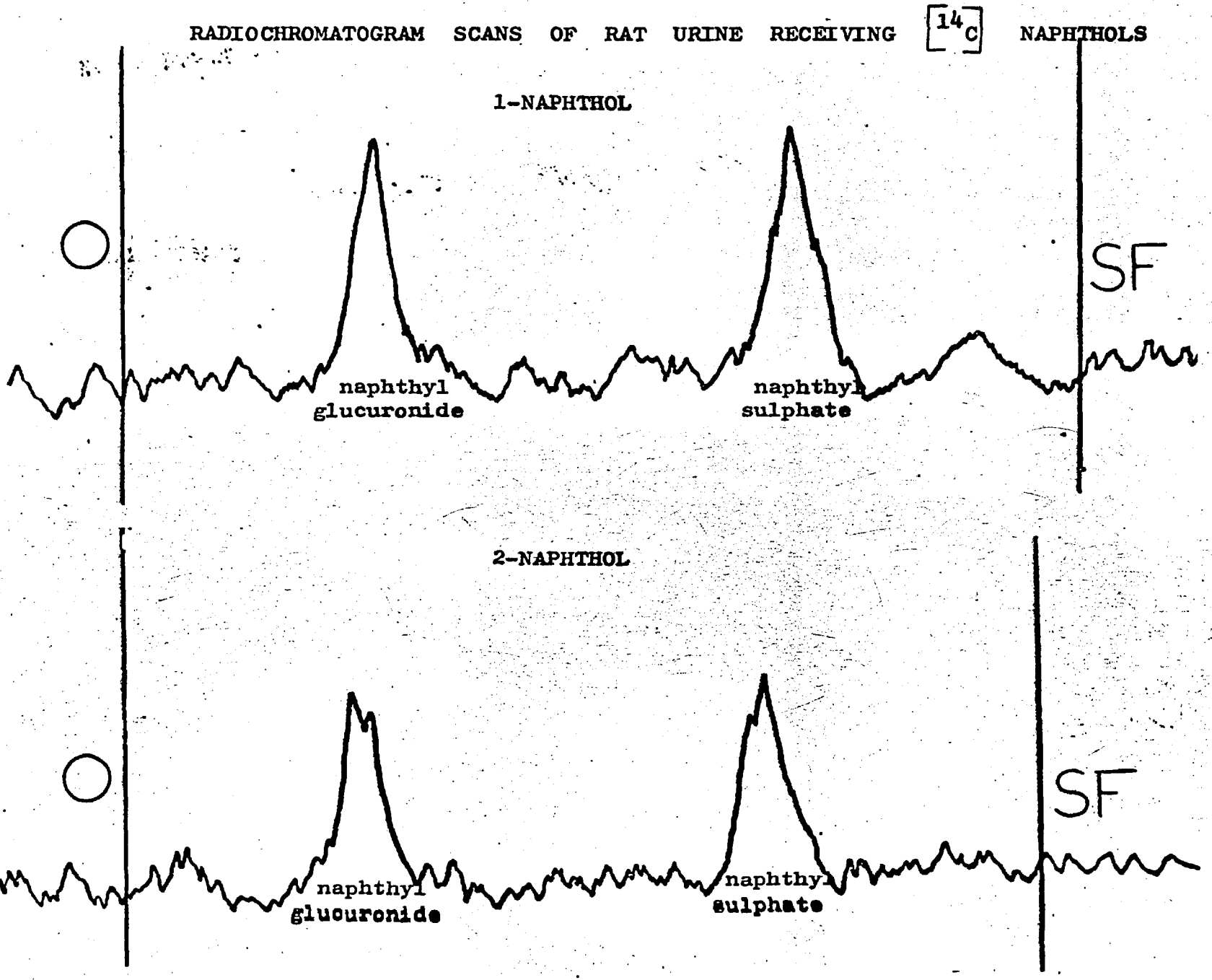

RADIOCHROMATOGRAM SCANS OF RAT URINE RECEIVING [14C] NAPHTHOLS
1-NAPHTHOL
O
naphthyl glucuronide
naphthyl sulphate
SF
2-NAPHTHOL
O
naphthyl glucuronide
naphthyl sulphate
SF

FIG 3-3

Fig 3.4
Histograms Showing the Distribution of [^{14}C] on Chromatograms
1-Naphthol in the Cat and Pig

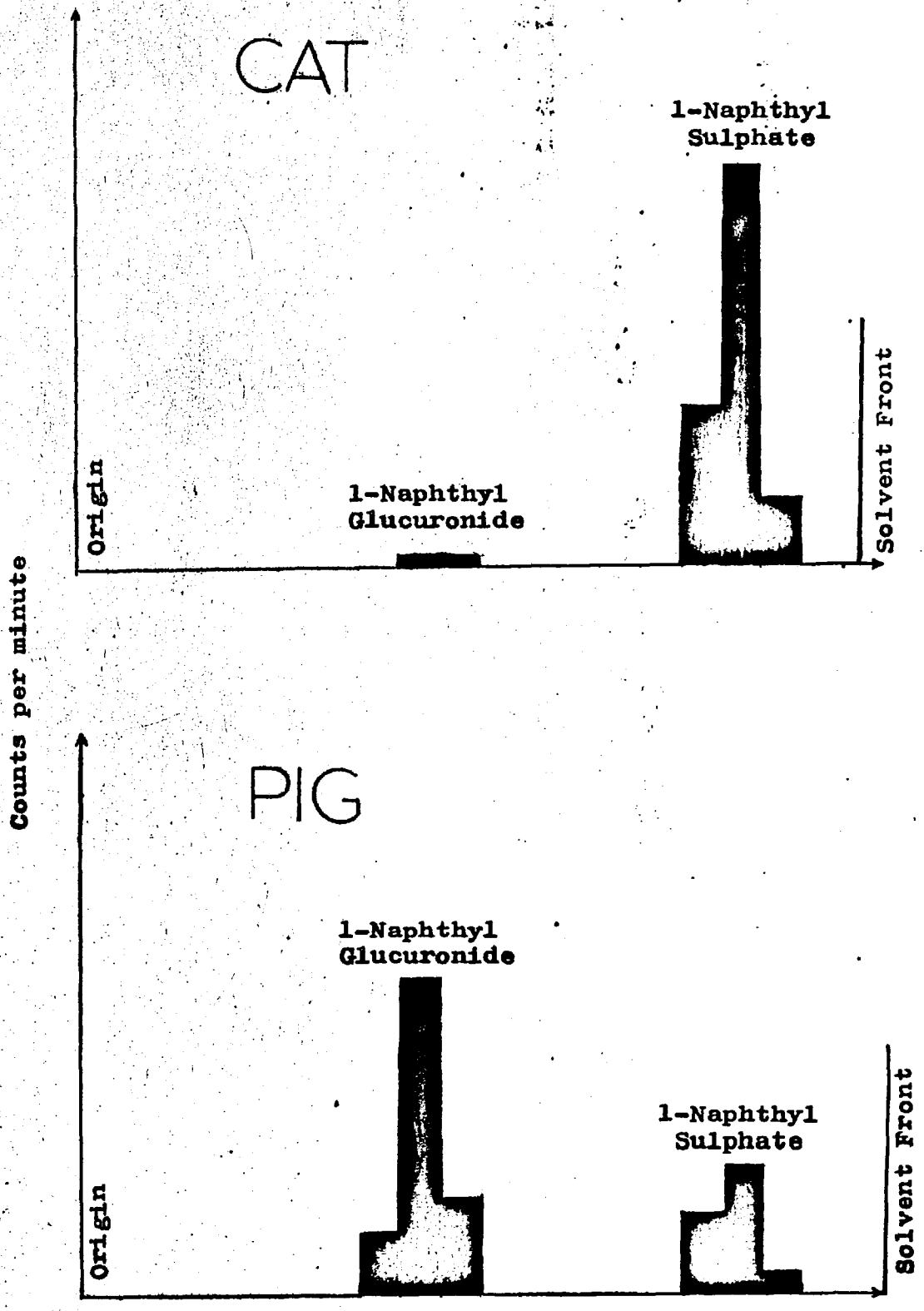
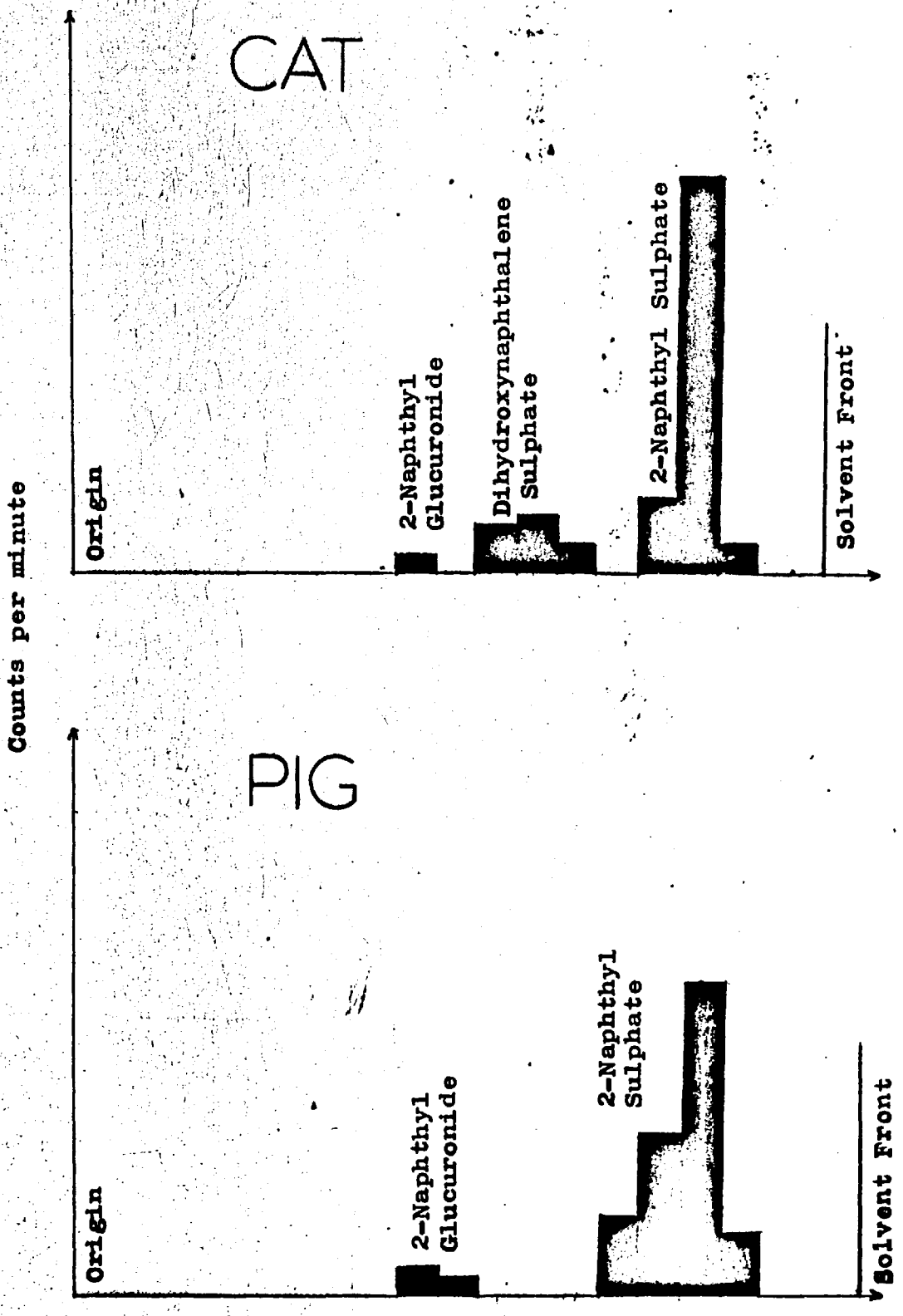


Fig 3.5
Histograms Showing the Distribution of $[^{14}C]$ on Chromatograms
2-Naphthol in the Cat and Pig



CHAPTER FOUR

THE METABOLISM OF SOME PHENOLS IN CAT AND PIG

Contents

Tables and Figures

Introduction

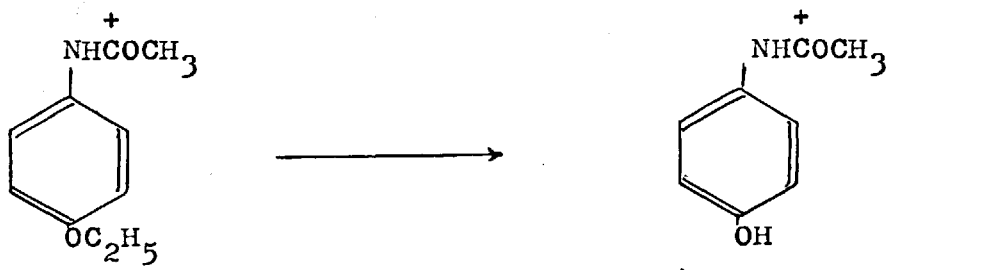
In Chapters Two and Three it was seen that in the cat phenol, 1-naphthol and 2-naphthol were excreted principally as sulphates and this species appeared to have little ability to form glucuronides of these compounds. In the pig phenol and 2-naphthol were excreted principally as glucuronides and this species had little ability to form sulphates of these compounds. However, the pig readily conjugated 1-naphthol with sulphate. It could be postulated that the formation of sulphate conjugates in the pig, and possibly glucuronides in cat could be substrate dependant. In this Chapter, therefore, a number of phenols were administered to pig and cat in order to determine whether this hypothesis was correct.

Phenacetin was administered to the pig and cat to determine the conjugation pattern of 4-acetamidophenol resulting from its dealkylation. 4-Hydroxyamphetamine was also administered to pigs. Both these compounds have basic groups in the β-position to the hydroxyl group of the phenol.

Morphine and phenolphthalein were administered to cats. The conjugates of these compounds have a molecular weight

in the range 350-500 and might therefore be excreted extensively in the bile (see Abou-El-Makarem, et al., 1967). Morphine and phenolphthalein have a larger molecular size than the previous phenols administered to cats. It is possible that if a family of UDP-glucuronyl transferases exist the structures of these compounds might be sufficiently different to invoke an alternative transferase to that conjugating phenol, 1- and 2-naphthol and 4-acetamidophenol.

[1^{14}C-acetyl] Phenacetin (acetophenetidine, p-ethoxyacetamilide) was administered to the pig and cat and both species formed the phenolic metabolite 4-acetamidophenol which could then be conjugated and excreted.



Phenacetin 4-Acetamidophenol

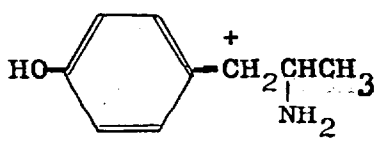
+ = indicates ^{14}C radioactive label

The major metabolic route of phenacetin is oxidative O-de-ethylation to 4-acetamidophenol (Brodie and Axelrod,1949). The urine of rats administered phenacetin contains 4-acetamidophenyl sulphate, 4-acetamidophenyl glucuronide,

4-phenetidine, 2-hydroxyphenetidine sulphate, and also 2- and 3-hydroxyphenacetin (see Buch, et al., 1967). The 2-hydroxy metabolite has been demonstrated in the urine of treated cats, dogs and man, (Klutch, et al., 1966). More recently the metabolism has been investigated in the rat by Nery, (1971) using [ethyl-^{14}C] phenacetin and [acetyl ^{3}H] phenacetin. In this study four other metabolites were detected, namely N-acetyl-S-ethylcysteine, quinol, acetamide and a substance which was probably N-acetyl-S-2,4-(ethoxyacetanilido) cysteine-S-oxide. The quinol was excreted usually as a glucuronide or sulphate. Recently the metabolism of [1-^{14}C acetyl] phenacetin was studied in various species including rabbit, guinea-pig, ferret, rat and man (see Smith and Timbrell, 1972). The [$^{14}CO_2$] liberated was monitored as a measure of deacetylation. This reaction was found to occur to a greater extent in ferret and rat (13% and 16% respectively) but was generally lower in the other species (up to 4%). In the rat the major metabolite was 4-acetamidophenyl sulphate but in all the other species it was 4-acetamidophenyl glucuronide.

4-Hydroxyamphetamine was administered to pigs. This compound is used therapeutically as a vasoconstrictor. In addition it arises from the metabolism of amphetamine

((±)2-amino-1-phenyl propane). In rat 4-hydroxyamphetamine is the major metabolite (60% of administered dose) of amphetamine, (Dring, et al., 1966) but it is a minor one in man and certain other species (Dring, et al., 1970). In man 4-hydroxyamphetamine formed as a metabolite of metamphetamine is excreted mainly as the sulphate conjugate (Caldwell, et al., 1972). Recently Sever et al., (1973) investigated the fate of 4-hydroxyamphetamine in man, rat and guinea-pig and found that this compound was excreted partly as the free drug and partly conjugated with glucuronic acid (in rat and guinea-pig). In man, however, 4-hydroxyamphetamine was excreted mainly as a sulphate conjugate. In the rat a stereospecific hydroxylation of (+) 4-hydroxyamphetamine to (+) 4-hydroxynorephedrine has been demonstrated (see Goldstein and Anagoste, 1965).

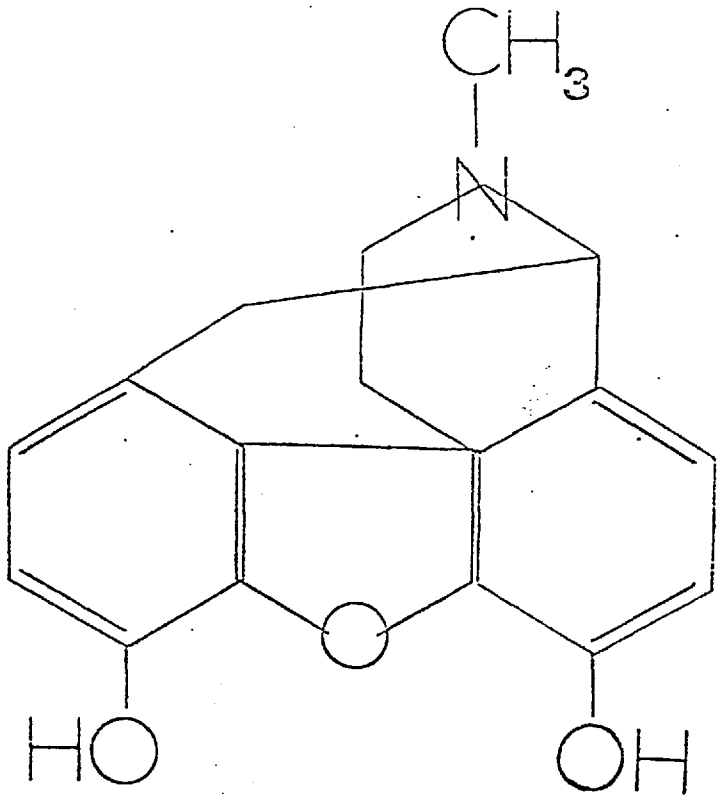


4-Hydroxyamphetamine
+ = position of ^{14}C-radioactive label

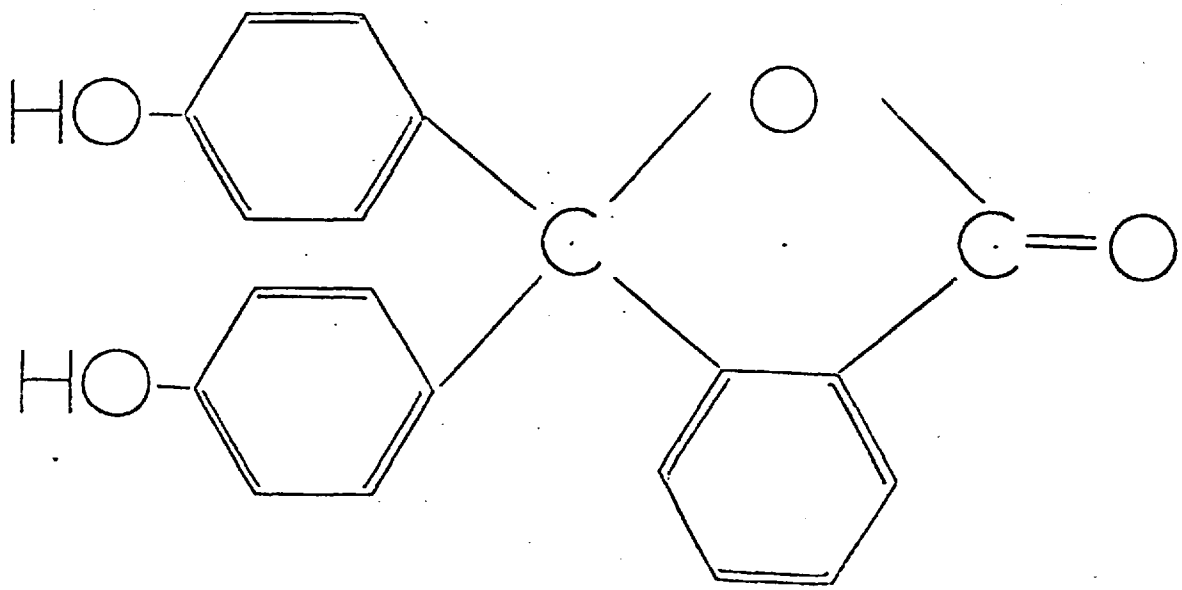
In 1896 Marquis postulated that 3 forms of morphine were excreted in the urine of animals receiving the drug; unchanged,

"converted morphine" and "coupled morphine". Later workers postulated the excretion of the glucuronide but this was not confirmed until 1954 by Seibert, et al. Later Yoshimura, et al., (1969) demonstrated that in addition to morphine-3-glucuronide, morphine-6-glucuronide was excreted as a minor metabolite of this drug in the urine of rabbits. Morphine-3-sulphate has been demonstrated as a major urinary metabolite in the chicken and cat, (Fujimoto and Haarstad, 1969). Morphine-3-glucuronide and morphine-6-glucuronide were shown recently to be metabolites of morphine in rabbits, guinea-pigs, rats, mice, and humans. Normorphine resulting from N-demethylation of the drug was also found to be a metabolite in these species.

Phenolphthalein usually undergoes conjugation at one of the phenolic hydroxyl groups forming phenolphthalein monoglucuronide (Flieg, 1909; Abel and Rowntree, 1909). The monoglucuronide of phenolphthalein has been isolated from the urine of rabbits following injection of this drug (Di Somma, 1940). Millburn, et al., (1967a), demonstrated in rat that phenolphthalein was excreted in the bile as its glucuronic acid conjugate. Abou-El-Makarem, (1967) demonstrated that 13% of a dose of phenolphthalein was excreted in the bile of



MORPHINE



PHENOLPHTHALEIN

cat in three hours. Irrespective of whether phenolphthalein was given orally or intravenously more than 50% of the radioactivity was excreted by dogs in the faeces, while some 36-37% was excreted in the urine in 3 days (cited by Williams, 1959). Brodie and Maickel, (1962) reported that the cat had negligeable UDPGA transferase activity towards phenolphthalein.

Materials and Methods

Phenacetin (m.p. 134°) was obtained from Hopkin and William Chadwell Heath, Essex. [1-^{14}C acetyl] Phenacetin was synthesised by Mr. J. A. Timbrell in this laboratory and had a specific activity of 0.05 μCi/mg. 4-Acetamidophenyl sulphate was synthesized by Mr. Timbrell by the method of Feigenbaum and Neuberg, (1941). This sulphate gave one spot in the solvent systems employed (see Table 4.1) and on hydrolysis with dilute acid (see Chapter Two) yielded 4-acetamid -phenol.

[^{14}C] 4-Hydroxyamphetamine [(±)-2-amino-1-(4'-hydroxyphenyl) [1-^{14}C] propane; Paredrine] hydrochloride was a sample prepared by Dr. L. G. Dring in this laboratory and had a specific activity of 0.9 μCi/mg.
(±)4-Hydroxyamphetamine hydrobromide and (±)4-hydroxy-norephedrine, [(±)2-amino-1-(4'-hydroxyphenyl)propan-1-ol]

hydrochloride were samples available in this laboratory (see Caldwell, et al., 1972), 4-hydroxybenzoic acid was purchased from the Sigma Chemical Co., London., 4-hydroxyhippuric acid was a sample prepared by Dr. L. G.Dring.

Morphine hydrochloride was obtained from Macfarlan Smith Ltd., Edinburgh, U.K. Morphine-3-sulphate, (m.p. 298°) was prepared by the method of Fujimoto and Haarstad, (1969). [^{14}C N Me] Morphine was purchased from the Radiochemical Centre, Amersham, Bucks, U.K.

Phenolphthalein, (m.p. 255-257°), was purchased from British Drug Houses Ltd., Poole, Dorset, U.K. Phenolphthalein monoglucuronide, sodium salt, phenolphthalein disulphate tripotassium salt, were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Phenol (m.p. 43°) and phthallic anhydride (m.p. 131°) used in the following experiment were purchased from British Drug Houses, Poole, Dorset, U.K.

Tritiated phenolphthalein was prepared by the condensation of phenol with tritiated phthallic anhydride essentially as described by Vogel, (1956). 400 μCi of [G-^{3}H] phthallic anhydride, specific activity 665 mCi/mM, was placed in a small round bottomed flask. To this was

added 1 g. of phthallic anhydride, 5 g. of phenol and 5 g. of anhydrous zinc chloride. The mixture was heated with stirring in an oil bath at 115° for 24 h. Then 5 ml. of warm water was added to the reaction mixture and the contents of the flask poured into 50 ml. of warm water. The resulting solution was boiled to remove all traces of phenol, water was added to replace that lost by evaporation, the mixture was cooled and the resulting precipitate filtered. This solid was added to 10% sodium hydroxide solution and the insoluble reaction bi-products filtered. The filtrate was acidified with 2N-HCl, cooled and the crude phenolphthalein filtered. The phenolphthalein was dissolved in ethanol 25 ml, 5 g. of decolourising charcoal added and the mixture refluxed for 30 min. The charcoal was filtered and washed with ethanol. The volume of the ethanol was reduced by half on the rotary evaporator and mixed with 200 ml. of water. The resulting solution was gently heated and then left to cool. White crystals of phenolphthalein were obtained, filtered and weighed. The yield was 1.25 g. which is 63.8% of the theoretical yield, m.p. = 256°, mixed melting point with the authentic compound = 256°. The radioactive phenolphthalein gave one ^{3}H labelled peak on chromatography in the solvents listed in Table 4.7 which corresponded to authentic phenolphthalein.

Enzyme preparations: β-glucuronidase (Ketodase; Warner-Chilcott Laboratories, Eastleigh, Hampshire, U.K.; sulphatase, which has both sulphatase and β-glucuronidase activity, (Helix pomatia preparation) and saccharo-1, 4-lactone were purchased from Sigma Chemical Co. Ltd., London.

Animals

The cats and pigs (see Appendix 1) were obtained from animal dealers in the London area and maintained on appropriate diets. [1 ^{14}C acetyl] phenacetin (3.5 μCi/animal) together with sufficient carrier phenacetin to give a dose level of 25 mg/kg was dissolved in 5 ml of dimethylsulphoxide and administered intraperitoneally to each pig. In cats a similar dose of radioactive phenacetin (3.5 μCi/animal and 25 mg/kg) was dissolved in 1 ml of dimethylsulphoxide and administered intraperitoneally.

[^{14}C] 4-Hydroxyamphetamine, dissolved in water (4 mg; 3.6 μCi/ml) was administered (0.8 or 1 ml/animal) intraperitoneally to pigs, giving a dose level of 0.1 mg/kg (0.66 μmol/kg).

[N- ^{14}C methyl] morphine (10 μCi/ml in water) was administered (1 ml/animal) intraperitoneally to cats together

with sufficient carrier morphine hydrochloride dissolved in 0.5 ml of water to give a total dose of 5 mg/kg.

Tritiated phenolphthalein together with sufficient carrier phenolphthalein was dissolved in 0.5 ml of absolute ethanol to give a total dose of 25 mg/kg which was administered intraperitoneally to each of 3 cats.

All animals were placed in suitable metabolism cages and their urine collected for 24h.

Chromatography

For paper chromatography Whatman No. 1 chromatography paper (5 cm strip) and the descending technique were employed. For T.L.C. silica gel G, HF 254, Merck (U.K. distributers Anderman and Co. Ltd., London) 0.25 mm thick on glass plates was used, the silica being activated by heating at 105° for 30 min before use.

The Rf values and visualisation techniques are given as follows:-

phenacetin, Table 4.1; 4-hydroxyamphetamine, Table 4.3; morphine, Table 4.6; phenolphthalein, Table 4.8.

Counting Procedure

^{14}C was estimated in the usual manner (see Chapter 2).

Urine (0.1 - 0.2 ml samples) containing tritiated phenolphthalein was counted in quadruplicate in a scintillation fluid comprising;
Naphthalene 200 g., PPO (2,5-diphenyloxazole) 10 g., POPOP (1,4-Di [2-(5-phenyloxazolyl)] benzene 0.6 g., dioxan 1440 ml, toluene 270 ml and methanol 90 ml, using the Packard Tri Carb Scintillation Spectrometer (model 3320). The samples were counted at an efficiency of 30-35%, determined by the twin-channel ratio method. Radiochromatograms of urine were prepared and scanned as previously described (see Chapter Two). After scanning the paper strip chromatograms were cut into 1 cm pieces and placed in dioxan scintillation fluid and the associated radioactivity determined.

Results and Discussion

Phenacetin

Pig

When [1-^{14}C-acetyl] phenacetin was administered to pigs one large radioactivity peak was seen on radiochromatogram scanning of urine chromatographed in solvent I of Table 4.1 (see Fig 4.1). This peak had an Rf value of 0.10 which is

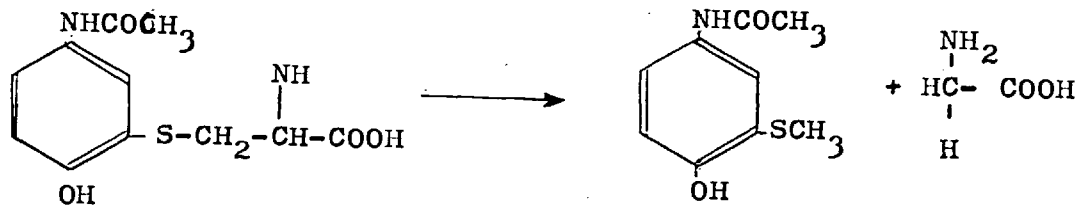
identical to that quoted for 4-acetamidophenyl glucuronide by Timbrell, (1973). This radioactivity peak was removed on incubation of the urine with β-glucuronidase and this hydrolysis was inhibited by saccharo-1, 4-lactone (which is a specific inhibitor for this enzyme). This metabolite of phenacetin was not hydrolyzed by heating with 2N-HCl under which conditions sulphate conjugates are hydrolysed (see Garton, et al., (1949). It was concluded that this radioactivity peak was 4-acetamidophenyl glucuronide.

Hydrolysis of 4-acetamidophenyl glucuronide with β-glucuronidase revealed another small ^{14}C metabolite of Rf value 0.20 in solvent I (Table 4.1) which had been overshadowed by the larger glucuronide peak. The radioactivity peak corresponding to this unknown metabolite was not hydrolysed by either β-glucuronidase or sulphatase. In solvent II (see Table 4.1) a better separation between 4-acetamidophenyl glucuronide and this unknown metabolite was obtained (Rf values 0 and 0.21 respectively).

The percentage of the metabolites present were accurately determined when chromatograms developed in Solvent II were cut into 1 cm pieces and the associated ^{14}C determined with a Tricarb Liquid Scintillation spectrometer (model 3320). 4-Acetamidophenyl glucuronide accounted for 54% of the ^{14}C

in the urine (i.e. 16.2% of the dose) of pigs administered radioactive phenacetin (see Table 4.2), while the unknown metabolite accounted for 20% of the ^{14}C in the urine (6% of the dose).

The Rf value (0.20) of the unknown metabolite in solvent I was similar to that quoted for a metabolite of phenacetin in a number of species identified by Timbrell, (1973) as the cysteinyl conjugate of 4-acetamidophenol, i.e. 3[(5-acetamido-2-hydroxyphenyl)]thio alanine. Timbrell, isolated this metabolite from the urine of rats dosed with [^{14}C] phenacetin and it was purified and crystallised, but the purified compound decomposed into 2-thiomethyl-4-acetamidophenol and glycine.



The identity of 2-thiomethyl 4-acetamidophenol was confirmed by mass spectroscopy and the glycine liberated identified by chromatography. It is possible therefore that

the unidentified metabolite of phenacetin formed in pigs was 3[(5-acetamido-2-hydroxyphenyl)]thio alanine.

When chromatograms of pig urine developed in solvent II were cut up and the associated ^{14}C estimated as described above a small (10% of ^{14}C in the urine; 3.1% of the dose) but clearly defined ^{14}C peak of Rf value 0.53 corresponding to authentic 4-acetamidophenyl sulphate was observed. This peak was hydrolysed when urine was treated with sulphatase but not β-glucuronidase and it was also removed by heating the urine with 2N HCl as described by Garton, et al., (1949). It was concluded therefore that this radioactivity represented 4-acetamidophenyl sulphate.

A small amount (16%) of the ^{14}C on the chromatograms (i.e. 4.8% of dose) was identified as free (unconjugated) 4-acetamidophenol since its Rf values corresponded to an authentic sample chromatographed in the solvents of Table 4.1. The total recovery of ^{14}C (31% - see Table 4.2) in the urine of pigs administered ^{14}C-labelled phenacetin was lower than that found for other phenols administered to pigs (see Chapters Two and Three). This could be due to the removal of the [acetyl ^{14}C] label by deacetylation reactions or by the metabolism of the [^{14}C] phenacetin to non urinary metabolites.

Cat

Radiochromatogram scanning of the urine of cats administered [^{14}C] phenacetin showed only one radioactivity peak (see Fig. 4.1). This metabolite had the same Rf value as an authentic sample of 4-acetamidophenyl sulphate in the solvents of Table 4.1. This peak was not present in cat urine which had been treated with sulphatase but it was not attacked by a β-glucuronidase (Ketodase) preparation. Heating a urine sample with dilute acid as described by Garton, et al., (1949) also caused the removal of this ^{14}C peak corresponding to 4-acetamidophenyl sulphate, thus confirming its identity as a sulphate conjugate.

Chromatograms developed in both solvents listed in Table 4.1 were cut into 1 cm pieces and the associated ^{14}C-determined using the liquid scintillation spectrometer. Histograms of the distribution of ^{14}C along the chromatograms were constructed and two ^{14}C-radioactivity peaks were detected in addition to the ^{14}C associated with 4-acetamidophenyl sulphate. The first of these ^{14}C peaks to be considered ran very close to the origin in solvents I and II (Rf values 0.1 and 0, respectively). From the experimentation in pig it seems likely that this metabolite might be a glucuronic acid

conjugate. It was found that this metabolite was hydrolysed by β-glucuronidase since it was not detected on cutting up chromatograms of urine treated with this enzyme and estimating the associated ^{14}C. The hydrolysis with β-glucuronidase was specifically inhibited by saccharo-1,4-lactone. This radioactivity peak was also hydrolysed by sulphatase which contains β-glucuronidase activity. It was concluded that this metabolite was probably 4-acetamidophenyl glucuronide. In cat only small quantities (3%) of the ^{14}C on the chromatogram (1.7% of dose) was present as this glucuronide (see Table 4.2).

The other ^{14}C metabolite detected on cutting up the chromatograms was distinct from the ^{14}C peak ascribed to 4-acetamidophenyl glucuronide in both solvents listed in Table 4.1 It had Rf values of 0.20 and 0.22 in solvents I and II respectively and was present in similar quantities (7% of the ^{14}C in the urine; 4% of dose) to the metabolite of similar Rf value found in the pig. This metabolite was not hydrolysed by enzyme or acid treatment and it is possible that it is the 3 [(5-acetamido-2-hydroxyphenyl)]thio alanine metabolite detected by Timbrell,(1973) described above.

Free 4-acetamidophenyl was only detected on cutting up

the developed chromatograms into 1 cm pieces and counting the associated ^{14}C. Free 4-acetamidophenol was excreted in lower quantities in the cat than in the pig (see Table 4.2).

The total recovery of ^{14}C-radioactivity was relatively low in cat (as in the pig). It is possible that this could be due to deacetylation reactions and subsequent loss of the ^{14}C label since Krebs, et al., (1947) showed that deacetylation reactions occur extensively in cat.

4-Hydroxyamphetamine in Pig

Table 4.4 shows that most (89%) of an intraperitoneal dose of [^{14}C] 4-hydroxyamphetamine is excreted in the urine in 24 h by the pig. Radiochromatograms of this urine were prepared using solvents B and G (see Table 4.3). These radiochromatograms were scanned and showed two ^{14}C peaks. In solvent B these peaks had Rf values of 0.15 and 0.80, (see Table 4.5), the latter corresponding to authentic 4-hydroxyamphetamine (see Table 4.3). Treatment of the urine with β-glucuronidase caused the peak at Rf 0.15 to disappear with a corresponding increase in the 4-hydroxyamphetamine peak at Rf 0.80. Similarly, in solvent G, two ^{14}C peaks were detected corresponding to 4-hydroxyamphetamine (Rf 0.79) and its glucuronic acid conjugate (Rf 0.26), the

latter being hydrolysed by β-glucuronidase to free 4-hydroxyamphetamine. This hydrolysis was inhibited by saccharo-1, 4-lactone.

On cutting chromatograms developed in solvent B into suitable pieces, which were then counted for radioactivity, a small amount (5.6% of the ^{14}C excreted in 24 h (5% of dose) see Table 4.4) of the ^{14}C was found at Rf 0.54. The ^{14}C material at Rf 0.54 (solvent B) appears to be 4-hydroxyamphetamine sulphate as it is hydrolysed by the sulphatase preparation (which contained both sulphatase and β-glucuronidase activity). This peak was not hydrolysed by Ketodase (β-glucuronidase).

These results show that the pig excretes 4-hydroxyamphetamine largely as the unchanged compound and its glucuronic acid conjugate, which each account for approximately 40-50% of the urinary radioactivity, Only small amounts (5.6% of the urinary ^{14}C) are formed of the sulphate conjugate of 4-hydroxyamphetamine, Thus the pig forms only very small amounts of sulphate conjugates of 4-hydroxyamphetamine, at the same level as sulphate conjugates of phenol, 2-naphthol and phenacetin.

Authentic samples of other possible metabolites of 4-hydroxyamphetamine which would arise by the metabolism of the side chain, namely 4-hydroxynorephedrine (β-hydroxylation) and 4-hydroxybenzoic acid (deamination) and its glycine

conjugate, 4-hydroxyhippuric acid, were chromatographed in solvent B. In this solvent these compounds have different Rf values from those of 4-hydroxyamphetamine and its glucuronide and sulphate conjugates (see Tables 4.3 and 4.5). On the chromatograms cut up in solvent B no radioactivity was detected at the Rf values of 4-hydroxynorephedrine, 4-hydroxybenzoic acid and 4-hydroxyhippuric acid. This means that less than 1% of the ^{14}C in the urine was present in each of these compounds.

Morphine in Cat

When $\boxed{^{14}C}$ morphine was administered to cats two radioactivity peaks were seen on radiochromatogram scanning of thin-layer chromatograms, (see Fig. 4.3). The larger of these peaks No. 2, (see Fig. 4.3), has the same Rf value as authentic morphine-3-sulphate in the solvents of Table 4.6. The other radioactivity peak had the same Rf value as that of the free drug in these solvents. When urine was incubated with sulphatase, but not Ketodase (β-glucuronidase) and then chromatographed the radioactivity peak corresponding to morphine-3-sulphate was observed to have been hydrolysed and all the ^{14}C radioactivity corresponded in Rf value to free

morphine. The peak ascribed to morphine-3-sulphate was also hydrolyzed by 2N HCl as described by Garton, et al., (1949) thus confirming its identity as a sulphate conjugate.

The silica on the plates was divided into 0.5 cm segments and then scraped into separate vials containing scintillation gel and the associated ^{14}C determined by use of the liquid scintillation spectrometer. Using this technique the percentage of the metabolites present in the urine was accurately determined (see Table 4.7).

When cat urine was chromatographed on paper using solvent E of Table 4.6, an additional small ^{14}C peak was detected (see Fig. 4.3). It was found that this peak was hydrolyzed by sulphatase and dilute acid as described above but not by β-glucuronidase. It is likely therefore that this ^{14}C peak was also a sulphate conjugate, possibly morphine-6-sulphate which had been described (see later). This peak was only detected on paper chromatography in solvent E, it is likely that in the TLC systems it was not separate from the larger morphine-3-sulphate peak. Paper chromatograms were also cut into 1 cm pieces and the associated ^{14}C estimated in the liquid scintillation spectrometer. The percentage of the metabolites present was accurately determined and 1% of the activity was ascribed to morphine-6-sulphate. No other

significant ^{14}C radioactivity other than that represented by the metabolites seen on radiochromatogram scanning was detected on cutting up the paper chromatograms. Thus no glucuronic acid conjugates were detected either by T.L.C. or paper chromatography.

Therefore in these experiments no glucuronide conjugate present in quantities greater than 2.4% was detected in the urine of the three cats after morphine administration. Possibly this is due to technical difficulties involved in the use of T.L.C. systems since less radioactivity can be applied to thin-layer than paper chromatograms. With the separations of metabolites obtained in these solvent systems it is possible that any small amounts of glucuronide present might be overshadowed by the principal conjugate, morphine-3-sulphate.

Approximately 88% of the injected ^{14}C was recovered in the urine (see Table 4.7), it is possible that some of the morphine conjugates were excreted in the bile. Possibly the higher molecular weight glucuronide conjugate (molecular weight of morphine sulphate = 419, morphine glucuronide 548) is excreted preferentially in the bile. Any N-demethylation would result in the loss of the ^{14}C label and it is possible

that this reaction accounted for some of the radioactivity which was not recovered.

Fujimoto and Haarstad, (1969) isolated morphine-3-sulphate as the principal metabolite from cat urine when they administered this drug. The results from the above experiments are therefore in agreement with these workers. Mori, et al., (1972) synthesised morphine-6-sulphate and found that it had greater analgesic properties than morphine, however morphine-6-sulphate was not detected in the urine of cats even after careful examination by T.L.C. In the experiments described here a metabolite which may have been morphine-6-sulphate was only detected on paper chromatography in solvent E not in the T.L.C. systems (see Fig.4.3) which is in agreement with the findings of Mori, et al.

Yeh, et al (1971) reported that 14% of morphine was excreted as a conjugate in the bile; however, these workers did not identify the biliary conjugate(s). It is possible that some [^{14}C] morphine was excreted in the bile as a glucuronide. However, these workers were able to detect morphine-3-glucuronide on chromatography of the urine only after total hydrolysis of the sulphate conjugate. Thus we

see that morphine glucuronide is formed in small quantities in cat although it was not detected in the experiments described in this chapter even after total hydrolysis of sulphates with dilute acid.

Summarising in this and other studies of the metabolism of morphine in cat the principal metabolite is morphine-3-sulphate which is excreted via the kidneys.

Phenolphthalein in cat

Two ^{3}H radioactivity peaks were seen on radiochromatogram scanning of urine of cats dosed with ^{3}H phenolphthalein (see Fig. 4.4). From Table 4.9 it is seen that the main urinary metabolite of phenolphthalein was phenolphthalein monoglucuronide. The amount of glucuronide and other metabolite present was accurately determined by cutting up the chromatograms into pieces and determination of the associated radioactivity by Liquid Scintillation counting. No free phenolphthalein was detected in the urine.

The glucuronide peak had a similar Rf value to an authentic sample in all the solvents employed (see Table 4.8). The radioactivity peak corresponding to phenolphthalein glucuronide was hydrolysed by treating cat urine with Ketodase

(see Fig. 4.4) but not in the presence of saccharo-1,4-lactone which is a specific inhibitor of β-glucuronidase activity. This peak was also hydrolysed by sulphatase (which contains β-glucuronidase activity) when it gave a radioactivity peak corresponding in Rf value to free phenolphthalein. This peak seen on radiochromatogram scanning of cat urine was not removed by heating the urine with dilute acid, (a treatment described by Garton, et al., (1949) which selectively hydrolyses sulphate conjugates. It was concluded that the largest ^{3}H peak seen on radiochromatogram scanning of urine, corresponding to the principal metabolite of phenolphthalein in cat urine, was phenolphthalein glucuronide.

When cat urine was treated with sulphatase and dilute acid as described above the other ^{3}H metabolite was not detected on radiochromatogram scanning. This metabolite which had Rf values of 0.80, 0.80, 0.75, 0.68 and 0.90 in solvents 1, 2, 3, 4 and 5 of Table 4.8, respectively, was not hydrolysed on incubation with β-glucuronidase. It was concluded therefore that the other metabolite of phenolphthalein in cat urine was probably a sulphate conjugate.

An authentic sample of phenolphthalein disulphate was available and was chromatographed in the solvents of Table 4.8.

The Rf values of phenolphthalein disulphate in these solvents does not correspond to the unknown metabolite which the hydrolytic techniques indicated was a sulphate conjugate. It is likely, therefore, that the unknown metabolite present in cat urine was phenolphthalein monosulphate.

Attempts to synthesize the monosulphate by the technique of Fiegenbaum and Neuberg, (1941) were unsuccessful, the disulphate being the final product. Attempts to liberate the monosulphate from the disulphate by mild acid hydrolysis were also unsuccessful. Thus, although this compound was likely to be a monosulphate without authentic reference compounds its identity could not be confirmed.

The mean recovery of radioactivity in the urine was low. This might be predicted since Abou-El-Makarem, et al., (1967) showed that, when administered, phenolphthalein glucuronide is excreted extensively (37%) in the bile of cats. It was also demonstrated by Abou-El-Makarem,(1967) that 13% of the dose of phenolphthalein administered to cat was excreted as phenolphthalein glucuronide in the bile in 3 hours. It is likely, therefore, that the low urinary recovery of ^{3}H radioactivity is a consequence of the fact that phenolphthalein

monoglucuronide is preferentially excreted by a biliary route in this species. Approximately 60% of the recovery was shown to be a glucuronide conjugate, so that 17% of the dose of phenolphthalein was excreted as a glucuronide conjugate in the urine of cats. As seen in the previous chapters cats usually conjugate less than 2% of the dose of potentially glucuronogenic phenols with glucuronic acid so that in comparison with the other phenols administered cat is not defective in forming glucuronic acid conjugates of phenolphthalein.

Summary

1. Cat excretes phenacetin mainly as 4-acetamidophenyl sulphate and only traces of the glucuronide conjugate were formed.

2. Pig excretes phenacetin mainly as 4-acetamidophenyl glucuronide and only traces of the sulphate conjugate were formed.

3. Pig excretes 4-hydroxyamphetamine mainly as the glucuronide and only traces of the sulphate conjugate were formed.

4. Cat excretes morphine mainly as morphine-3-ethereal sulphate and glucuronide conjugates were not detected in the urine.

5. The major urinary metabolite of phenolphthalein in cat was phenolphthalein glucuronide which accounted for approximately 17% of the dose.

Table 4.1 The Chromatography of Phenacetin and its Metabolites

For chromatography, Whatman No. 1 paper and the descending technique were used. Solvent I: Butan-1-ol: Propan-1-ol: Ammonia (2M)(2:1:1) by vol.; Solvent II: Butan-1-ol: Pyridine: Water: Benzene (5:3:2:1 by vol.) 4-Acetamidophenol was visualized by spraying the chromatograms with freshly diazotized p-nitroaniline followed by 0.5M KOH in ethanol when it gave a blue spot. 4-Acetamidophenyl sulphate was visualized by spraying with 2 M HCl and heating at 100°C for 2 min followed by spraying with freshly diazotized p-nitroaniline and KOH (see above). (The Rf values quoted for phenacetin is that of the ^{14}C peak given by the labelled compound).

	Solvent I	Solvent II
Phenacetin	0.90	0.90
4-Acetamidophenol	0.81	0.85
4-Acetamidophenyl sulphate	0.43	0.53

Table 4.2 The Urinary Metabolites of [^{14}C] Phenacetin in the Cat and Pig

Three male, but sexually immature pigs (large White): 18-20 weeks, 40 kg body wt. were used. Three female mongrel cats; 3 kg body wt. were used. [^{14}C] Phenacetin was injected intraperitoneally as described in the text. Urine was collected for 24 h and chromatographed on Whatman No.1 paper (5 cm strips) in the solvents of Table 4.1. Average values are given with, in parentheses, the value for each animal in order found in column 1.

Species	Dose mg/kg	Dose μCi/animal	^{14}C excreted in 24 h	% of ^{14}C excreted in 24 h found as 4-Acetamidophenyl sulphate*	4-Acetamidophenyl glucuronide=	Other±	4-Acetamidophenol
Cat (B,G,C)	25	3.5	56(93,38,38)	86(87,88,84)	3(2,1,6)	7(7,8,7)	4(4,5,3)
Pig (5,6,4)	25	3.5	31(23,35,37)	10(17,1,14)	54(50,56,60)	20(19,17,25)	16(21,12,15)

* This metabolite was hydrolysed by sulphatase and dilute acid (see text).

= This metabolite was hydrolysed by β-glucuronidase (see text).

± An unidentified metabolite of phenacetin which was not hydrolysed by either sulphatase or β-glucuronidase preparations. This metabolite may be 3[(5-acetamido-2-hydroxyphenyl)]thio alanine (see text).

Table 4.3 Rf values and colour reactions of 4-hydroxyamphetamine and related compounds

For paper chromatography, Whatman No. 1 paper and the descending technique were used. Solvents: B, pyridine-pentan-1-ol-water (7:7:6 by vol): G, butan-1-ol-acetic acid-water (4:1:2). Phenolic compounds were visualised by spraying the chromatograms with freshly diazotized p-nitroaniline followed by 0.5 M KOH in ethanol.

Compound	Rf value in solvents B	G	Colour with diazotized p-nitroaniline
4-Hydroxyamphetamine	0.82	0.79	purple
4-Hydroxynorephedrine	0.68	-	red
4-Hydroxybenzoic acid	0.61	-	dark red
4-Hydroxyhippuric acid	0.50	-	cherry red

Solvents B and G were used in the study of 1- and 2-Naphthol (see Chapter 4)

Table 4.4 Urinary Metabolites of [^{14}C] 4-Hydroxyamphetamine in the Pig

Three male, but sexually immature pigs (large White; 12 - 18 weeks old; 29-36 kg body wt.) were used. [^{14}C]-4-Hydroxyamphetamine was injected intraperitoneally (0.1 mg/kg; 2.9 or 3.6 μCi/animal). Urine was collected for 24 h and chromatographed on Whatman No. 1 paper using solvent system B. (see Table 1). The radiochromatograms were cut into suitable pieces which were then counted. Values are given for individual animals together with the mean values.

Pig	^{14}C excreted in 24 h (% dose)±	% ^{14}C excreted in 24 h found as 4-Hydroxyamphetamine conjugates: glucuronide≠	sulphate+	Unchanged 4-hydroxyamphetamine
4	97	43	8.4	48
5	97	51	0.6	48
6	73	60	7.8	32
Mean	89	51	5.6	43

± In the case of pigs 4, 5 and 6, 61, 35 and 32% of dose respectively in urine in 3 h and for pigs 4 and 6, 84 and 47% of dose respectively in urine in 6 h.

≠ This ^{14}C- peak hydrolysed by β-glucuronidase (Ketodase) to give a ^{14}C-peak with Rf value of 4-hydroxyamphetamine. This hydrolysis by β-glucuronidase was inhibited by saccharo-1,4-lactone.

+ This ^{14}C-peak hydrolysed to free 4-hydroxyamphetamine by the sulphatase preparation (contains both sulphatase and -glucuronidase activity) in the presence of saccharo-1,4-lactone, which inhibited the β-glucuronidase activity of this enzyme preparation.

Table 4.5 Compounds found in pig urine after the administration of 4-Hydroxyamphetamine

The method of administration of 4-hydroxyamphetamine and the collection of urine are described in the text.

Metabolites found in urine+	Rf value (solvent‡) of metabolite
4-Hydroxyamphetamine	0.80 (B), 0.79 (G)
4-Hydroxyamphetamine sulphate	0.54 (B)
4-Hydroxyamphetamine glucuronide	0.15 (B), 0.26 (G)

+ See Table 2 for % ^{14}C excreted in 24 h found in each metabolite

‡ The letters in this column refer to solvents given in Table 1

Table 4.6 Chromatography of Morphine and its Metabolites

For paper chromatography, Whatman No. 1 paper and the descending technique were used; for T.L.C. plates were prepared with fluorescent silica gel (see text). Solvents; paper chromatography; E, Benzene: Methanol: Amyl alcohol: Water: Pyridine, (9:7:3:2:1 by vol); T.L.C.:A, Butan-1-ol: Acetone: 5% Acetic Acid: Ammonium hydroxide 5%: Water (45:15:10: 10:20 by vol.); B, Ethanol:dioxan:benzene:con.NH_4OH (5:40:50:5 upper phase); C, n-butanol: Acetic acid (glacial): Water (4/1/5 v/v. upper phase); D, Chloroform: methanol, (3:1 v/v.) Morphine and its metabolites were visualized by spraying the chromatograms with freshly diazotized p-nitroaniline followed by 0.5 M KOH in ethanol when a reddish-brown colour was obtained.

	Rf Values in Solvents				
	T.L.C.				Paper Chromatography
Compound	A	B	C	D	E
Morphine	0.42	0.1	0.44	0.22	0.76
Morphine-3-glucuronide+	0.15	-	0.35	-	-
Morphine-6-glucuronide+	0.25	-	0.45	-	-
Morphine-3-sulphate	0.33	0.0	0.24	0	0.22

+ The values quoted for these glucuronide conjugates were obtained from the literature (see text).

Table 4.7 Urinary metabolites of [^{14}C] morphine in Cat

Two female and one neutered male mongrel full grown cats were used. [N-^{14}C methyl] morphine hydrochloride (10μCi/animal), together with sufficient carrier morphine to give a dose of 5 mg/kg all dissolved in 1 ml of water was injected intraperitoneally. Urine was collected for 24 h then chromatographed in the solvents of Table 5.1.

Cat	Sex	Wt. (Kg)	^{14}C excreted in 24 h (% dose)	% ^{14}C excreted in 24 h found as:- morphine conjugates: glucuronic acids	morphine-3-sulphate‡	Other+	Morphine
1	F	3	85	nd	67	1	33
2	M	3	91	nd	73	1	27
3	F	3	87	nd	74	1	24
MEAN			88	nd	71	1	28

‡ This peak was hydrolysed by a sulphatase preparation from (Helix pomatia) and also by dilute acid as described by Garton and Williams,(1949). From its chromatographic values concluded it was morphine-3-sulphate.

+ This peak was only detected in solvent E (paper chromatography). It was hydrolyzed under the conditions above suggesting it was a sulphate, it was concluded that it was morphine-6-sulphate.

Table 4.8 The Chromatography of Phenolphthalein and its Metabolites

For paper chromatography Whatman No. 1 paper and the descending technique were used. Solvents: (1) Butan-1-ol-Acetic Acid (glacial)-Water (4/1/2); (2) Butan-1-ol-Ethanol-Acetic acid (glacial) water (32/8/1/12); (3) Propan-1-ol-Ammonia (7/3); (4) Pyridine-Pentan-1-ol-Water (7/7/6); (5) Propan-1-ol-Pyridine-Water (1/1/1). Phenolphthalein was identified by spraying with glycine buffer (pH 10.4) when they gave a red colour. Phenolphthalein disulphate gave this colour after hydrolysis by spraying with dilute HCl., warming at 100°C for 2 mins. then spraying with buffer as above. Phenolphthalein glucuronide was identified by spraying with 1% Naphtho-resorcinol in 33% Trichloracetic acid when it gave a blue colour on warming at 100° for 15 min. Phenolphthalein, and hydrolyzed phenolphthalein disulphate were also visualised by using freshly diazotized p-nitroaniline followed by 0.5 M KOH when they appeared as yellow-orange spots.

Compound	Rf value in Solvent 1	2	3	4	5
Phenolphthalein	0.91	0.93	0.90	0.91	0.90
Phenolphthalein monoglucuronide	0.89	0.85	0.50	0.55	0.78
Phenolphthalein disulphate	0.55	0.46	0.44	0.25	0.50

Table 4.9 Urinary Metabolites of [^{3}H] Phenolphthalein in the Cat

Two female and one neutered male full grown mongrel cats (3-3.5 kg) were used. The phenolphthalein (20 μCi/animal, 25 mg/kg) was administered intraperitoneally (see text). The urine was collected for 24 h then chromatographed in the solvents of table 4.7. The chromatograms were then cut into 1 cm pieces and the associated ^{3}H determined by the Tricarb Scintillation Spectrometer.

Cat	Sex	Wt (Kg)	^{3}H excreted in 24 h (% dose)	% ^{3}H excreted in 24 h found as Phenolphthalein	glucuronide+	‡Phenolphthalein monosulphate
3	M	3.5	19	67		33
4	F	3	45	53		47
5	F	3	17	59		41
MEAN			27	60		40

+ Hydrolysed by Ketodase, but not in the presence of saccharo-1,4lactone.

‡ Hydrolysed by sulphatase or dilute acid as described by Garton, et al., (1949) only

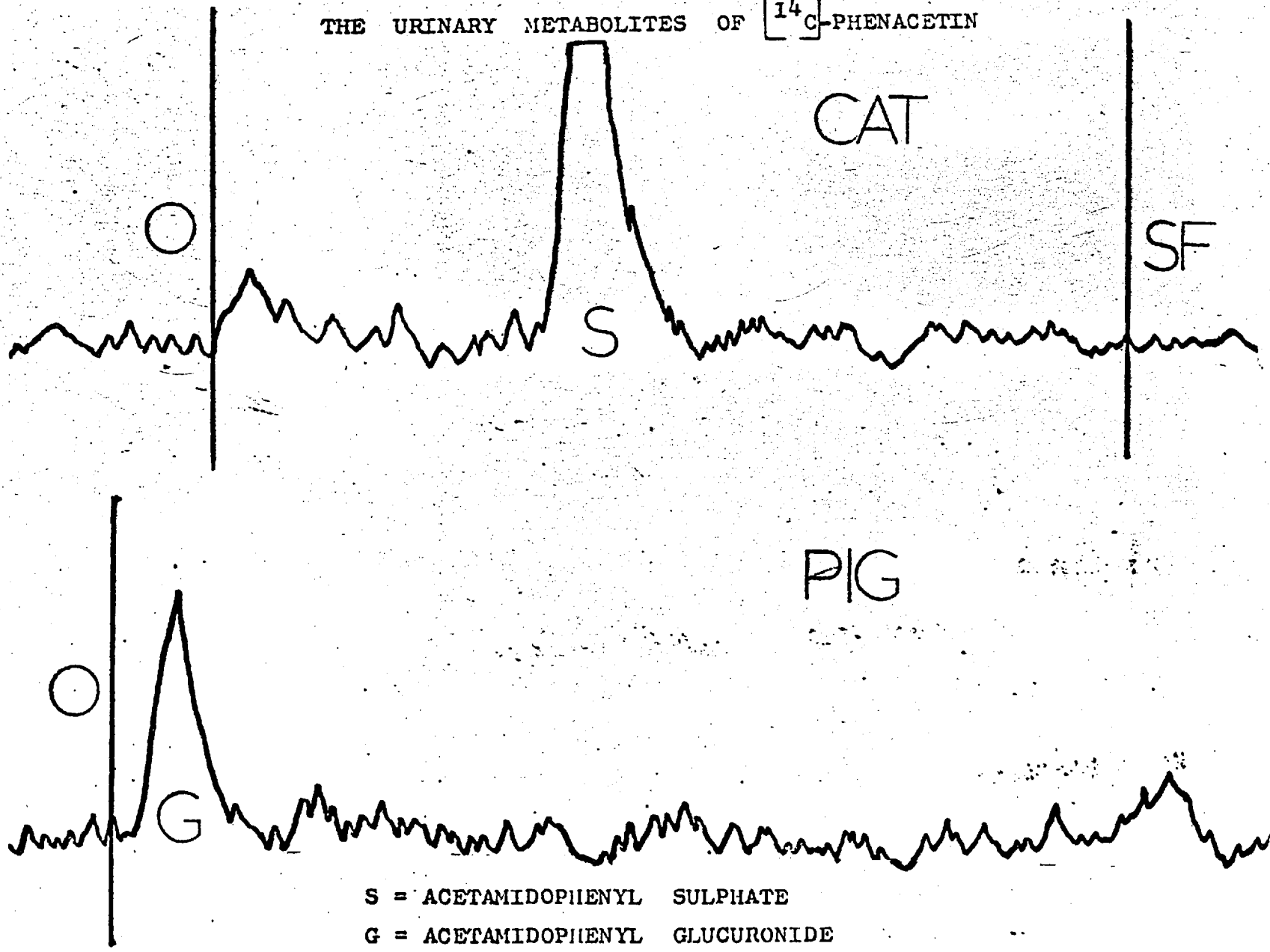
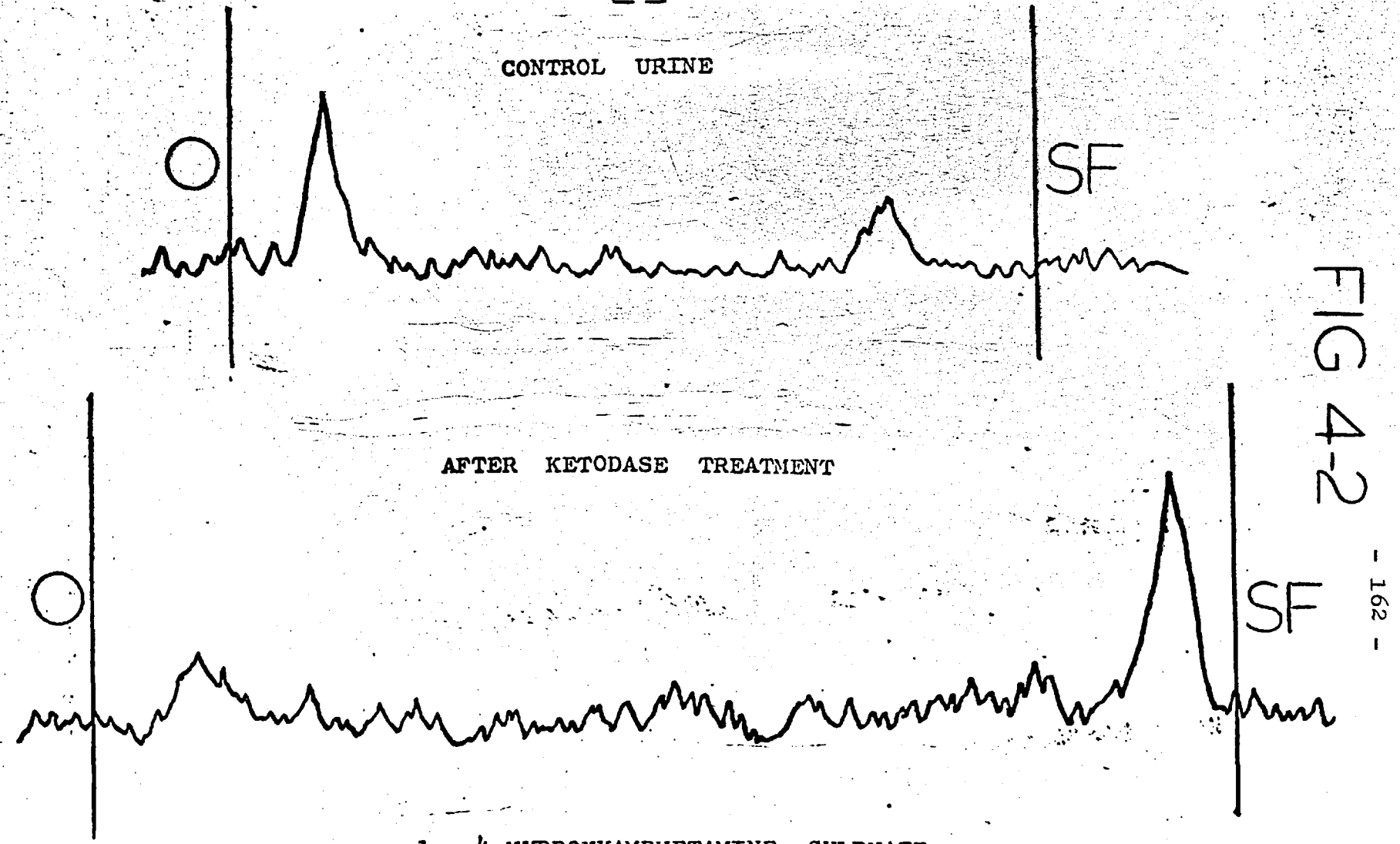

THE URINARY METABOLITES OF [14C]-PHENACETIN
CAT
O
S
SF
PIG
O
G
SF
S = ACETAMIDOPHENYL SULPHATE
G = ACETAMIDOPHENYL GLUCURONIDE

FIG 4-1

THE URINARY METABOLITES OF [^{14}C] 4-HYDROXYAMPHETAMINE IN PIG



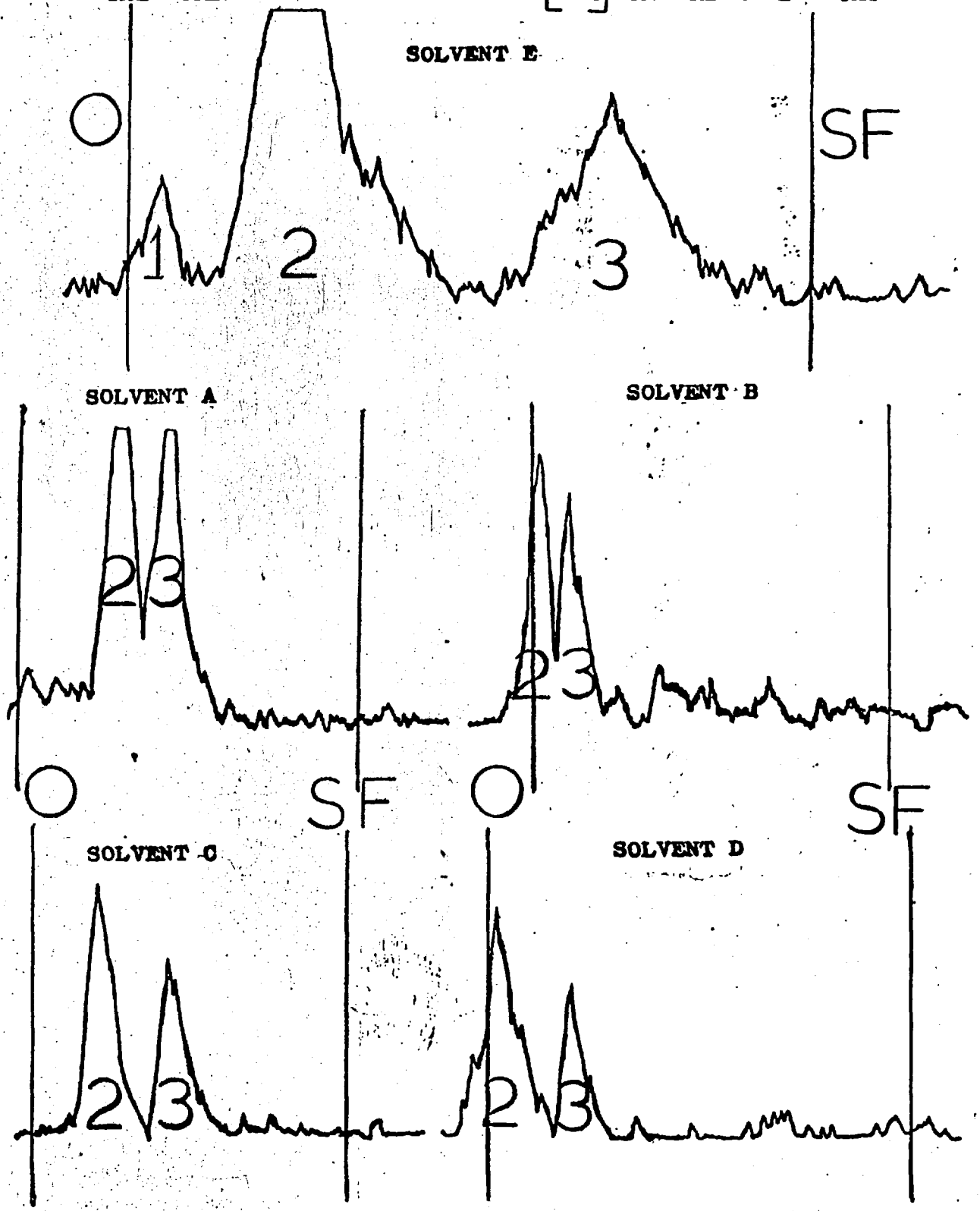
1 = 4-HYDROXYAMPHETAMINE SULPHATE

2 = FREE 4-HYDROXYAMPHETAMINE

FIG 4-2

FIG 4-3

THE URINARY METABOLITES OF [^{14}C] MORPHINE IN CAT

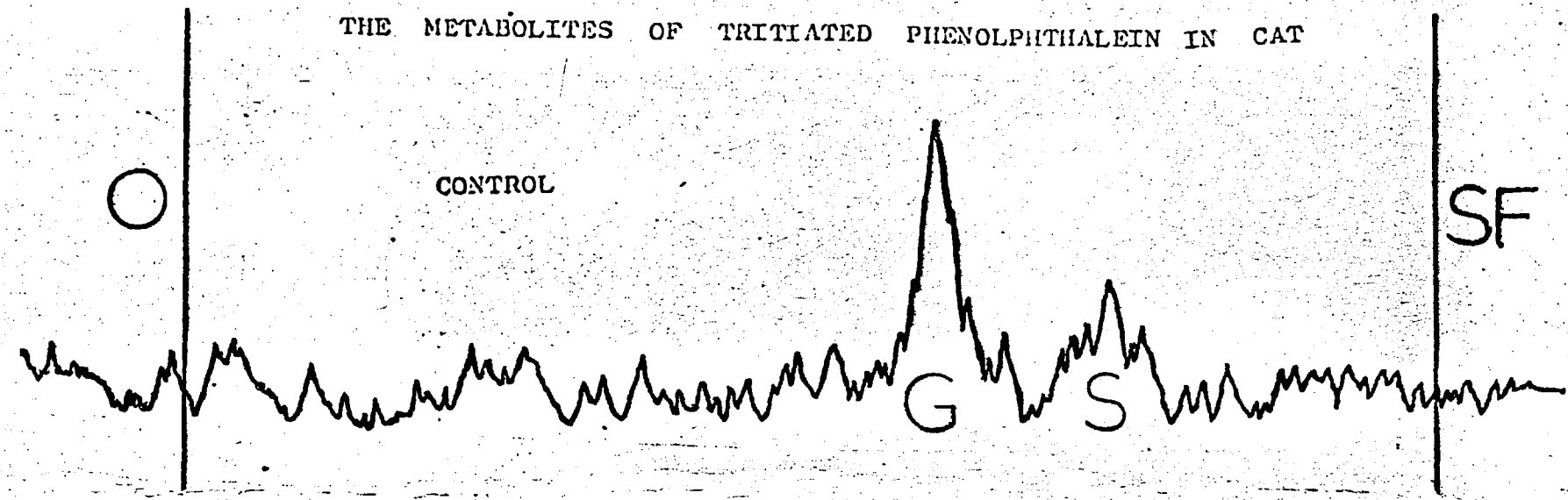


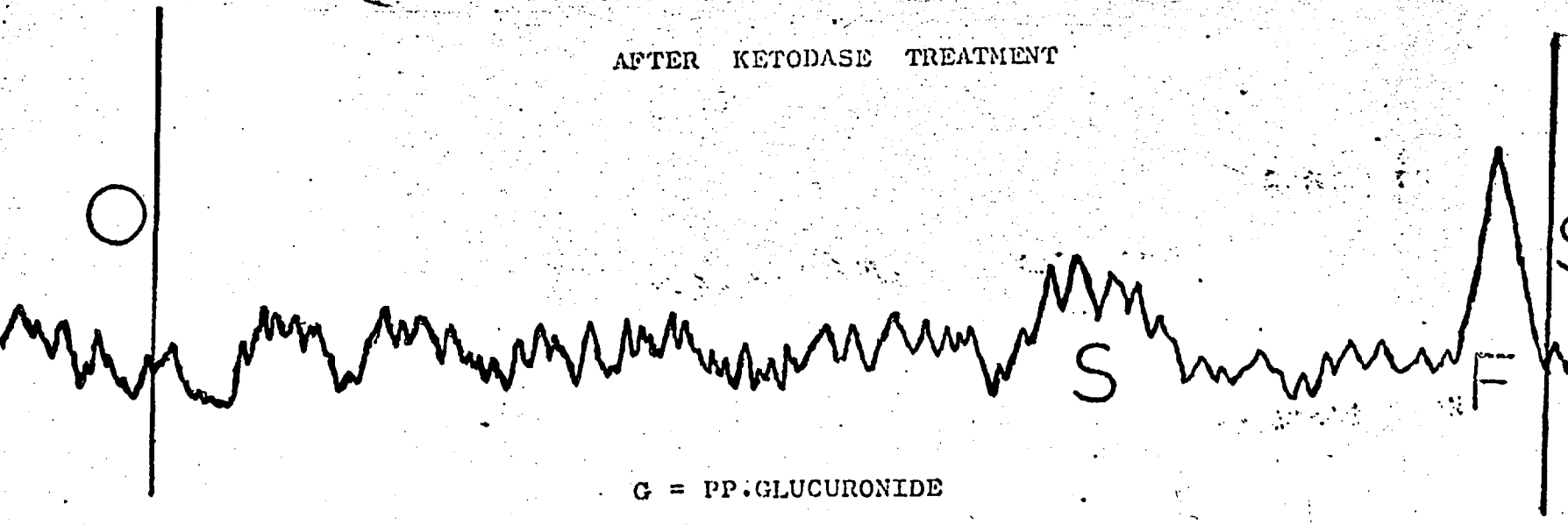
1 = MORPHINE-6-ETHEREAL SULPHATE

2 = MORPHINE-3-ETHEREAL SULPHATE

3 = FREE MORPHINE

THE METABOLITES OF TRITIATED PHENOLPHTHALEIN IN CAT





G = PP.GLUCURONIDE

S = PP.SULPHATE

F = FREE PHENOLPHTHALEIN

FIG 4-4

CHAPTER FIVE

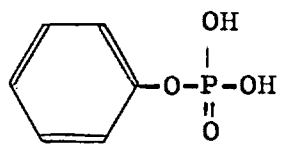
PHENYL DIHYDROGEN PHOSPHATE, A NEW METABOLITE OF PHENOL IN THE CAT

Contents

Tables and Figures

Introduction

Chapter Two shows that in the cat [^{14}C] phenol is excreted in the urine mainly as phenyl sulphate with some quinol sulphate and just detectable amounts (<1% of the dose) of phenyl glucuronide. However, there was also found an appreciable amount (up to 8% of the dose) of an unknown metabolite. Thus, radiochromatogram scans of cat urine showed, when the chromatograms were developed in the solvents of Table 5.1, a small unidentified ^{14}C peak at the origin. This ^{14}C peak was detected when [^{14}C] phenol was administered both orally and intraperitoneally at 5 and 25 mg/ml (see Table 2.3, Chapter Two). This Chapter describes the identification of this unknown metabolite of phenol in the cat as phenyldihydrogen phosphate.



phenyl dihydrogen phosphate

The formation *in vivo* of the phosphates of foreign organic compounds is a conjugation reaction which has been rarely observed in the past. Troll, *et al.*, (1959) detected a new metabolite of 2-naphthylamine in the urine of dogs.

Enzymic studies indicated that this was di(2-amino-1-naphthyl) hydrogen phosphate. This was confirmed by Boyland, et al, (1961) who synthesized the new metabolite. Radomski, et al, (1967) administered 1- and 2-naphthylamine to dogs and although di(2-amino-1-naphthyl) hydrogen phosphate was detected, the comparable diester was not present in the urine of dogs treated with 1-naphthylamine.

Di(2-amino-1-naphthyl) hydrogen phosphate has aroused some interest as a detoxication product because 2-naphthylamine has been implicated as a causitive agent of bladder cancer in dogs and humans exposed to this chemical. In these two species 2-naphthylamine is hydroxylated to 2-amino-1-naphthol, which is excreted as the sulphate and glucuronide conjugates and also as the phosphate derivative. 2-Amino-1-naphthol is thought to be the active agent in the induction of bladder tumours by 2-naphthylamine and hydrolysis of the above conjugates in the bladder would release this known carcinogen. The sulphate ester of 2-amino-1-naphthol is apparently not hydrolysed by any known naturally occuring sulphatase and the glucuronide conjugate, although hydrolysed by β-glucuronidase which is present in the urine, has also been found in the urine of animals not

susceptible to bladder tumour formation when fed 2-naphthylamine (Troll, et al., 1963). However, it has been suggested that di(2-amino-1-naphthyl) hydrogen phosphate is the probable cause of the bladder cancer because this conjugate is excreted in dogs but not in rabbits, which do not develop bladder tumours in response to 2-naphthylamine, and furthermore the bladder epithelium is rich in phosphatases (Troll, et al., 1959;1963),(Schmidt, 1961).
Di(2-amino-1-naphthyl) hydrogen phosphate contains two molecules of 2-amino-1-naphthol to one of phosphoric acid. Boyland, et al.,(1961) examined the urine of animals treated with 2-naphthylamine for the monoaryl phosphate conjugate containing one molecule each of 2-amino-1-naphthol and phosphoric acid (i.e. 2-amino-1-naphthyl dihydrogen phosphate) and failed to detect it.

Lotlikar & Wasserman,(1970) showed that N-acetyl-N-hydroxy-2-aminofluorene interacts with phosphate buffer at pH7 to produce water-soluble material which was characterised as the N-O-phosphate. The phosphate ester of N-acetyl-N-hydroxy-2-aminofluorene was also formed by an ATP and Mg^{2+} dependant enzyme-catalyzed system from rat liver in vitro. The evidence for the formation of this phosphate in

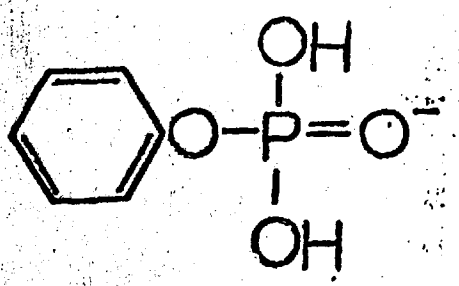
the above *in vitro* system is, however, equivocal and has been reviewed by Irving,(1970). This phosphate conjugate has never been detected in rat urine following administration of *N*-acetyl-*N*-hydroxy-2-aminofluorene to rats and it has been suggested that this is due to the activity of phosphatases in bladder epithelia (Schmidt, 1961).

Monoethyl phosphate has been isolated from the livers of rats given large doses of ethanol intraperitoneally. Using ^{14}C labelled ethanol and $[^{32}P]$ phosphate the phosphorylation was demonstrated *in vitro* with a rat liver preparation, subsequent hydrolysis yielding radioactive ethanol and ^{32}P-labelled phosphoric acid (Tomaszewski & Buchowitz, 1972).

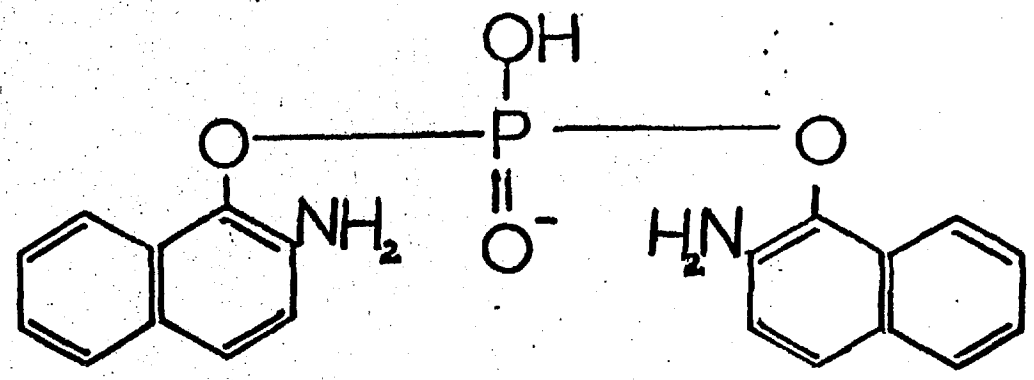
Phosphate conjugates have been found in some insects. 1-Naphthol, for example, as well as being conjugated with glucose and sulphate, also appears to be conjugated with phosphate. Thus, houseflies, blowflies and New Zealand grass grubs (but not locusts) all excrete 1-naphthyl dihydrogen phosphate as a metabolite of 1-naphthol, this conjugate being the major metabolite in grass grubs (Binning, *et al.*, 1967). Heenan and Smith,(1967) showed that *p*-nitrophenol and 1-naphthol form glucoside-6-phosphates in houseflies and

SOME PHOSPHATE CONJUGATES OCCURRING IN NATURE

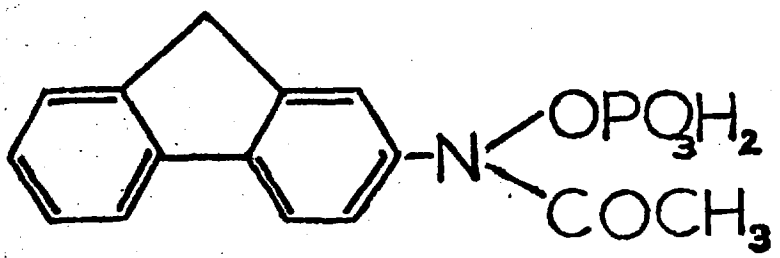
PHENYL PHOSPHATE (CATS)



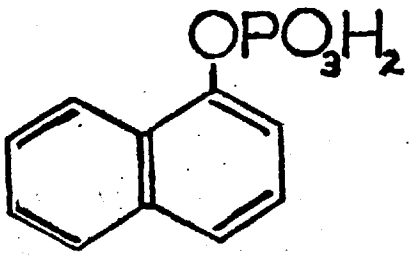
DI(2 AMINO-1-NAPHTHYL) HYDROGEN PHOSPHATE (DOGS)



N-ACETYL-N-HYDROXY-2 AMINOFLUORENE (AAF) (RATS)



1-NAPHTHYL PHOSPHATE (SOME INSECTS)



blowflies, but in these conjugates the phosphate group is not attached directly to the phenol.

Materials and Methods

Phenol m.p. 43° was purchased from British DrugyHouses, Poole, Dorset and recrystallised from aqueous ethanol. Amberlite XAD-2 resin and disodium phenyl phosphate were also purchased from British Drug Houses. [U-^{14}C] Phenol specific activity 266 μCi/mg, and disodium hydrogen [^{32}P] phosphate, specific activity 200 μCi/ mol., were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Acid and alkaline phosphatase were purchased from the Sigma Chemical Company, London. Phenyl β-D-glucoside was obtained from the Sigma Chemical Co. Ltd., London. The other compounds are as described for the study of phenol in Chapter 2.

Animals

Details of cats used are given in appendix 1. [^{14}C] Phenol dissolved in water was administered orally or intraperitoneally, (10 μCi per animal) together with sufficient carrier phenol to give a final dose of 25 mg/kg.

Two cats were each given intraperitoneally in 1 ml of water

1 mg of disodium hydrogen [^{32}P] phosphate on two consecutive days. On the second day they were also given in 1 ml of water neutralised by 2.5 M NaOH, 25 mg of phenol per kg.

The animals were placed in suitable metabolism cages and their urine collected daily in receptacles containing a few drops of 2 N-HCl to lower the pH and prevent bacterial breakdown of conjugates.

Radiochemical Techniques

^{14}C was estimated as described in Chapter Two. ^{32}P radioactivity was determined using a Packard Tricarb Scintillation Spectrometer (Model 3320). The settings of the instrument were optimally adjusted (Gain 13.5%, window C→D 50→∞) and the radioactivity reading in this channel (Blue) was taken. It was found that counting efficiencies of 95-100% were obtained. In some experiments the paper strip chromatograms were cut into 1 cm pieces and placed in dioxan scintillatior and the associated ^{32}P radioactivity determined.

Chromatography

The unknown metabolite was detected on radiochromatogram

scanning of the urines of cats which had received [^{14}C] phenol at 5 or 25 mg/kg orally or intraperitoneally when it appeared as a small peak at the origin of the chromatograms. The largest quantity of the unknown ^{14}C peak was observed on radiochromatogram scanning of the urine of cats receiving [^{14}C] phenol intraperitoneally. Greater quantities of the unknown metabolite were observed following intraperitoneal rather than oral administration at both 5 and 25 mg phenol/kg.

With the solvents employed for the detection of phenyl or quinol sulphates and glucuronides (see Chapter Two, Table 2.2) the unknown metabolite remained at the origin of the chromatogram. Six other solvents were used and the Rf value of the unknown metabolite increased (see Table 5.1 and Fig 5.1).

When urine containing ^{32}P was chromatographed a very large peak was found at the origin due to inorganic [^{32}P] phosphate (see Fig 5.2). This would have "overshadowed" any smaller peak due to phenyl phosphate, so that a means of reducing the inorganic phosphate content of the urine was required; Amberlite XAD-2 resin was employed. A column of this resin, 2 cm in diameter and 40 cm long was constructed. This column was washed with 3 x 25 mls of distilled water. The

urine was then added to the column and the inorganic $[^{32}P]$ phosphate washed through with water. The aromatic conjugate was then eluted from the column with analar methanol (3 x 25 ml), the volume of which was reduced to 1 ml, using the rotary evaporater. Samples (0.1 ml) of this concentrated methanol eluate were then chromatographed (in solvent 1, Table 5.1; see also Fig 5.2).

Enzyme Hydrolyses

The unknown metabolite of $[^{14}C]$ phenol observed on radiochromatogram scanning had an Rf value of 0.25 in Solvent 1 of Table 5.1. This peak was not observed however in urine which had been incubated with alkaline phosphatase under the following conditions, 1 ml of cat urine + 1 ml of alkaline phosphatase (20 mg/ml in 0.1 M glycine buffer, pH 10.4). In the control incubate boiled enzyme was used. Both incubations were at 37° for 18 h. The incubated urines (0.2 ml) were then chromatographed as described above and the distribution of ^{14}C metabolites determined with the radiochromatogram scanner. (Under these conditions standard disodium phenyl phosphate (100 mg in 1 ml of fresh normal cat urine) was hydrolysed and the resultant phenol detected with

the Folin-Coicalteau reagent).

Similar incubations under appropriate conditions were performed with other enzymes; β-glucuronidase (Ketodase), sulphatase and β-glucosidase (emulsin). In all the cases, the relevant authentic phenol conjugates for these enzymes were hydrolysed to phenol which was detected as above. All the urines used for these incubations contained no $HgCl_2$ (as a bacteriostat) since this might inhibit enzyme activity.

Hydrolysis of the unknown metabolite liberating [^{14}C] phenol and its identification by reverse isotope dilution

Cat urine (0.3 ml) was chromatographed on Whatman No.1 paper (5 cm strip) in solvent 1 (Table 5.1) and the unidentified ^{14}C-labelled metabolite located by radiochromatogram scanning. The area of the paper chromatogram corresponding to the unknown metabolite was eluted with water (10 ml). Twelve chromatograms were treated in this way and the eluates pooled. The combined eluates were concentrated at reduced pressure at 40° to a small volume. A 1ml aliquot of this concentrated eluate was then removed and incubated with alkaline phosphatase as described above. A second aliquot (1 ml) was incubated with boiled enzyme as a control. Phenol (100 mg) was added to the incubates,

followed by sufficient bromine-water (approximately 8 ml) to cause 2,4,6-tribromophenol to precipitate immediately. The 2,4,6-tribromophenol was filtered, washed with cold ethanol, and recrystallised from a mixture of petroleum-ether (b.p.60-80°) and 1,2-dichloroethane. The radioactive recrystallised product had m.p. 94° which agrees with the reported melting point of 2,4,6-tribromophenol.

Isolation of the Unknown Metabolite from Urine and Mass Spectroscopy

The combined urines collected for 24 h. from 3 cats which had received [^{14}C] phenol (25 mg/kg intraperitoneally) was filtered through Whatman No.44 paper. The urine was then concentrated at reduced pressure to a small volume using a rotary evaporator; the pH of the urine was alkaline (8-9) and the temperature maintained below 55°. The viscous urine concentrate was cooled in ice and then acidified by the slow addition of 11M-HCl (5 ml). After keeping at 4° for 1 h, the acidified concentrate was extracted by prolonged shaking with chloroform (3 x 50 ml). The chloroform extracts were evaporated to a brown oil which crystallized on keeping at 4° for 5 days in an evacuated desiccator. The radioactive

product was extracted from the brown oil with a small quantity of chloroform to give colourless crystals which were repeatedly recrystallized from chloroform to a constant m.p. of 98°. Phenyl-phosphoric acid has a m.p. 97-98° (Jacobsen, 1875).

An authentic sample of disodium phenyl phosphate (1 g) was dissolved in water (5 ml), 11 M-HCl (5 ml) added, and phenyl phosphoric acid crystals (m.p. 98°) isolated in a similar fashion to the crystals prepared above from cat urine.

Preparation of the dimethyl ester of phenyl phosphoric acid

The ^{14}C-labelled phenylphosphoric acid isolated from the urine of cats dosed with $\boxed{^{14}C}$ phenol and the phenyl phosphoric acid prepared from authentic disodium phenyl phosphate were converted to their dimethyl esters by methylation with diazomethane. Both acids were dissolved in methanol (1 mg/ml). 1 ml of each solution was placed in separate vials and the methanol evaporated with a stream of nitrogen. When the volume had been reduced to dryness, 1 ml of a freshly prepared solution of diazo-methane in ice-cold ether was added to each vial (see Vogel, 1956).

Mass Spectroscopy

The methanolic solutions of the urinary and standard phenyl phosphoric acids and their dimethyl derivatives were examined in a Varian MAT CH5 mass spectrometer. The mass spectra were determined by direct insertion; probe temperature 170° for phenyl dihydrogen phosphate and 135° for its dimethyl ester; source temperature 180°. The electron-beam energy was 70 eV.

Results and Discussion

From Table 5.1 we see that the unknown metabolite of $[^{14}C]$ phenol has an Rf value almost identical to that of standard disodium phenyl phosphate in the six solvents used.

Cat urine containing this metabolite was heated with dilute acid (under which conditions sulphate conjugates are hydrolysed as described in Chapter Two see Garton, et al., (1949). Following this hydrolysis the unknown metabolite was not detected on chromatography and radiochromatogram scanning of the urine. Under these conditions authentic disodium phenyl phosphate dissolved in cat urine was also hydrolysed and the phenol liberated was detected using the Folin-Coicalteau reagent.

The unknown ^{14}C-metabolite was not detected on radiochromatogram scanning of urine incubated with alkaline phosphatase. Furthermore, using this enzyme a solution of standard disodium phenyl phosphate in control cat urine was also hydrolysed, the free phenol liberated being detected with Folin-Coicalteau's reagent. Boiled alkaline phosphatase was also incubated with a sample of the cat urine as a control and the unknown metabolite was not hydrolysed (see Fig 5.1). Incubations with β-glucuronidase, sulphatase or β-glucosidase did not hydrolyze the unknown metabolite. Thus it appears from these preliminary enzyme hydrolyses that the unknown metabolite is a phosphate conjugate. It is likely that this conjugate was a monophosphate since alkaline phosphatase only attacks monophosphates.

Wong , (1972) suggested that glucosides might be formed as alternative metabolites to glucuronides in cat. However, the unknown metabolite was shown not to be a glucoside because it was not attacked by β-glucosidase (emulsin) and the Rf value of phenyl glucoside is different from that of phenyl dihydrogen phosphate (see Table 5.1).

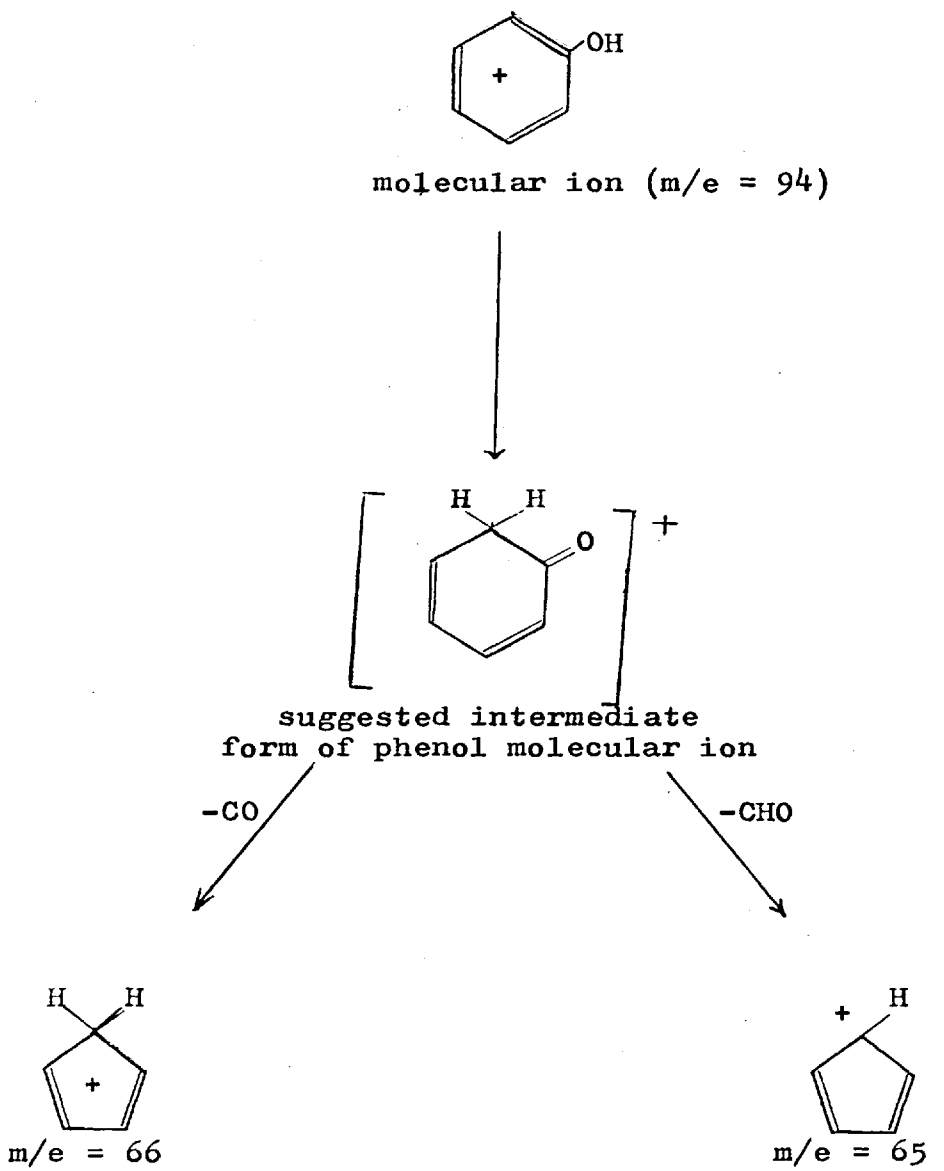
The liberation of $\boxed{^{14}C}$ phenol from the radioactive metabolite by alkaline phosphatase was demonstrated as follows. The unidentified ^{14}C-metabolite was eluted from twelve

chromatograms and the unidentified combined eluates concentrated to a small volume and this concentrated eluate hydrolysed with alkaline phosphatase. This gave $[^{14}C]$ phenol which was isolated as radioactive 2,4,6-tribromophenol (see methods). In control experiments using inactivated alkaline phosphatase no radioactivity was detected in the 2,4,6-tribromophenol isolated as above.

The ^{14}C-labelled phenyl phosphoric acid extracted from cat urine (see Methods) was shown to have the same m.p. (98^{o}) as the acid prepared from disodium phenyl phosphate. This agrees with the reported m.p. of monophenyl phosphoric acid (see Jacobsen, 1875).

When the phenyl phosphoric acid crystals isolated from cat urine and the authentic sample were investigated by mass spectrometry the spectra of both samples were almost identical (see Fig 5.3). The phenyl phosphoric acid isolated from cat urine showed a very small molecular ion at m/e 174. Ions characteristic of phenol were observed in both spectra (see Fig 5.3). (The mass spectrum of phenol shows peaks with m/e values of 94, 66, 65, 55, 51 and 50 respectively, see Beynon, et al., 1968). The peak with m/e 94 corresponds to the parent ion. The peaks at m/e 66 and 65 probably arise

by loss of CO and CHO respectively from the phenol molecule.



The peak at m/e 77 could be a phenyl $(C_6H_5)^+$ ion

The methylated derivative of authentic phenyl dihydrogen phosphoric acid and the sample isolated from cat urine were almost identical (see Fig 5.4). Both spectra showed a peak at m/e 202 (parent ion). Some of the peaks at m/e, 168, 141, 139, 128 and 115 are also found in the spectra of the free acid.

Thus from the mass spectroscopy studies we see that the unknown ^{14}C metabolite isolated from cat urine is phenyl dihydrogen phosphoric acid. Also the presence of phenol in this compound was confirmed by the recognition of some of the molecular fragments.

When the amount of the inorganic ^{32}P-radioactivity present in the urine was reduced in the manner described in Methods subsequent chromatography and radiochromatogram scanning showed a distinct peak corresponding to monophenyl phosphoric acid in the solvents of Table 5.1. This peak was not detected in day one cat urine before phenol had been administered (see Fig 5.2). From this experiment it is concluded that in cat phenol is conjugated with phosphate in vivo. It was not possible to calculate the percentage of the dose of phenol conjugated with phosphate, because endogenous phosphate would also be available for conjugation with phenol.

From these results it is concluded that monophenyl phosphoric acid is formed as a minor metabolite of phenol in the cat. Phosphate conjugates are rare in nature and it is interesting to note that in the cat a phosphate conjugate was only detected after phenol was administered; phosphate conjugates of 1- and 2-naphthol, phenacetin, morphine or phenolphthalein were not detected (see Chapters Two, Three and Four). It could be postulated that the enzymes forming phosphates (in the case of cat presumably a "phenol kinase") are relatively substrate specific. This would be expected since the endogenous substrates for kinases are often substrates of intermediary metabolism, and the reactions catalysed are irreversible and important in that they "activate" many compounds (e.g. glucose-6-phosphate, fructose-1,6-diphosphate). It is to be expected therefore that the active sites of these enzymes have evolved to determine relatively high substrate-specificity and consequently kinases might not be suitable as detoxication enzymes. In the case of cat the presence of other groups on the ring might cause steric hinderance at the active site of the enzyme and thus only phenol would be suitable as a substrate for the kinase.

Table 5.1 The Chromatography of Phenyl Phosphate

Paper chromatography using 5 cm strip of Whatman No.1 paper and the descending technique were used. Disodium phenyl phosphate was visualised (a) by spraying chromatograms with concentrated nitric acid, heating the paper at 100°C for 2 minutes followed by spraying with 4% ammonium molybdate and warming the paper until a dark blue-brown colour on a yellow background appears, and (b) by spraying with cobalt chloride (1% solution in acetone) followed by gentle heating until blue spots develop (see Donner and Lohs, 1965).

Solvent	Propan-1-ol Ammonia (S.G.0.88) (1/1)v/v (1)	Ethanol Water (7/3)v/v (2)	Butan-1-ol: Acetic Acid: (Glacial) Water (12:3:5)by vol (3)	Butan-1-ol Ethanol Water (17:3:20)by vol (4)	Pyridine: Pentan-1-ol: Water (7:7:6)by vol (5)	Butan-1-ol Acetic Acid (Glacial): Water (4:1:2)by vol (6)
UNKNOWN Rf by radiochromatogram scanning	0.25	0.40	0.16	0.24	0.03	0.25
Disodium Phenyl Phosphate	0.24	0.44	0.17	0.24	0	0.25
Phenyl β-D glucoside	0.65	-	-	-	0.50	0.70

Table 5.2

The Urinary Excretion of ^{32}P following the administration of disodium hydrogen [^{32}P] phosphate to cats

Two cats were each given intraperitoneally in 1 ml of water 1 mg of disodium hydrogen [^{32}P] phosphate on two consecutive days. On the second day they were also given in 1 ml of water neutralized by 2.5 M NaOH, 25 mg of phenol/kg. Urine was collected over 48 h and counted as described in the text.

	% Excretion of ^{32}P radioactivity over 48 h			
	0-4	4-6	6-24	Total
DAY 1 Cat One	64	8	12	85
Cat Two	56	22	19	97
DAY 2 Cat One	-	48	32	79
Cat Two	49	23	33	105

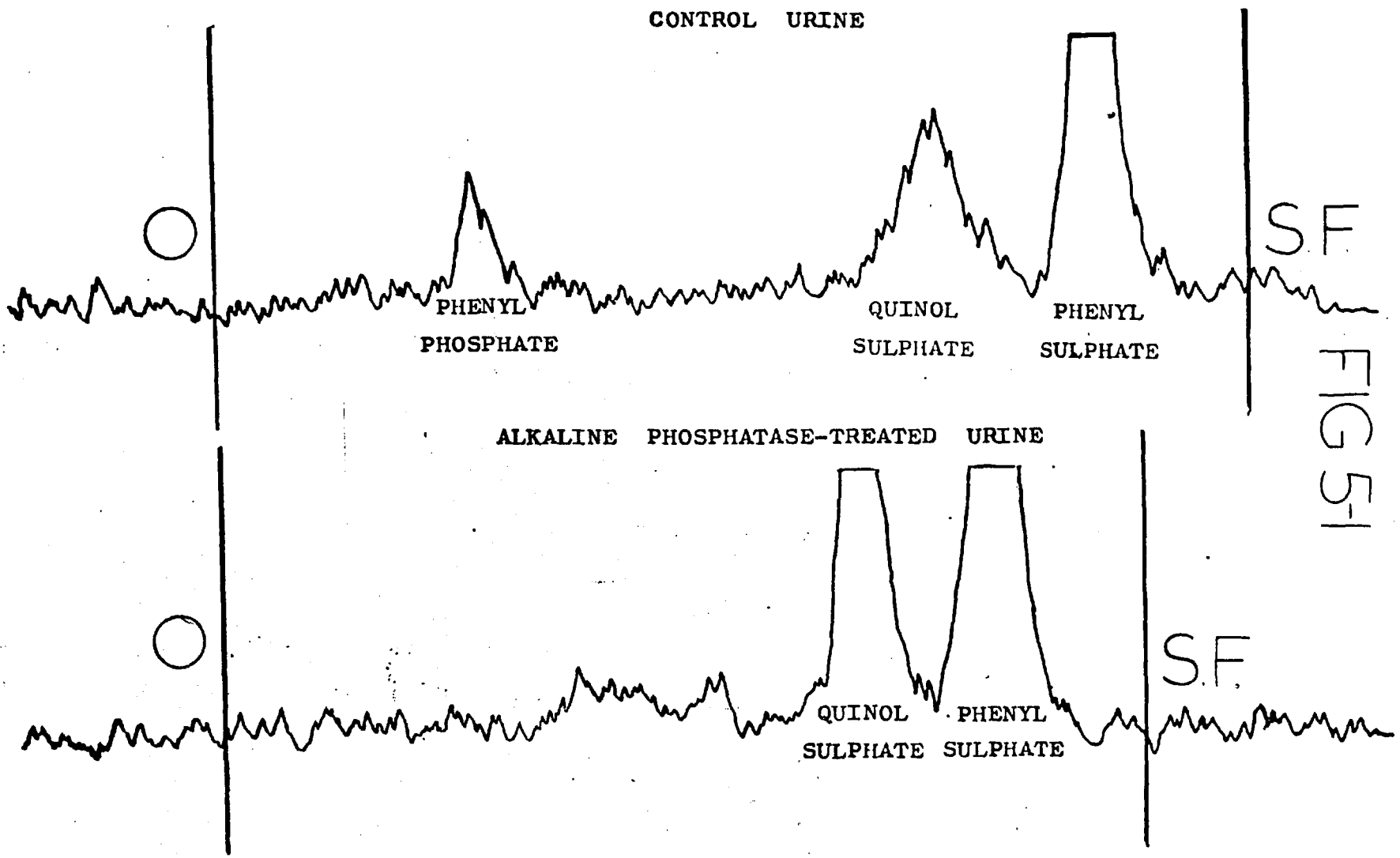
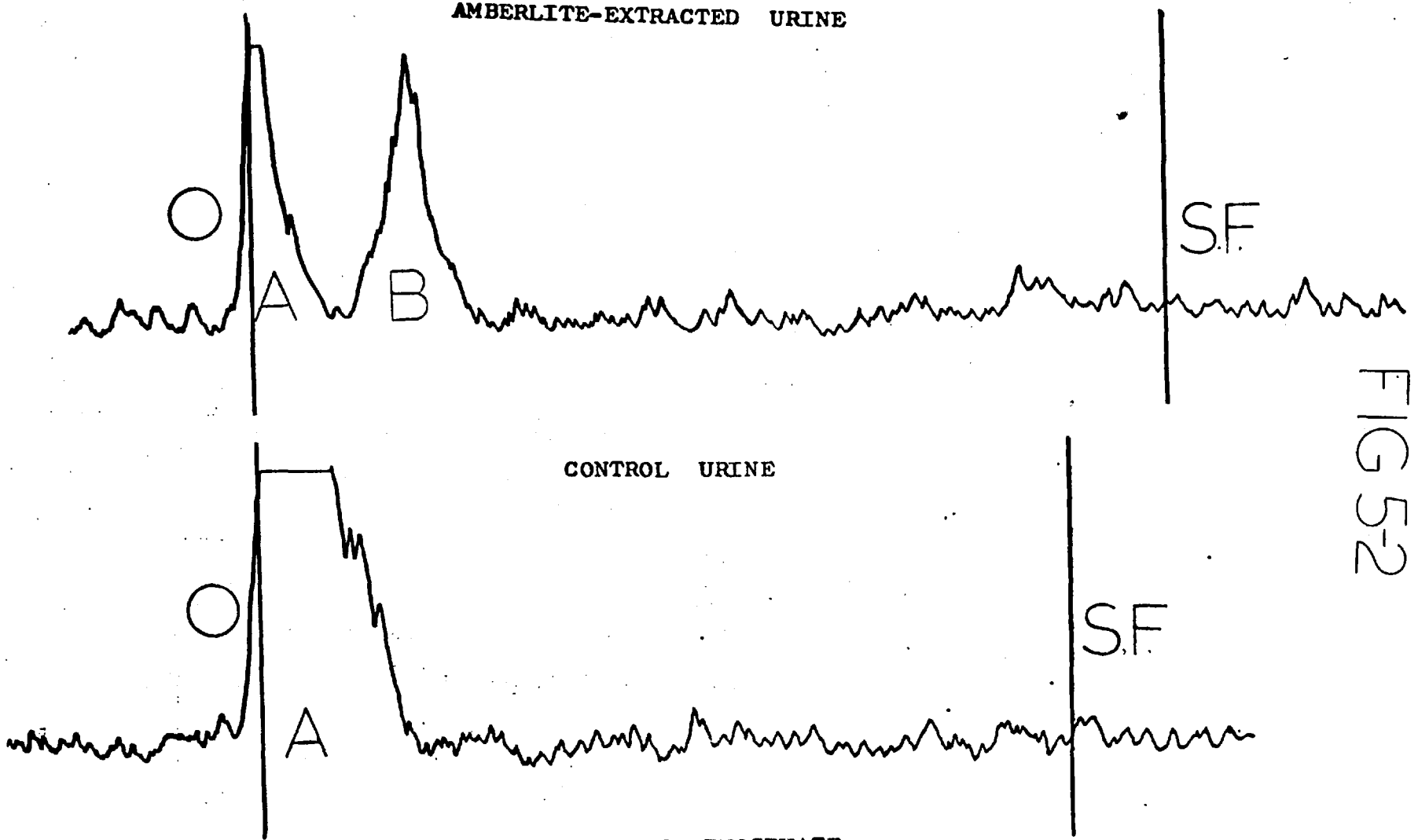

IDENTIFICATION OF [14C] UNKNOWN PHENOL CONJUGATE IN CAT URINE
CONTROL URINE
O
PHENYL PHOSPHATE
QUINOL SULPHATE
PHENYL SULPHATE
S.F.
ALKALINE PHOSPHATASE-TREATED URINE
O
QUINOL SULPHATE
PHENYL SULPHATE
S.F.
SOLVENT = PROPANOL: AMMONIA (1:1, v/v.)

FIG 5-1

DETECTION OF [^{32}P] PHENYL PHOSPHATE IN CAT URINE



A = INORGANIC PHOSPHATE

B = PHENYL PHOSPHATE

FIG 52

Fig 5.3 Mass Spectra of Phenyl Phosphoric Acids

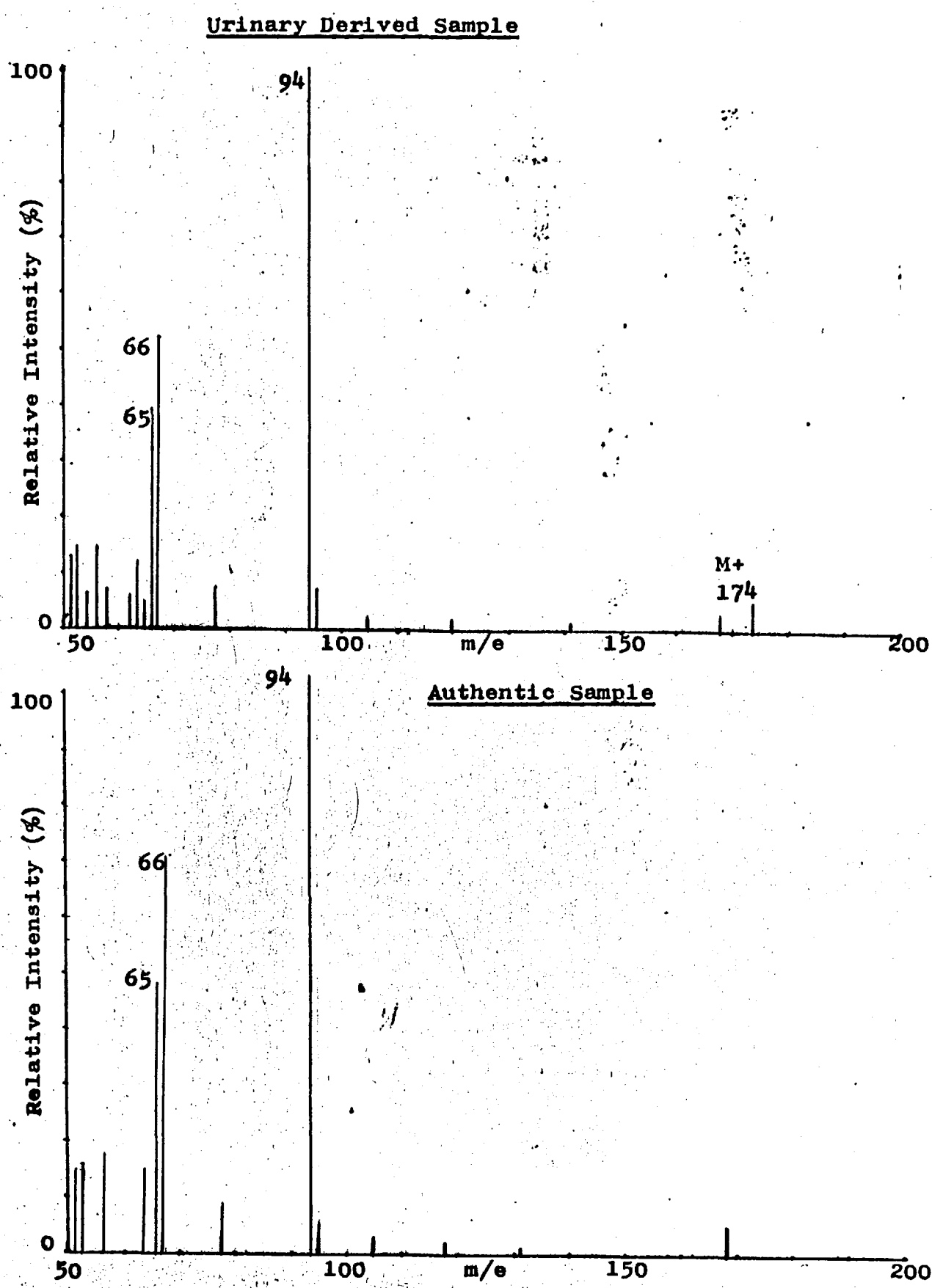
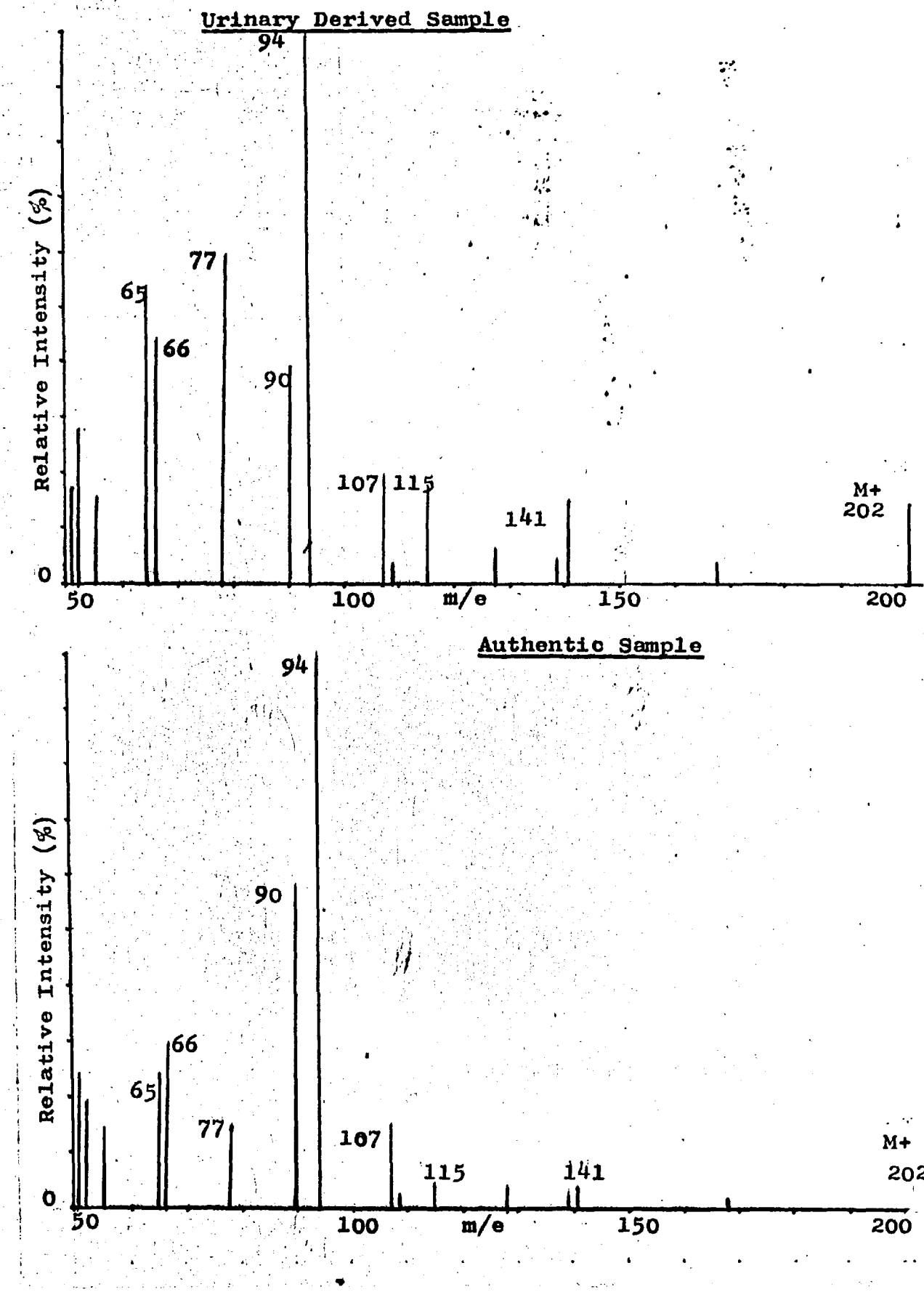


Fig 5.4 Mass Spectra of Dimethyl Phenyl Phosphoric Acids



CHAPTER SIX

PRELIMINARY STUDIES ON METABOLISM OF PHENOLS IN OTHER SPECIES

Contents

Tables and Figures

Introduction

As well as conducting the experiments on the metabolism of phenols in cats and pigs described in the previous chapters some preliminary investigations with other species were carried out. These were the effect of the route of administration on the excretory pattern of phenol in the hen and the metabolism of phenol and 4-hydroxyamphetamine were examined in four primate species.

The majority of species studied to date are reported to conjugate morphine with glucuronic acid. One exception is the cat which excretes morphine principally as morphine-3-sulphate (see Chapter Four). Hen also excretes this drug principally as morphine-3-sulphate, Fujimoto and Haarstad, (1969) isolated this conjugate from the excreta of hens which had been injected intramuscularly with morphine. Thus the hen appears to conjugate morphine in a similar fashion to cat which forms glucuronic acid conjugates only at a very low level. From these experiments it could be speculated that the hen is defective in forming glucuronic acid conjugates of morphine. It has been reported that hen is unable to form glucuronic acid conjugates of bilirubin (Dutton and Burchell, 1969). The hen excretes orally administered phenol, D- or L-borneol, 4-chloro-3,5-xylenol, benzoic acid

and o-methoxybenzoic acid partly as glucuronic acid conjugates (see Baldwin, 1961). It is possible, therefore, that in the hen the route of administration of the foreign compound might influence the type of conjugate excreted. In the experiments described in this Chapter [^{14}C] phenol was administered to hens by various routes in order to determine whether the mode of administration of this compound can influence the type of conjugate excreted.

French,(1970) administered [^{14}C] phenol orally to man and three species of primates; Rhesus monkey (Old World monkey species) and two species of New World monkey (squirrel and capuchin monkey). It appeared that man and Old World monkeys conjugated phenol mainly with sulphate, whereas the herbivorous New World monkeys conjugated phenol mainly with glucuronic acid. However, man received a much lower dose of phenol (0.01 mg/kg) than the other primates (25 mg/kg). These primates were thus dosed orally with phenol at a level comparable with man to ascertain whether this excretory pattern was still applicable. The primates dosed with phenol were also dosed with 4-hydroxyamphetamine by the same route and at a comparable dose level to determine whether any conclusions drawn from the results of the phenol studies would apply to other phenolic compounds.

Materials and Methods

The materials used in the experiments on hen, and the methods used for their estimation are fully described in Chapter One. The materials and methods used for the investigation of phenol metabolism in primates are also described in Chapter One. The materials used for the investigation of 4-hydroxyamphetamine in primates are described in Chapter Four.

Animals

The animals used (see Appendix 1) were obtained from dealers in the London area and maintained on appropriate diets. [^{14}C] Phenol (10 μCi/animal) dissolved in water (0.5 mls), together with sufficient carrier phenol neutralised with 2M NaOH (1 ml/animal), to give a final dose of 25 mg/kg was administered to hens by various routes. For oral administration a crop tube was employed, otherwise phenol was injected either intraperitoneally, or intramuscularly (leg muscle).

[^{14}C] Phenol (10 μCi/animal, 0.01 mg/kg) dissolved in water (1 ml) was administered to the primates in their food.

[^{14}C] 4-Hydroxyamphetamine (3.2 μCi/animal, 0.01 mg/kg) dissolved in water (1 ml) was also administered to these primates in their food.

For collection of urine animals were housed in suitable metabolism cages and their excreta collected on galvanised metal trays. The trays were washed with distilled water (3 x 50 ml) and the faeces removed from the combined washings by centrifugation at 2,000 r.p.m. for 10 min. The washings were then counted and chromatographed as described above for urine samples.

Results and Discussion

Hen

Following the administration of [^{14}C] phenol intraperitoneally, orally or intramuscularly to hens, about 50% of the dose was recovered in the excreta in 24 h (Table 6.1). When [^{14}C] phenol was administered orally or intraperitoneally to hens two large radioactivity peaks at Rf values 0.44 and 0.75 in solvent A of Table 2.2, (see Chapter Two) were seen on radiochromatogram scanning of the excreta (see Fig. 6.1). When [^{14}C] phenol was administered intramuscularly to hens

only one large radioactive peak at Rf 0.75 was seen on radiochromatogram scanning (see Fig. 6.1). Chromatography of authentic samples showed that these peaks at Rf 0.44 and 0.75 corresponded to phenyl glucuronide and phenyl sulphate respectively. The identity of the radioactivity peaks was confirmed by acid and enzymic hydrolysis. It was found that a radioactivity peak of Rf 0.44 was not detected on radiochromatogram scanning of excreta incubated with β-glucuronidase or sulphatase (which contains β-glucuronidase activity), whereas the radioactivity peak at Rf 0.75 was h hydrolysed on sulphatase incubation of the excreta. The radioactivity peak at Rf 0.75 was not detected on radiochromatogram scanning of excreta heated with 2N HCl under which conditions sulphates, but not glucuronides are hydrolysed (see Garton, et al., 1949). It was concluded, therefore, that the radioactivity peaks at Rf values 0.44 and 0.75 in Solvent 1 represented phenyl glucuronide and phenyl sulphate respectively.

Radiochromatograms representing hens which had received [^{14}C] phenol by each route were cut into 1 cm pieces and the associated ^{14}C determined using a liquid scintillation

spectrometer. Using this technique the percentage of the metabolites present were accurately determined. It was found that a greater quantity (41% of ^{14}C recovered) of glucuronide was formed after intraperitoneal than oral (28% of ^{14}C recovered) administration of phenol (see Table 6.1). In the case of hens dosed intramuscularly with phenol a small but distinct amount (4%) of ^{14}C-radioactivity, of Rf value 0.44, corresponding to authentic phenyl glucuronide was present. This radioactivity was not detected on cutting up radiochromatograms of urine which had been incubated with β-glucuronidase. Thus hens dosed intramuscularly with phenol excreted only small quantities of phenyl glucuronide and therefore conjugated this compound in a similar fashion to cat which is defective in forming glucuronides.

Generally when a foreign compound is administered to an animal intramuscularly it is absorbed gradually into the blood stream from where it will be circulated to the principal site of drug metabolism, the liver. When a similar dose of foreign compound is administered intraperitoneally it is usually rapidly absorbed and passes directly to the liver so that the hepatic enzymes are in effect challenged with a greater dose of xenobiotic, presumably for a shorter interval

of time. Therefore from the metabolic aspect intramuscular administration could be likened to a lower dose over a prolonged period. Williams, (1938) stated, and it is generally accepted, that as the dose of a foreign compound increases the amount of sulphate conjugation decreases and the amount of glucuronide conjugation increases correspondingly. Thus following intraperitoneal administration a greater amount of glucuronidation of a compound would be expected, whereas following intramuscular administration a greater amount of sulphate conjugation may occur. This was seen to be the case in the experiments performed on hen. Therefore the observation that in hen phenyl glucuronide excretion depends on the route of administration of this compound could be explained in terms of differing absorption rates.

Furthermore, however, there is an anatomical consideration when considering these experiments on the hen. The hen has a renal portal blood system so that blood from the legs is returned to the heart via the kidneys. Thus before reaching the liver compounds administered to hens via the leg will traverse the kidneys. It is possible that the kidney of the hen is responsible for the extensive sulphation of the phenol administered so that the compound is conjugated

before reaching the liver where it might be conjugated with glucuronic acid. In order to determine which of these hypotheses is correct further experimentation is necessary. A similar dose of phenol must be administered to hens via the breast muscle. If appreciable quantities of phenyl glucuronide are formed then the lack of glucuronide conjugation observed when the phenol was administered via the leg would be due to the unusual renal portal system of the hen. If very little phenyl glucuronide formation occurs whether the phenol is administered via the breast or leg muscle then this is likely to be due to the slow rate of absorption following intramuscular administration.

Primates

(a) Phenol $[^{14}C]$ Phenol was administered to Old World monkeys (2 rhesus monkeys and one cynomolgus) and to New World monkeys (2 squirrel monkeys and 2 capuchins). The recovery of ^{14}C radioactivity in 24 h. monkey urine was not as high as in experiments performed with other species. This was probably due to technical difficulties encountered in working with these primates. Small amounts of urine were produced which dried on the galvanised collection trays.

Bacterial contamination from faeces might result in deconjugation of metabolites and the [^{14}C] phenol (m.p. 43°) liberated would vaporize under these conditions, or possibly be oxidised to polyhydroxyphenols. Unidentified ^{14}C-material believed to be oxidation products of phenol was observed at the origins of chromatograms of the urine of these primates. On radiochromatogram scanning of the urine from Old World monkeys one main radioactivity peak of Rf value corresponding to an authentic sample of phenyl sulphate in the solvents of Table 2.2, Chapter Two, was detected. On radiochromatogram scanning of the urine of New World monkeys three radioactivity peaks, the Rf values of which corresponded to authentic samples of quinol glucuronide, phenyl glucuronide and phenyl sulphate in the solvents of Table 2.2, Chapter Two. The identity of these radioactivity peaks was confirmed by specific enzyme hydrolysis. The conjugates assigned glucuronides were hydrolysed by β-glucuronidase since they were not observed on radiochromatogram scanning of monkey urine which had been incubated with β-glucuronidase or sulphatase (which contains β-glucuronidase activity). The sulphate conjugates were hydrolysed by sulphatase only.

Radiochromatograms developed in solvent A, representative of each type of primate used, were cut into 1 cm pieces and the associated ^{14}C-radioactivity determined using the liquid scintillation spectrometer. By this technique the percentage of each of the metabolites present were determined (see Table 6.2). It is seen that in the case of New World monkeys phenol is conjugated extensively with glucuronic acid (squirrel monkey 66% and capuchin 52%). Old World monkeys however formed greater amounts of sulphate conjugates (rhesus 62%, cynomolgus 82%). Thus it appeared that Old World monkeys conjugated phenol preferentially with sulphate whereas New World monkeys utilise glucuronic acid. These results are, therefore, in agreement with the findings of French, 1970.

(b) 4-Hydroxyamphetamine $[^{14}C]$ 4-Hydroxyamphetamine was administered orally to the same primates which had received phenol. On radiochromatogram scanning of the urine one ^{14}C-radioactivity peak of Rf values 0.54 and 0.48 in solvents B, G (see Table 4.5, Chapter 4) was detected in the urine of all the primates. This radioactivity peak did not correspond to any of the authentic samples of Table 4.3 (Chapter Four). However, it corresponded to the Rf value ascribed to 4-hydroxyamphetamine sulphate (see Table 4.5).

In the case of the cynomolgus monkey a large amount of radioactivity which corresponded in Rf value to authentic 4-hydroxyamphetamine in solvents and was detected in addition to the ^{14}C peak detected in all the other primates. The ^{14}C peak of Rf value 0.54 in solvent B was not seen on radiochromatogram scanning of monkey urine which had been incubated with sulphatase. This peak was not hydrolysed by β-glucuronidase. It was concluded therefore that this radioactivity peak corresponded to 4-hydroxyamphetamine sulphate.

Chromatograms of urine developed in the solvents of Table 4.5 representative of each of the primates were cut into 1 cm pieces and the associated radioactivity determined using a liquid scintillation spectrometer. A small but distinct amount of ^{14}C of Rf value 0.15, 0.26 in solvents B and G respectively was detected. This peak had an Rf value in these solvents similar to that ascribed to 4-hydroxy-amphetamine glucuronide (see Table 4.5). This ^{14}C was not present on chromatograms of urine incubated with β-glucuronidase It was concluded therefore that this small amount of ^{14}C-radioactivity corresponded to 4-hydroxyamphetamine glucuronide. Thus in all the primates receiving

[^{14}C] 4-hydroxyamphetamine the principal conjugate formed was 4-hydroxyamphetamine sulphate and only traces (<2%) of 4-hydroxyamphetamine glucuronide were excreted. Thus the difference in conjugative pattern between New and Old World monkeys seen with phenol does not apply to all other phenols for, in the case of 4-hydroxyamphetamine, extensive sulphate conjugation predominates for all the monkeys. This is comparable with metabolism of 4-hydroxyamphetamine in man, where a dose of this drug is also excreted principally as 4-hydroxyamphetamine sulphate (Sever, et al, 1973).

From these studies on hens and primates some useful parameters relating to species with defective conjugation reactions are obtained. In order to describe a species as defective in a conjugation reaction it must have a limited ability to form this conjugate regardless of the mode of administration of the substrate. Hens receiving phenol by intramuscular injection via the leg appear to have little ability to form glucuronide conjugates. However, when phenol is given orally or intraperitoneally to hens considerable amounts of phenylglucuronide are formed. Furthermore, from the results of phenol metabolism in the primates (see Table 6.2)

it appears New World monkeys are poor at conjugating even very low doses of phenol with sulphate (preferentially utilising glucuronic acid). However these species are not defective in sulphate conjugation since all the monkeys studied conjugated 4-hydroxyamphetamine with sulphate rather than glucuronic acid.

In conclusion a species with a defect in a conjugation mechanism must be one where a number of potential substrates administered at similar dose levels do not undergo the reaction in question when administered by various routes.

Summary

1. The extent of glucuronic acid conjugation of phenol in the hen is dependent upon the mode of administration of this compound.

2. Old World monkeys appear to conjugate phenol mainly with sulphate whereas New World monkeys form glucuronic acid conjugates more extensively.

3. Both Old and New World monkeys excreted 4-hydroxy-amphetamine almost exclusively as the sulphate.

Table 6.1 The Metabolites of [^{14}C] Phenol in the Hen

Three Rhode Island Red Hens (2 - 2.5 kg body wt) were used. 25 mg of phenol/kg was administered by injection orally using a crop tube. 24 hr excreta was collected on galvanised trays and the faeces removed by centrifugation (see text). Samples were chromatographed on Whatman No. 1 paper (5 cm strip) by the descending technique developed in the solvents of Table 2.2 (see Chapter 2). Average values are given with, in parentheses, the range of values for the animals found in column 5.

Hens	Dose Phenol mg/kg	Dose ^{14}C μCi/animal	Route of Administration+	^{14}C excreted in 24 h (% of dose)	% ^{14}C excreted in 24 h found as: Phenyl Sulphate	Quinol Sulphate	Phenyl Glucuronide	Quinol Glucuronide
3	25	10	i.p.	55.4(50-60)	56(53-62)	3(0-9)	41(37-47)	-
3	25	10	p.o.	57 (53-60)	72(64-80)	-	28(20-34)	-
3	25	10	i.m.	45.2(33-69)	93(87-98)	3(0-4)	4(2- 9)	-

+ i.p. = intraperitoneal injection
p.o. = oral (by feeding tube)
i.m. = intramuscular injection

Table 6.2 The Metabolism of Phenol in Some Primates

The details of the primates used are given below. Phenol was administered orally in the food. The urine was collected for 24 h. and then chromatographed in the solvents of Table 2.2 (see Chapter 2) on Whatman No. 1 paper (5 cm strip) and using the descending technique. The Rf values given are the mean for 2 animals, with, in parenthesis, each individual value where appplicable.

Species No., Sex and Trivial Name	Family	Dose Phenol mg/kg	Dose ^{14}C µCi/animal	^{14}C excreted in 24 h (% dose)	% ^{14}C excreted in 24 h found as :- Phenyl Sulphate	Quinol Sulphate	Phenyl Glucuronide	Quinol Glucuronide	+ Other
Rhesus (2F) Monkey	Macaca	0.01	10	40(51,29)	58(57,58)	4(2,5)	6(2,10)	2(0,4)	30(23,37)
Cynomolgus Crab-Eating monkey (1F)	Macaca	0.01	10	31.2	82	n.d.	9		9
Squirrel Monkey (2F)	Saimiri	0.01	10	40(37,43)	7(0,14)	n.d.	58(42,68)	8(0,16)	27(23,31)
Capuchin Monkey (2F)	Cebus	0.01	10	50(62,38)	33(31,34)	n.d.	45(43,51)	7(6, 8)	16(7,22)

+ Other = Unidentified ^{14}C-material at the origin believed to be an oxidation product of phenol

n.d. = Not detected

Table 6.3 The Metabolism of 4-Hydroxyamphetamine in Some Primates

The details of the primates used are given below. 4-Hydroxyamphetamine was administered orally in the food. The urine was collected for 24 h. and then chromatographed in the solvents of Table 4.3 (see Chapter 4) on Whatman No. 1 paper (5 cm strip) and using the descending technique. The values given are the mean for 2 animals, with in parenthesis, each individual where applicable.

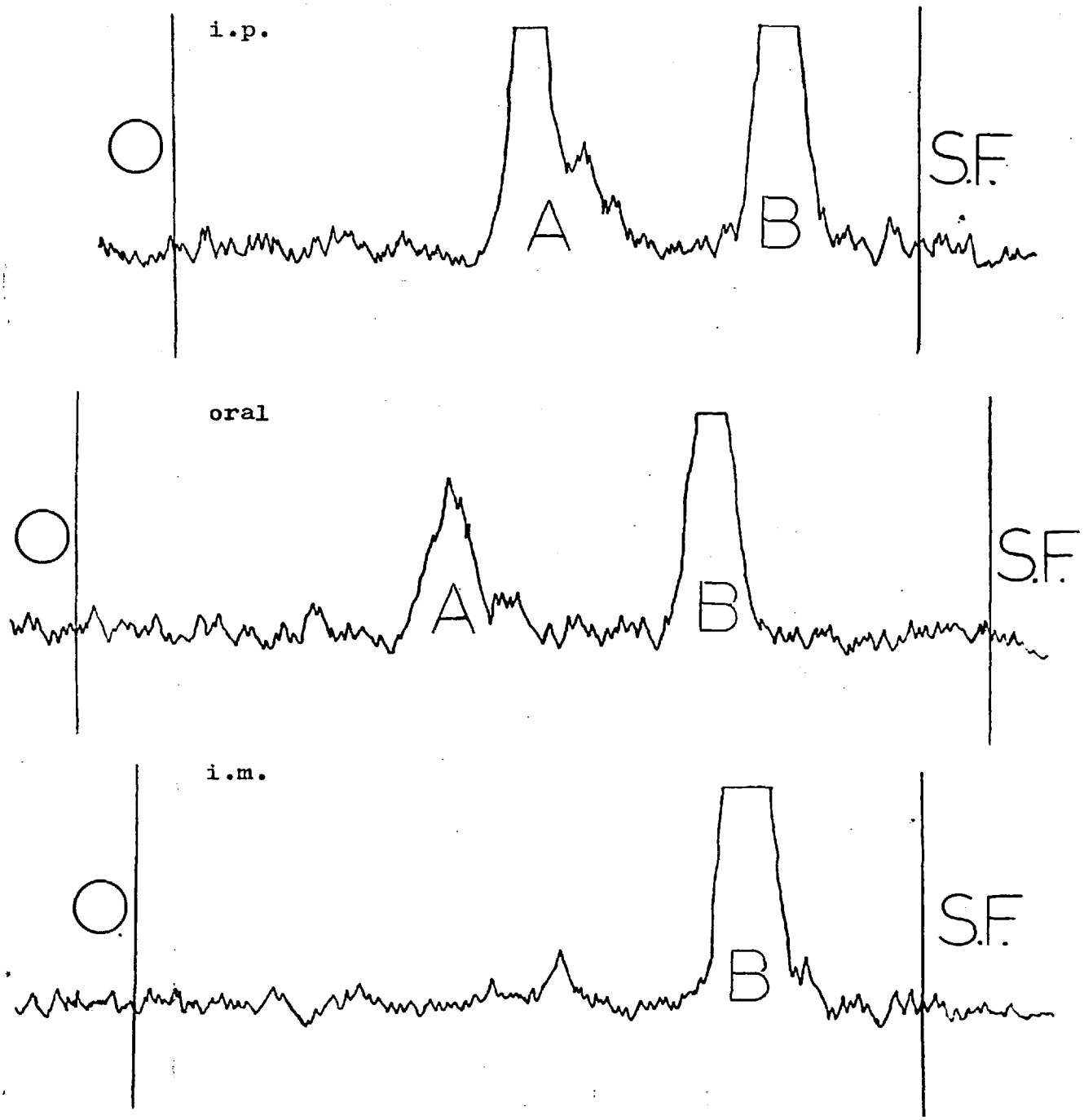
Species No. Sex and Trivial name	Family	Dose 4-Hydroxy-amphetamine mg/kg	^{14}C μCi/ animal	^{14}C excreted in 24 h (% dose)	% ^{14}C excreted in 24 h found as:- 4-hydroxy-amphetamine sulphate	4-hydroxy-amphetamine glucuronide	Free 4-Hydroxy-amphetamine
Rhesus Monkey (2F)	Macaca	0.01	3.2	72(70,74)	93(91,97)	2(1,2)	4(2,6)
Cynomolgus Crab-Eating Monkey (1F)	Macaca	0.01	3.2	60	12.6	2.8	84.6
Squirrel Monkey (2F)	Saimiri	0.01	3.2	21(20,21)	92(97,87)	1(1,1)	7(3,12)
Capuchin (2F)	Cebus	0.01	3.2	65(45,84)	97(99,95)	1(1,1)	2(0,4)

No other metabolites were present in quantities greater than 1% of the dose.

FIG 6-1

METABOLITES OF [^{14}C] PHENOL IN THE CHICKEN

(ADMINISTERED BY VARIOUS ROUTES)

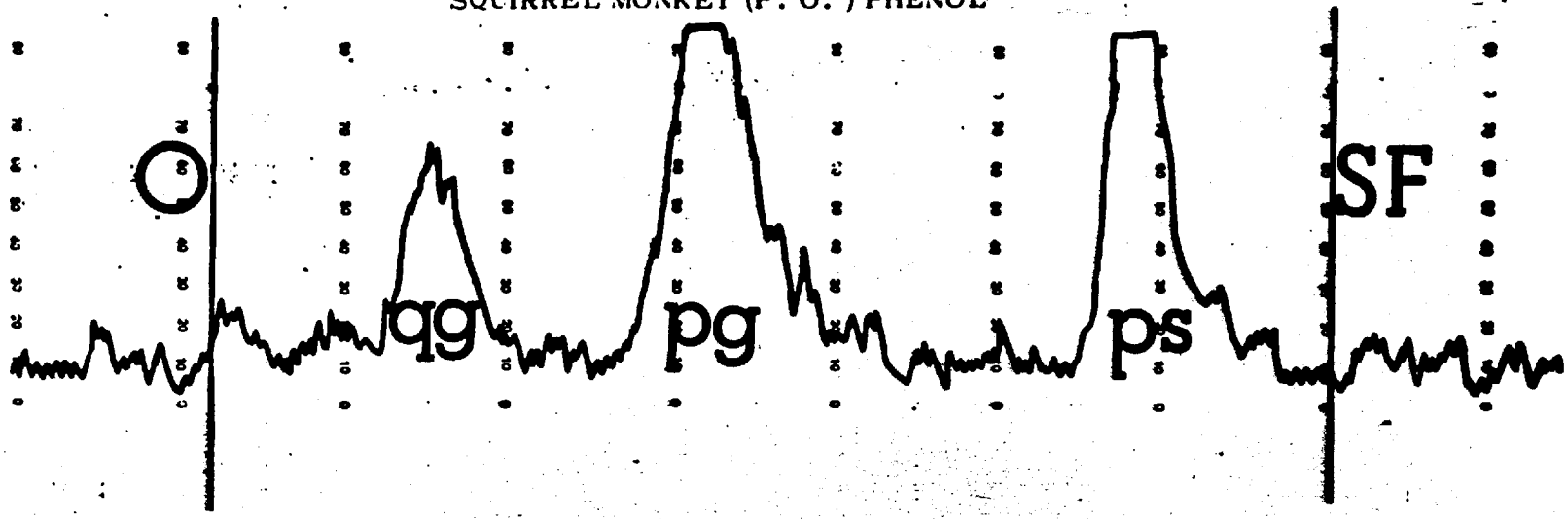


A = PHENYL SULPHATE

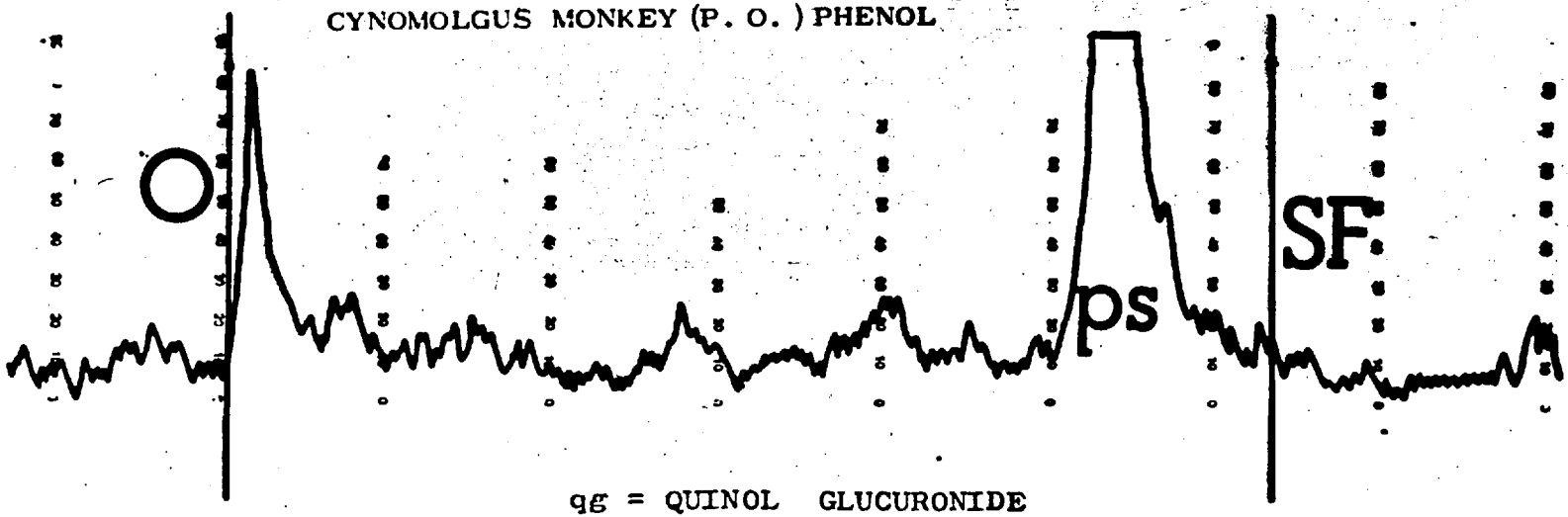
B = PHENYL GLUCURONIDE

RADIOCHROMATOGRAM SCANS OF PRIMATE URINE

SQUIRREL MONKEY (P. O.) PHENOL



CYNOMOLGUS MONKEY (P. O.) PHENOL



qg = QUINOL GLUCURONIDE
pg = PHENYL GLUCURONIDE
ps = PHENYL SULPHATE

FIG 6-2

THE URINARY METABOLITES OF [^{14}C] 4-HYDROXYAMPHETAMINE IN PRIMATES

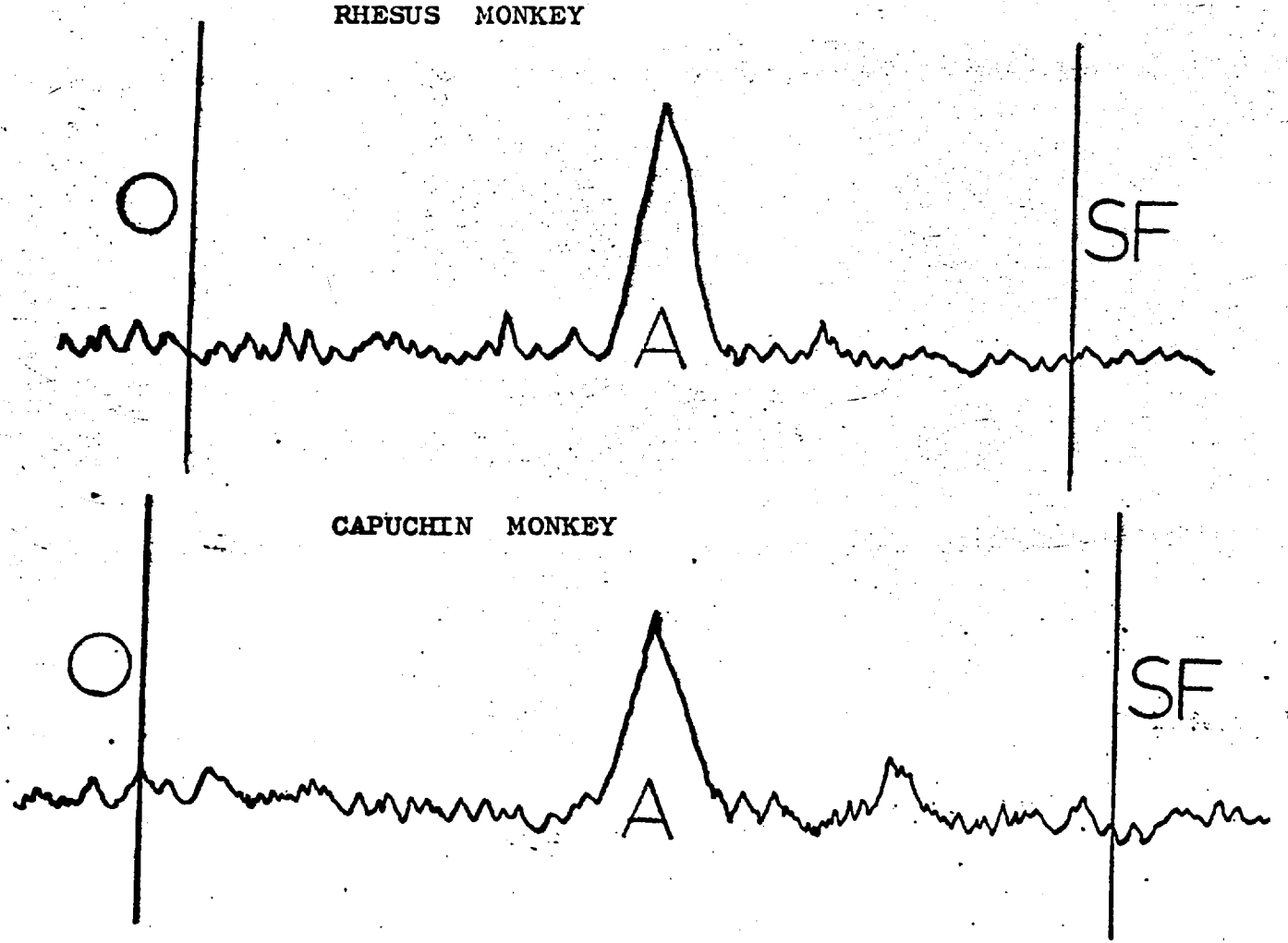


FIG 6-3

A = 4-HYDROXYAMPHETAMINE SULPHATE

CHAPTER SEVEN

GENERAL DISCUSSION

Contents

General Discussion

The investigation has been carried out in order to obtain information concerning the extent and specificity of some defects in detoxiciation mechanisms. Sulphate and glucuronic acid conjugation are phylogenically the most widespread, and therefore important, of the synthetic detoxication mechanisms and these have been investigated in the pig and cat respectively. From the preliminary studies in other species discussed in Chapter Six a species with a defect in a conjugation (detoxication) mechanism is one where the enzyme performing the detoxication reaction has little ability to metabolise various potential substrates administered at similar dose levels by various routes.

Cat: Glucuronic Acid Conjugation

When phenol was administered to cats either orally or intraperitoneally detailed examination of the urine showed that small amounts (0-2% of the dose) of phenyl glucuronide were formed. It appears therefore that the cat is not totally incapable of conjugating phenol with glucuronic acid. This is in accordance with the original work of Robinson and Williams, (1958) who reported that the amount of

4-methylumbelliferone glucuronide formed in response to the administration of the fluorescent aglycone was almost negligible, but not totally absent.

When radioactive 1- and 2-naphthol and phenacetin were administered to cats a similar excretory pattern to that of phenol was found. Small quantities (<2% of the dose) of the corresponding glucuronic acid conjugates were formed. In the case of cats receiving morphine no glucuronic acid conjugates were detected, but it is likely that this metabolite might have been present in very small quantities since Yeh, et al., (1971) were able to detect morphine-3-glucuronide in the urine of cats following total hydrolysis of the sulphate conjugate. Thus, with the above phenols cat is not totally unable to form glucuronic acid conjugates, minor quantities of these metabolites being excreted in the urine.

When $[^{14}C]$ phenolphthalein was administered to cats, however, 60% of the ^{14}C recovered in the urine was found to be a monoglucuronic acid conjugate, the remainder was probably phenolphthalein monosulphate. The recovery in the urine was low (approximately 30%) so that approximately 20% of the dose of phenolphthalein was excreted in the urine as the monoglucuronide conjugate. In the case of the other phenols

administered to cat no greater than 2% of the dose was excreted in the urine as a glucuronic acid conjugate so that in comparison with the other compounds administered cat was not defective in forming glucuronic acid conjugates of phenolphthalein. It was concluded, therefore, that the cat may form appreciable quantities of glucuronic acid conjugates of some phenols and it might be possible to predict the phenolic likely to be excreted as a glucuronide.

In Chapter One the possible existence of a "family" of UDP glucuronyl transferase enzymes was discussed. If such a multiplicity of UDPG transferase enzymes with differing, possibly overlapping, specificities occurs and if some of the transferase enzymes are not defective then the extent of the defect in glucuronic acid conjugation in cat will depend upon the specificity of the defective UDPG transferase enzymes(s). Enzymes involved in detoxication reactions are relatively non-specific (compared with those of intermediary metabolism). It is likely therefore that the UDPG transferase enzyme (or isoenzyme) which has little ability to glucuronidate phenol in cats will also be unable to form glucuronic acid conjugates of a large number of other related phenols. Thus, to estimate the specificity of the defective UDPG transferase enzyme(s)

of the cat various phenols of differing structure were administered and the levels of glucuronic acid conjugate excreted in the urine determined. It appeared the presence of another planar aromatic ring (as with 1- and 2-naphthol) was not sufficient to invoke an alternative UDPG transferase enzyme although there could be an alternative enzyme for these compounds which was also defective in the cat. Similarly 4-acetamidophenol, which was formed by the dealkylation of phenacetin, was also excreted principally as a sulphate and was therefore metabolised in a similar fashion to phenol. Thus, the basic group in the p-position to the phenolic -OH also does not invoke a non-defective UDPG transferase enzyme.

Morphine and phenolphthalein were also administered to cats. These compounds have a larger molecular size than the other phenols. It is likely that the molecular size of these compounds would cause steric hindrance of the enzyme responsible for the conjugation of the smaller phenols, and an alternative UDPG transferase would be necessary for these larger phenols. Since morphine was excreted principally as a sulphate in the urine it appears that the defective UDPG transferase of cat which has little ability to glucuronidate phenol can also utilise morphine as a substrate.

Phenolphthalein, however, was excreted mainly as a glucuronic acid conjugate in cat. It was concluded that the cat was not defective in forming glucuronic acid conjugates of phenolphthalein. Therefore, an alternative non-defective UDP-glucuronyl transferase enzyme might be responsible for the conjugation of this compound. Gram, et al., (1968) studied the intracellular distribution of UDPG transferase in rat liver preparations using phenolphthalein, o-aminophenol and p-nitrophenol as substrates. Activity towards phenolphthalein appeared equally in both rough and smooth microsomes but activity towards o-aminophenol and p-nitrophenol was predominantly in the rough microsomes. This was postulated as evidence for the possible existence of different transferase enzymes for the glucuronidation of phenolphthalein and the other smaller phenols. The in vivo results described in Chapter Four are in agreement with this idea.

From these limited studies it is difficult to speculate upon the type of compound which could be conjugated with glucuronic acid in the cat. It is likely, however, that larger phenols with a number of aromatic rings such as steroids will be more likely to be excreted as glucuronides. The compounds with the basic groups in the p-position to the

phenol -OH group administered to the cat were not readily glucuronidated. As discussed in Chapter One since phenols are not readily glucuronidated in the cat their toxic effects are prolonged and thus the cat will have a lower LD_{50} for these compounds than other species. Table 7.1 compares the LD of the o- and p- isomers of some phenols in the cat. It is seen that o-cresol and o-aminophenol are more toxic to cat than the respective p-isomers, o-nitrophenol, however is less toxic than p-nitrophenol. Both nitrophenols are less toxic than the aminophenols or cresols. This could indicate that nitrophenol is conjugated more extensively with glucuronic acid than either aminophenol or cresol.

Table 7.1

Compound	Animal	Dose	Dosage mg/kg	Time of death after the administration of the compound
Aminophenol	Cat	LD		
o-	"	"	37	1.75 h
p-	"	"		30 h
Cresol	Cat	LD		
o-	"	"	55	60 h
p-	"	"	80	120 h
Nitrophenol	Cat	LD		
o-	"	"	600	-
p-	"	"	197	-

Route of administration was subcutaneous injection

This is in accordance with the findings of Mowatt and Arias, (1970) who working with cat liver preparations showed that p-nitrophenol was conjugated with glucuronic acid at a slightly greater rate than o-aminophenol.

Thus far the effect of the molecular size and shape of the compound has been discussed in terms of invoking a specific UDPG transferase. The nature of the chemical group which is conjugated will have an important bearing on the specificity of the UDPG transferase. All the groups of Table 8-1, Chapter One are potentially capable of forming glucuronic acid conjugates. Therefore various compounds which contain these groups in their structure should be administered to cats to determine whether non-defective UDPG transferase(s) exist for the conjugation of groups other than the phenol -OH. Various phenols have been administered to cats and the results are summarised in Table 7.2.

Glucuronic acid conjugation in cats was reviewed by Dutton, (1966). The cat had reportedly negligible UDPG transferase activity towards o-aminophenol, menthol, phenolphthalein, 8-hydroxyquinoline, 1-naphthol and p-nitrophenol French, (1970) demonstrated the apparent inability of the cat to form glucuronic acid conjugates of phenol, benzoic acid and

chlorobenzene. More recently Miller, et al., (1973) has shown that only traces (<2%) of 2,6-dimethoxyphenol, quinol and chlorophenol are excreted as glucuronic acid conjugates in the urine of cats. Some compounds have been detected in the bile of cats as glucuronides including progesterone, testosterone, corticosterone, thyroxine, tyropanoic acid, iodopanoic acid, and hydroxy derivatives of N-2-fluorenylacetamide. These glucuronides were detected but were not quantitated because they were difficult to assay in bile. Information relating to the excretion of some steroids in the bile of the cat was reviewed by Taylor, (1971). In the case of corticosterone and progesterone approximately 20% of the dose of these steroids was excreted in the bile in 4 hours as glucuronic acid conjugates. Some endogenous compounds are excreted partly as glucuronic acid conjugates in the cat these include metanephrine which is excreted in the urine (Axelrod, et al., 1959) and bilirubin which is excreted in the bile, Dutton, (1966)

Thus the cat can conjugate both endogenous and foreign compounds with glucuronic acid forming ethereal and ester glucuronides. It was reported by Dill, et al., (1960) that the glucuronic acid conjugate of the antibiotic chloramphenicol was excreted in the urine of cats. When this urine was chromatographed an area of the chromatogram which corresponded

Table 7.2 Glucuronide Conjugation in Cats

Vehicle of Excretion	Compounds reported not to be excreted as glucuronides in cats		Compounds reported excreted as glucuronides in cats	
Urine	p-nitrophenol	1	lorazepam	6
	menthol	1	chloramphenicol	7
	salicylamide	1	metanephrine	8
	benzoic acid	2		
	phenol	3 & 4		
	chlorobenzene	3		
	amphetamine	1		
	2,6-dimethoxyphenol	4		
	quinol	4		
	4-methylumbelliferone	5		
	p-chlorophenol	4		
	o-aminophenol	1		
	1-naphthol	1		
Bile	phenolphthalein	9	progesterone	11
			testosterone	1
			corticosterone	11
			thyroxine	1
			tyropanoic acid	1
			iodopanoic acid	1
			bilirubin	1
			hydroxy derivatives of N-2-fluorenyl-acetamide	1
In Vitro	phenolphthalein	1	p-nitrophenol	10

The number behind each compound is a reference to the author(s) reporting the work. See key overleaf.

KEY TO TABLE 7.2

No.	Author(s)
1	Dutton, (1966)
2	Bridges, et al., (1970)
3	French, (1970)
4	Miller, et al., (1973)
5	Robinson and Williams, (1959)
6	Schillings, et al., (1971)
7	Dill, et al., (1960)
8	Axelrod, et al., (1959)
9	Abou-El-Makarem, (1967)
10	Mowatt and Arias, (1970)
11	Taylor (1971)

Full details of references appear on page beginning

to chloramphenicol glucuronide had no antibiotic activity against Sarcina lutea. Treatment of this area with β-glucuronidase hydrolysed the glucuronic acid conjugate and the antibiotic action against the bacteria was restored. The amount of chloramphenicol excreted as a glucuronic acid conjugate was not determined. Recently Schillings, et al., (1971) has demonstrated that the principal metabolite of Lorazepam (7-Chloro-5-(o-chlorophenyl)-1, 3-dihydro-3-hydroxy-2H-1, 4-Benzodiazepin-2-one) in cats and 3 other species was a glucuronide the other metabolites accounting for less than 1% of the dose. The percentage of the dose of Lorazepam excreted as a glucuronic acid in cat was not reported although the authors had isolated this metabolite from the pig and cat and no other conjugates were detected in these species.

An appreciable amount of quinol was formed in cats following the administration of phenol and this was excreted in the urine as quinol sulphate. Greater quantities of quinol were formed following intraperitoneal than oral administration of phenol. When phenol was administered orally to cats some of the compound was probably conjugated with sulphate in crossing the gut wall and thus less phenol is available for hydroxylation by the liver. When 2-naphthol was administered intraperitoneally as with phenol appreciable oxidation to dihydroxynaphthalene occurred. No dihydroxynaphthalene was detected following the intraperitoneal administration of 1-naphthol however.

Phosphate Conjugation

Wong, (1972) after comparing the activities of bilirubin UDP transglucosidase and UDP transglucuronidase postulated the importance of the formation of β-glucosides as an alternative conjugate in a species such as cat which is defective in forming glucuronides. No glucoside conjugates have been detected in cat urine following the administration of the phenols discussed in the previous chapters. However, phenyl phosphate was detected as a minor metabolite in the urine of cats following administration of phenol and this could be an important conjugate in a species with low glucuronic acid conjugation ability.

It appears likely that the enzyme concerned in phosphorylating phenol was a hepatic one. A larger quantity of phenyl phosphate was formed following intraperitoneal injection than oral administration of phenol. The larger the dose of phenol the greater was the amount of phenyl phosphate formed, this could reflect the limitation of the sulphating capacity of cat, the phosphate being formed as an alternative metabolite to the defective glucuronic acid conjugate.

Phosphate is more plentiful in the animal body than is sulphate, large quantities of phosphate being excreted in the urine. A phosphate conjugate would be ideal for detoxication purposes since it would be ionized at physiological pH and

therefore readily water-soluble and excreted. However phosphate metabolites have proved remarkably rare in nature (see Chapter Five). It has been suggested that this is because bladder epithelia (and many other tissues) are rich in phosphatases. Thus phosphates might be deconjugated and then possibly recirculated and excreted as different conjugates. The question is, therefore, why is the phosphate conjugate detected in cat? It is possible that the bladder epitheleum of cat contains less phosphatase enzymes than most other species, alternatively the absence of the efficient glucuronic acid conjugating mechanism facilitates easier detection of any other minor metabolites.

Whether phosphate conjugates were formed as minor metabolites in other species is technically difficult to determine with the presence of other metabolites not found in the cat (phenylglucuronide and often quinol conjugates also). Phosphate conjugation might be important in pregnancy, for example, where both glucuronide and sulphate formation are reduced.

The importance of the phosphate metabolite to the cat as an alternative conjugation mechanism is difficult to assess since it was formed in the greatest quantities (up to 8% of the dose) only with larger doses of phenol. The effectiveness of the combined phosphate and sulphating capacity must be limited since

the LD_{50} of cat for phenol is lower than most other species (see Table d.1 Chapter One). This low LD_{50} in the cat was ascribed to the defective glucuronic acid conjugation in cat and this must be more effective in detoxicating phenols in other species than the combined phosphate and sulphate conjugation mechanisms. Furthermore, the failure to detect phosphate conjugates of any of the other phenols suggests the enzyme responsible for the phosphate formation is relatively specific. This renders phosphate metabolites less suitable for detoxication purposes since a detoxication enzyme should have activity against a broad range of substrates. The kinase enzyme which phosphorylates endogenous substrates are by necessity specific, assuming it is such an enzyme which conjugates the phenol hydroxyl group then the other phenols administered to the cats might be sterically unsuitable for the enzyme. This, therefore, could not only explain why only phenol was phosphorylated in the cat but also the comparative rarity of phosphate conjugates in nature.

Pig

When phenol was administered either orally or intraperitoneally to pigs it was excreted mainly as phenyl glucuronide. However, detailed examination of the urine

showed that small quantities of phenyl sulphate were excreted.

In the pig it is likely that the intestinal mucosa is defective in forming sulphate conjugates as is the liver since phenol is excreted almost exclusively as a glucuronic acid conjugate whether it is administered orally or intraperitoneally. In the cat which is not defective in sulphate conjugation less hydroxylation occurs following oral than intraperitoneal administration of phenol since this compound was partly sulphated in crossing the gut wall (see above).

Williams, (1938) suggested, and it is generally accepted that as the dose of a xenobiotic increases the amount excreted as a sulphate conjugate decreases while the amount excreted as a glucuronide increases correspondingly. A greater amount of phenyl sulphate should be excreted, therefore, when a low dose of phenol is administered to pigs. A very low dose of phenol (μg quantities) was administered to pigs but the amount of phenyl sulphate excreted was of the same order as when phenol is administered at 5 and 25 mg/kg. Thus, in the pig glucuronic acid conjugation predominates regardless of the dose of phenol administered.

In Chapter One the possible reason for the poor ability of the pig to form sulphate conjugates was described. This was ascribed to a defect in the phenol sulphotransferase of the pig.

The number of sulphotransferases known to occur were reviewed by Roy, (1971). The specificity of a phenolsulphotransferase extracted from guinea-pig liver and the existence of a multiplicity of sulphotransferase enzymes is described in Chapter One. In the cat it was likely that the UDPG transferase enzyme was a multiple enzyme consisting of isoenzymes with differing, possibly overlapping, specificities, for a very wide range of substrates. It is possible that the phenol sulphotransferase enzymes of the pig are similar. If more than one sulphotransferase enzyme capable of conjugating phenols exists in the pig then these enzymes might not all be defective. Therefore some phenols might form appreciable sulphate conjugates in the pig.

In order to determine whether some compounds might be excreted as sulphate conjugates in the pig phenacetin, 4-hydroxyamphetamine, 1-naphthol and 2-naphthol were administered. Phenacetin, 4-hydroxyamphetamine and 2-naphthol were all excreted principally as the glucuronic acid conjugates; as with phenol only traces of the sulphate conjugates were detected on detailed examination of the urine. Williams, (1938) discussed the effect of some groups adjacent (ortho-) and opposite (para-) to the phenol -OH group in the sulphation of

some phenols in the rabbit. Some groups increased, while others decreased sulphate conjugation. The basic group of 4-acetamidophenol (which is formed by the metabolism of phenacetin) and of 4-hydroxyamphetamine do not have any effect on the amount of sulphate conjugation in the pig, since these compounds are sulphated at a similar level as phenol. In the pig 2-naphthol was excreted almost entirely as a glucuronic acid conjugate in the urine.

However, when 1-naphthol was administered to pigs approximately one third of the dose was recovered in the urine as 1-naphthyl sulphate. With this compound, therefore, the pig was not defective in sulphate conjugation. The phenols were administered to test the specificity of the conjugating transferase enzymes. One could conclude that in the case of the pig a non-defective phenol sulphotransferase exists which can accept 1-naphthol but not 2-naphthol or phenol as substrates. The isomer difference between 1- and 2-naphthol appears therefore to be sufficient to invoke the activity of this non-defective phenol sulphotransferase. To establish that the results were owing to an isomer difference between these naphthols and not to a difference between animals, one pig received doses of 1- and 2-naphthol respectively and only traces of 2-naphthyl sulphate were formed, 1-naphthol was conjugated readily with sulphate.

It is likely that metabolically 2-naphthol is more similar to phenol than is 1-naphthol. This is possibly indicated from the experiments performed with cats. When phenol was administered intraperitoneally to cat a considerable amount of hydroxylation (resulting in quinol formation) occurred. 2-Naphthol, but not 1-naphthol was hydroxylated to a similar degree when administered to cats by this route. Thus with respect to this Phase I oxidation reaction the metabolism in the cat of 2-naphthol is certainly more similar to phenol than is 1-naphthol.

In Table d.1 (see General Introduction) the phenol sulphotransferase isolated from guinea-pig liver had a similar V max and km values when challenged with a variety of phenols including 1- and 2-naphthol. In the case of pig however this enzyme is defective and another sulphotransferase, possibly a steroid sulphotransferase, "accepts" 1-naphthol, but not its isomer as a substrate.

A number of interesting observations arise from the in vitro studies of pig and rat liver homogenates. Firstly the rate of synthesis of naphthyl glucuronide (assayed in μmoles/g. of liver/h) was higher in pig than rat. Pig therefore appeared to be a faster glucuronide former than rat with the substrates used.

The rat formed sulphate conjugates of 1-naphthol and 2-naphthol in roughly similar quantities and at similar rates *in vitro*. The pig formed little of the sulphates of these phenols at a very slow rate *in vitro*. 1-Naphthol was not sulphated at a greater rate than its 2-hydroxy isomer. This is apparently difficult to reconcile with the *in vivo* findings; it might be expected that the rate of 1-naphthyl sulphate formation in pig should be comparable with the rate in rat. If however, a different sulphotransferase from that required for phenol were responsible for conjugating the 1-naphthol then the lack of sulphation *in vitro* might be explained in terms of differing optimum conditions. The incubation conditions are the optimum for phenol sulphotransferase (which is defective in the pig); the isoenzyme or related enzyme conjugating 1-naphthhl with sulphate might have differing pH optima and/or activators, etc. so that it would not be active under these conditions. It is also possible that this enzyme is located in a different site in the cell and is possibly removed in the preparation of the homogenate, e.g. when removing the microsomes. Further studies varying the conditions of the incubation are therefore necessary to determine if such another enzyme exists and if so to elucidate its kinetic parameters.

Thus it was concluded that a non-defective sulphotransferase

enzyme exists in pig capable of sulphating 1-naphthol. It is difficult to speculate on any other phenols than 1-naphthol likely to be conjugated with sulphate in the pig since a compound as similar as 2-naphthol was not sulphated. The metabolism of few compounds have been investigated in the pig and thus the only information available on the specificity of phenol sulphotransferase is indirect. No phenol sulphotransferase has been obtained as a single protein. The most highly purified enzyme of this type is that from guinea-pig liver described in Chapter One, which had no detectable steroid, alcohol or arylamine sulphotransferase activity. The compounds conjugated by this enzyme are listed in Table d.1, Chapter One, which shows that this enzyme conjugates phenol, 1-naphthol, 2-naphthol and other phenols. It is likely that if a similar phenol sulphotransferase enzyme is defective in the pig 1-naphthol is conjugated by one of the other sulphotransferase enzymes possibly steroid or alcohol sulphotransferase.

A comparable study to the investigation carried out in the pig has been made in the Marsupial opossum (Trichosorus vulpecula) by Roy, (1963). In vitro experiments with liver preparations from opossum revealed that it formed no ethereal sulphates of p-nitrophenol, naphthylamine, androstenolone,

oestrone and testosterone. The defect was not apparently due to an inability to synthesise PAPS, the addition of which did not stimulate sulphate conjugation. It appears therefore that the transferase enzymes are defective here. In vivo experiments showed that the Marsupial opossum had negligible activity towards a number of phenols including 1-naphthol. The opossum appears, as does the pig to be defective in forming sulphate conjugates of a wide variety of phenolic compounds.

Conclusions from the Investigations in the Cat and Pig

From the studies in the cat and pig two important points emerge. The first is that in both animals the defect is not total, minor quantities of glucuronic acids in cat and sulphate conjugates in pig being excreted in the urine. The second is that the conjugative defect does not apply to all the possible substrates for these transferase enzymes. What conclusions may be drawn from these observations on the possible cause of the defect? The fact that small quantities of the minor metabolites can be detected in the cat and pig suggests that the transferase enzymes responsible for their synthesis are active at very low levels.

The classical system of Jacob and Monod, (1961) might apply to the genetic control of the synthesis of such

transferase enzymes (see also Monod, Jacob and Gros, 1967). An operator zero (O^c) mutation (see Beckwith, 1964) would lead to the defect in conjugation as observed in the cat and pig. Such mutations cause greatly reduced levels of all the enzymes of the operon and are probably identical with polarity mutants, extreme with respect to the first gene in the operon and in extent. In all likelihood they are caused by deletions in this first gene and are expressed at the translational level. However, mutations in any part of the genome leading to a change in the essential amino acid sequence of the transferases might result in an enzyme with decreased affinity for its substrate. With the limited information available it is not possible to determine the nature of these defects at the genetic level, detailed amino acid-sequencing studies on purified enzymes being necessary.

In vitro studies on liver homogenates could yield information on whether any inhibitors were present. If a liver homogenate from the cat or pig is mixed with a similar rat liver homogenate, and a large decrease in the glucuronidating ability of the rat mixed with cat or sulphating ability of the rat mixed with pig homogenate, results, then it is likely an inhibitor of the respective conjugation

reactions is present in the cat or pig homogenate. No inhibitor has been found in cat liver preparations other than the usual nucleotide-inactivating enzymes (see Dutton, 1966).

The existence of more than one sulphotransferase is certain, (see Roy and Trudinger, 1970; Roy, 1971) and although much of the evidence is indirect, it appears to be overwhelming to support the postulated "family" of related UDP glucuronyltransferases (see Dutton, 1971). When attempting to determine the specificity of these enzymes great care must be taken in the interpretation of individual results. When compounds are administered which the species may never have encountered in its history, specifically evolved transferases are assailed with unfamiliar molecules; from the response of these enzymes the specificities towards these and other substrates are deduced. The "detoxication enzyme being assailed with varying and often unrelated compounds are by necessity unspecific with broad spectra of activity. In this case we are seeking information on the range of substrates for the transferase enzymes in species in which these enzymes are defective, so that more often we are looking at what is not formed, rather than what is. Furthermore foreign substrates no matter how useful pharmacologically are clumsy tools with

which to probe such intricately evolved 3-dimensional structures as the active sites of enzymes.

To summarise this study it should be said that the term "defective" is better defined as "has little ability" and the extent of the defect is dependent upon the specificity of the enzyme concerned. Bearing this in mind rules or generalisations concerning defects in detoxication mechanisms should not be attempted until the conjugation of many more xenobiotics has been investigated in these comparatively neglected species.

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Appendix

Details of Animals Used in Metabolism Studies

Animal	Order	Family	Species	Strain or Breed	Sex	Diet	Supplier
Rhesus Monkey (Bengal)	Primates	Cercopithecidae	Macaca mulatta	-	F	Dixons 41B supplemented with fruit	Shamrock Farms Ltd.
Squirrel Monkey	Primates	Cebidae	Saimiri sciureus	-	F	- do -	A.S.L.
Capuchin Monkey	Primates	Cebidae	Cebus apella	-	F	- do -	A.S.L.
Cynomolgus Crab-eating monkey	Primates	Macaca	Macaca fasicularis	-	F	- do -	A.S.L.
Cat	Carnivora	Felidae	Felis catus	Mongrel	M,F	Meat, milk	N. Stacey, Esq.
Rat	Rodentia	Muridae	Rattus norvegicus	Wistar albino	F	Dixons 41B	S.A.S.
Hamster (Golden)	Rodentia	Cricetidae	Mesocricetus auratus	-	F	Dixons 41B	A. F. Longmoor, Esq.
Pig	Artiodactyla	Suidae	Sus scrofa	Large White	M,F	Dixons 41B supplemented with vegetables	W. Harris, Esq.
Hen	Galliformes	Phasianidae	Gallus domesticus	Rhode Island Red	F	Pauls' Layers mash No.1	P. W. Needle, Esq.

cont/...

Abbreviations used in Appendix

M = Male

F = Female

Animal Suppliers

Shamrock Farms (Gt. Britain) Ltd.,
Upper Horton Farm,
Small Dole,
Henfield,
Sussex.

A.S.L. Farms Ltd.,
685 High Road,
N. Finchley,
London, N.12.

N. Stacey, Esq.,
Sidlow Nurseries,
Ironsbottom Lane,
Sidlow,
Nr. Reigate, Surrey.

S.A.S.,
Home Farm,
Aldenham Park,
Elstree, Herts.

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Species Variations in the Metabolism of Phenol

By I. D. CAPEL, M. R. FRENCH, P. MILLBURN, R. L. SMITH and R. T. WILLIAMS (*Department of Biochemistry, St. Mary's Hospital Medical School, London W.2, U.K.*)

Marked species variations in the pattern of metabolism of a number of simple organic compounds, including benzoic acid (Bridges *et al.*, 1970), amphetamine (Dring *et al.*, 1970), phenylacetic acid (James *et al.*, 1971) and quinic acid (Adamson *et al.*, 1970), have been reported. These studies have now been extended to an examination of the species variation in the metabolism of [^{14}C]phenol in 19 species of animals. The animals were dosed orally or injected intraperitoneally with [^{14}C]phenol (25mg/kg), and urine was collected for 24h and then analysed for metabolites by paper chromatography followed by radiochromatogram scanning. For man (three male subjects) the oral dose was 0.01mg/kg and for the rhesus monkey it was 50mg/kg. The recovery of ^{14}C in the 24h urine ranged from 30% for the squirrel monkey to 95% for the rat; for most species it was about 50–70%.

Eight of the species examined, namely, the rat, mouse, jerboa, gerbil, hamster, lemming, guinea pig and man, excreted four metabolites, these being the sulphate and glucuronic acid conjugates of phenol and its oxidation product, quinol.

Six species, namely the squirrel monkey, capuchin, ferrel, dog, hedgehog and rabbit, excreted only three metabolites. The two herbivorous New World monkeys excreted phenyl glucuronide, quinol glucuronide and phenyl sulphate, and, at the dose of phenol used, glucuronic acid conjugation appeared to be more effective than sulphate conjugation in these two species. The other four species, however, excreted phenyl sulphate, quinol sulphate and phenyl glucuronide, and these species varied according to the extent to which they utilized the two conjugation mechanisms and in the extent of oxidation of phenol to quinol.

Four species, the rhesus monkey, cat, fruit bat and chicken, excreted only two metabolites, and one species, the pig, only one. The rhesus monkey, fruit bat and hen excreted phenyl sulphate and phenyl glucuronide, but no quinol conjugates. The cat appeared to excrete only phenyl sulphate and quinol sulphate, whereas the pig appeared to excrete only phenyl glucuronide.

The apparent inability of the cat to utilize the glucuronic acid mechanism and the pig the sulphate mechanism for the conjugation of phenol was therefore investigated in greater detail. It was found that these mechanisms were not entirely absent as far as phenol is concerned, since they were found at a very low level. The pig given [^{14}C]phenol orally or intraperitoneally excreted more than 90% of the 24h ^{14}C excretion as phenyl glucuronide, but small amounts of phenyl sulphate (0.2–8.3%) were also detected. Similarly, in the cat, although phenyl sulphate and quinol sulphate accounted for over 90% of an oral or injected dose of [^{14}C]phenol, small amounts (0.1–2.0% of the 24h ^{14}C excretion) of phenyl glucuronide were also found in the urine.

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The Fate of [^{14}C]Phenol in Various Species

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1. [^{14}C]Phenol has been administered to man (dose, 0·01 mg/kg) and 18 animal species (25 mg/kg) and the urine examined for metabolites by radiochromatogram scanning.

2. In three men 90% of an oral dose was excreted in 24 h mainly as phenylsulphate (77% of 24 h excretion) and phenylglucuronide (16%) with very small amounts of quinol sulphate and glucuronide.

3. Four metabolites, the sulphates and glucuronides of phenol and quinol, were found in the urine of the rodents, the rat, mouse, jerboa, gerbil, hamster, lemming and guinea-pig after an oral dose of phenol.

4. Three metabolites were excreted by some species, namely, phenol and quinol glucuronides and phenylsulphate by the squirrel monkey and capuchin monkey, and phenol and quinol sulphates and phenylglucuronide by the ferret, dog, hedgehog and rabbit.

5. Two metabolites were excreted by the rhesus monkey, fruit bat and hen (phenylsulphate and phenylglucuronide) and by the cat (phenylsulphate and quinol sulphate). One metabolite (phenylglucuronide) only was excreted by the pig.

6. The cat appeared to form no phenylglucuronide and the pig no phenylsulphate, but detailed examination of radiochromatograms showed that the cat did excrete small amounts of the glucuronide and the pig excreted small amounts of the sulphate.

7. *In vitro* studies with rat and pig liver preparations showed that the rat preparations conjugate phenol with sulphate and glucuronic acid with roughly equal facility, but the pig liver preparations conjugated phenol with glucuronic acid at a much faster rate than with sulphate.

Introduction

Species variations in the metabolism of some relatively simple organic compounds which include benzoic acid (Bridges, *et al.*, 1970), quinic acid (Adamson, *et al.*, 1970 a), sulphadimethoxine (Adamson, *et al.*, 1970 b) and amphetamine (Dring, Smith & Williams, 1970) have been reported recently from this laboratory. The fate is now described of phenol in nineteen species of animals including man. Previous work on the metabolism of phenol (see Williams, 1959) has shown that its main metabolites are phenylsulphate and phenylglucuronide together with small amounts of conjugated quinol and lesser amounts of conjugated catechol (Parke & Williams, 1953). In this paper it is

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shown that, in most of the species examined, phenylsulphate and phenylglucuronide are indeed the main metabolites except in the case of the cat and the pig. The cat is known to have a defective glucuronic acid conjugation mechanism for certain compounds and it formed only small amounts (0·1–2·0% of the 24 h excretion) of phenylglucuronide when dosed with phenol. By contrast, the pig excreted only small quantities (0·2–8·3% of the 24 h excretion) of phenylsulphate and it may thus have a defective sulphate conjugation mechanism. Preliminary studies with pig liver homogenates showed that sulphate conjugation was not entirely absent but occurred at a much slower rate than glucuronic acid conjugation, whereas in rat liver preparations both conjugations occurred with equal facility.

Materials and methods

Chemicals

Phenol, m.p. 42°, catechol, m.p. 103°, resorcinol, m.p. 109° and quinol, m.p. 170°, were purchased and purified. Phenylglucuronide, m.p. 161–162°, $[\alpha]_D^{17}$ -78·5° (c, 2 in water) was a sample previously prepared in this laboratory (cf. Garton, Robinson & Williams, 1949). Potassium phenyl sulphate and potassium 4-hydroxyphenyl sulphate (quinol monosulphate) were prepared according to Garton (1949). These sulphates were characterized (see Table 1) by colour reactions before and after hydrolysis with 2M-HCl and with an arylsulphatase preparation from *Helix pomatia* (Sigma Chemical Company, London). 4-Hydroxyphenylglucuronide (quinol monoglucuronide) was obtained as a gum from the urine of rabbits fed with quinol according to Garton & Williams (1948). The gum was purified by t.l.c. in solvent A (see Table 1) in which the glucuronide had an R_F value of 0·10. It was characterized by colour reactions (see Table 1) before and after hydrolysis with beef liver β-glucuronidase preparation (Ketodase; Warner–Chilcott Laboratories, Eastleigh, Hampshire).

[U-^{14}C]Phenol (266 μCi/mg) (Radiochemical Centre, Amersham), uridine diphosphate glucuronic acid (UDPGA: Sigma Chemical Company, London) and adenosine triphosphate (ATP; British Drug Houses Ltd., Poole, Dorset) were purchased.

Animals

The animals used (see Table 2) were obtained from animal dealers in the London area, and were maintained on appropriate diets. [^{14}C]Phenol, dissolved in water, was administered orally or intraperitoneally. For the collection of urine, animals were placed in suitable metabolism cages and their urine was collected daily in receptacles containing a few ml of saturated aq. $HgCl_2$ soln. to prevent bacterial breakdown of conjugates. The excreta of chickens was collected on galvanized metal trays placed below their cages. These excreta were extracted with three separate portions (40 ml) of water to remove the water-soluble conjugates of phenol. The three extracts were pooled and made up to 150 ml with water. This soln. was then treated in the same way as the urines of other animals for the estimation of ^{14}C and for chromatography (see below). The solid excreta remaining after the above extraction procedure contained no detectable radioactivity.

Table 1. Chromatography of phenol and its metabolites

For paper chromatography, Whatman No. 1 paper and the descending technique were used; for t.l.c., plates were prepared with fluorescent silica gel (see text). Solvents, paper chromatography: A, propan-1-ol—ammonia soln. (s.g. 0·88) (7:3 by vol.): B, butan-1-ol—ammonia soln. (s.g. 0·88)—water (10:1:1): C, butan-1-ol—benzene—pyridine—water (5:1:3:2); t.l.c.: D, benzene—methanol—acetic acid (45:8:4): E, benzene—dioxan—acetic acid (90:25:4). Detecting reagents: freshly diazotized *p*-nitroaniline followed by 0·5 M-KOH in ethanol. $FeCl_3$+ ferricyanide: chromatograms were dipped in a mixture of equal vol. of $FeCl_3,6H_2O$(0·3% w/v) and $K_3Fe(CN)_6$(0·3% w/v) in water, then in 2 M-HCl and then water. Gibbs reagent is a spray of ethanolic 2,6-dichloroquinonechloroimide (1%) followed by saturated $NaHCO_3$. HCl then Gibbs reagent: the paper was sprayed with 5 M-HCl, dried and then sprayed with Gibbs reagent and $NaHCO_3$. Naphtharesorcinol is a spray of naphtharesorcinol (1% w/v) in aq. trichloroacetic acid (33% w/v) followed by heating the paper at 120° for 5–10 min. Phenols on the t.l.c. plates were detected as dark spots on illumination with u.v. light (254 nm) from a Hanovia Chromatolite lamp, s = streaks; –, not tested.

Compound	R_F values in solvents					Colour reactions on paper				
	Paper			Thin-layer						
	A	B	C	D	E	Diazotized *p*-nitroaniline	$FeCl_3$ + ferricyanide	Gibbs reagent	HCl then Gibbs reagent	Naphtharesorcinol
Phenol	0·92	0·94	0·97	0·68	0·82	Yellow red	Blue	Blue	Blue	None
Catechol	s	s	0·90	0·54	0·62	Yellow red	Blue	Blue	Blue	None
Resorcinol	—	—	—	0·45	0·55	—	—	—	—	—
Quinol	s	s	0·89	0·42	0·49	Red	Blue	—	—	—
Phenyl sulphate	0·75	0·52	0·60	—	—	None *	None	None	Blue	None
4-Hydroxyphenyl sulphate	0·55	0·25	0·55	—	—	Yellow red	Blue	Blue	Blue	None
Phenylglucuronide	0·45	0·10	0·25	—	—	None	None	None	—	Blue
4-Hydroxyphenyl-glucuronide	0·20	0·00	0·00	—	—	Yellow red	Blue	Blue	—	Blue

*Turns yellow red after 5–10 min.

Table 2. Urinary metabolites of [^{14}C]phenol in various species

[^{14}C]Phenol in water was administered by stomach syringe or tube except in the case of the primates, carnivores and pigs, when it was given incorporated in the food or drink. Urine was collected for 24 h and chromatographed (see text). Radiochromatograms were prepared and the areas corresponding to peaks were cut out and their ^{14}C determined by scintillation counting. Where 3 or more animals were used averages are given with ranges in parentheses. Individual values are given where only one or two animals were used. tr. means < 1% of urinary ^{14}C; — means no ^{14}C peak was detected by radiochromatogram scanning. m = male; f = female

Species (No., sex and trivial name)	Family or other description	Dose: Phenol (mg/kg)	Dose: ^{14}C (μCi/animal)	^{14}C excreted in 24 h (% dose)	%^{14}C excreted in 24 h found as: Phenyl sulphate	Quinol sulphate	Phenyl glucuronide	Quinol glucuronide	Total sulphate	Total glucuronide	Total quinol
Primates											
Man (3 m)	Homo	0·01	2·7	90(85–98)	77(69–90)	1†	16(4–23)	tr	78	16	1
Rhesus monkey (2 f)	Macaca	50	10	37, 49	60, 70	—	40, 30	—	65	35	—
Squirrel monkey (3 f)	Saimiri	25	8	31(18–52)	7(2–17)	—	68(55–86)	25(11–36)	7	93	25
Capuchin (1 f)	Cebus	25	8	73	14	—	65	21	14	86	21
Carnivores											
Ferret (3 f; mongrel)	Mustelidae	25	5	51(49–54)	28(19–34)	30(21–34)	40(22–52)	—	58	40	30
Cat (3 f; mongrel)	Felidae	25	8	59(52–61)	87(86–89)	13(11–14)	—	—	100	—	13
Dog (1 f, 1 m; mongrel)	Canidae	25	10	53, 62	33, 68	43, 20	24, 12	—	82	18	32
Miscellaneous											
Pig (3 f; large White)	Suidae	21	10	51(18–72)	—	—	100	—	—	100	—
Hedgehog (2 m; European)	Erinaceidae	20	7	34, 43	63, 86	17, 4	20, 10	—	85	15	10
Fruitbat (2 f; Indian)	Pteropidae	25	5	50, 58	9, 11	—	91, 89	—	10	90	—
Rabbit (3 f; New Zealand white)	Leporidae	25	10	48(35–60)	45(41–48)	9(7–13)	46(39–52)	—	54	46	9
Chicken (3 f; Rhode Island red)	Phasianidae	25	10	57(53–60)	78(57–91)	—	22(9–43)	—	78	22	—
Rodents											
Rat (3 f; Wistar albino)	Muridae	25	5	95(91–100)	54(44–65)	1(0–2)	42(35–55)	2(0–5)	55	44	3
Mouse* (3 × 10 f; ICI)	Muridae	25	2·5	66(64–68)	46(40–51)	5(3–8)	35(33–37)	15(12–17)	51	50	20
Jerboa (3 f; Egyptian)	Dipodidae	25	6·5	47(28–65)	61(51–71)	12(9–15)	26(20–33)	1(0–4)	73	27	13
Gerbil (2 f; 1 m)	Cricetidae	25	3	55(45–68)	42(22–53)	19(13–23)	35(30–49)	1(0–4)	61	36	20
Hamster (2 f; golden)	Cricetidae	25	5	73, 78	27, 24	1, tr	44, 41	28, 27	26	70	28
Lemming (3 f; steppe)	Cricetidae	25	5·6	40(15–67)	35(18–51)	10(8–12)	39(28–48)	15(10–23)	45	54	25
Guinea-pig (2 f; English)	Caviidae	25	2·7	64, 64	13, 22	tr, tr	82, 73	5, 5	18	82	5

*The urines of 10 mice were pooled for each determination. †One subject only.

Chromatography

The R_F values and colour reactions of phenol and its metabolites on paper and t.l.c. are given in Table 1. For radiochromatogram scanning the urine (0·1–0·5 ml) was placed as band on strips (5 cm wide) of Whatman No. 1 paper and developed with the solvents listed in Table 1. The paper was scanned in a Packard radiochromatogram scanner, model 7200.

Thin-layer chromatography was carried out with fluorescent silica gel (silica gel HF_{254}, Merck, Anderman & Co. Ltd. London), 0·1 mm layers being used for detection and 0·8 mm for preparative work.

Radiochemical techniques

^{14}C was determined using a Packard Tri-Carb Scintillation Spectrometer (model 3214). Urine samples (0·5–1·0 ml) were counted in quadruplicate in a dioxan scintillator fluid (20 ml) (Bridges, Davies & Williams, 1967). In some experiments the paper strip chromatograms were cut into 1 cm pieces and placed in dioxan scintillation fluid and the associated ^{14}C was determined.

Experiments with liver preparations

Liver samples (10 g cooled on ice) from several male rats (Wistar albino) and female pigs (large White), removed immediately after killing, were homogenized at 5° in a Waring blendor in a medium consisting of 0·154 M-KCl (78 ml), 0·154 M-$MgSO_4$ (2 ml) and 0·1 M-sodium phosphate buffer, pH 6·8 (20 ml), to give 10% w/v homogenates (see De Meio, Wizerkaniuk & Fabiani, 1953). The sub-mitochondrial fraction of the homogenate was prepared by centrifuging in an MSE 'high speed 17' refrigerated centrifuge at 10 000 ($g_{av.}$) for 10 min and part of this 10 000 ($g_{av.}$) supernatant was used for investigating glucuronide synthesis. The remaining sub-mitochondrial fraction was centrifuged at 100 000 ($g_{av.}$) for 1 h in an MSE Automatic 'Superspeed 40' centrifuge to sediment the microsomal fraction and the supernatant (soluble fraction) was used for investigating ester sulphate synthesis. De Meio, *et al.* (1953) have reported that the microsomal fraction inhibits the synthesis of arylsulphates.

Incubations

(*a*) For investigation of the glucuronide synthesis, the incubates in duplicate consisted of the 10 000 ($g_{av.}$) supernatant (2 ml), aq. [^{14}C]phenol soln. (0·3 ml, equiv. to 0·2 mg phenol and 0·5μCi) or *o*-aminophenol soln. (0·2 ml; 1 mg/ml) and aq. UDPGA soln. (0·5 ml; 10 mg/ml). Suitable controls in which the substrates were omitted were also prepared. The mixtures were then incubated in glass-stoppered tubes for 1 h at 37° in a shaking water-bath (H. Mickle, Gomshall, Surrey). At the end of the incubation time, [^{14}C]phenol soln. (0·3 ml as above) was added to one control incubate, and the reaction in all the tubes containing phenol terminated by adding 10% aq. sodium tungstate (0·5 ml). The reaction in the tubes containing *o*-aminophenol was terminated by the addition of a mixture of equal parts of 2 M-trichloroacetic acid and 2 M-orthophosphoric acid (2 ml). The incubates were adjusted to 5 ml with water and then centrifuged at 2000 rev/min for 10 min in the MSE Minor centrifuge to remove precipitated protein.

(*b*) For investigation of sulphate synthesis, the incubates (duplicates) consisted of the 100 000 ($g_{av.}$) supernatant (2 ml), aq. [^{14}C]phenol soln. (0·3 ml, equiv. to 0·2 mg phenol and 0·5μCi) and an aq. soln. of ATP (0·2 ml; 6·24 mg/ml). In the controls phenol was omitted. The mixtures were incubated in glass-stoppered tubes in a shaking water-bath for 1 h at 37°. At the end of the incubation time, [^{14}C]phenol (0·3 ml as above) was added to the controls and then all incubation mixtures were treated with 10% aq. sodium tungstate (0·5 ml), diluted to 5 ml with water and centrifuged, as above, to remove protein.

Samples (0·1 ml) of the protein free incubates were chromatographed as bands on paper strips (Whatman No. 1) in solvents A, B and C (Table 1) and scanned for ^{14}C. Phenol, phenylglucuronide and phenylsulphate were located using reference samples of the compounds which were chromatographed simultaneously. The conversion of phenol to phenylsulphate or phenylglucuronide was estimated by cutting out the areas of the chromatogram corresponding to phenol and its sulphate and glucuronic acid conjugates and counting in dioxan scintillator. The extent of formation of *o*-aminophenylglucuronide was determined colorimetrically by diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine according to Levvy & Storey (1949) using a Unicam SP 500 spectrophotometer and a standard curve.

Results and discussion

The radiochromatogram scans of the urines of some of the species (Table 2) receiving [^{14}C]phenol showed four peaks which corresponded to phenylsulphate, phenylglucuronide, quinol monosulphate and quinol monoglucuronide. Catechol conjugates did not appear to be formed in sufficient quantities to be detected on any of the radiochromatograms under the conditions of these experiments and at the dose level of phenol (usually 25 mg/kg) used. Parke & Williams (1953) had reported the presence of minor amounts of catechol conjugates (1% or less of the dose) in the urine of rabbits receiving doses of [^{14}C]-phenol of 50 mg/kg.

The above four metabolites of phenol were detected in the urine of eight species, namely the rat, mouse, jerboa, gerbil, hamster, lemming, guinea-pig and man. Apart from man, all these are rodent species. The results for man however are not strictly comparable with any of the other species listed in Table 2 since the dose of phenol taken by the three human subjects was only 0·01 mg/kg which is 2500 times smaller than that given to the other species. However, this shows that man can conjugate phenol with sulphate and glucuronic acid and oxidize it to quinol. In six of the species listed in Table 2 only three of the four metabolites were detected. This means that the fourth metabolite is either absent or more probably is present at levels too low for detection by the methods used. The two New World monkeys, the squirrel monkey and capuchin, excreted the two glucuronides and phenylsulphate. At the level of phenol administered, glucuronic acid conjugation is much more effective than sulphate conjugation in these herbivorous monkeys, and the ability to oxidize phenol to quinol is also quite significant (25 and 21% of the 24 h excretion). The other animals which excrete three metabolites are the ferret, dog, hedgehog and rabbit, but this time the metabolites are the two sulphates and phenylglucuronide. In the dog and hedgehog, sulphate conjugation at the

level of phenol administered (25 mg/kg) is much more effective than glucuronic acid conjugation, while in the ferret and rabbit the two conjugation mechanisms are almost equally effective. The ferret and dog appear to oxidize phenol to quinol (about 30%) more effectively than the hedgehog and rabbit (about 10% of the 24 h excretion).

There are four species in Table 2, namely the rhesus monkey, cat, fruit bat and chicken which excrete only two of the metabolites, and one species, the pig, which excretes only one metabolite. The rhesus monkey, fruit bat and hen excrete phenylsulphate and phenylglucuronide but did not appear to oxidize phenol to quinol. The sulphate conjugation is more effective than the glucuronic acid conjugation in the rhesus monkey and hen, whereas in the fruit bat there is nine times as much glucuronide excreted as sulphate. It will be recalled (Bridges, *et al.*, 1970) that whereas most species conjugate benzoic acid mainly with glycine, the fruit bat conjugates the acid almost exclusively with glucuronic acid. The cat appears to conjugate phenol and quinol only with sulphate whereas the pig appears to excrete phenol entirely as phenylglucuronide.

The cat and the pig

The results in Table 2, which were obtained by radiochromatogram scanning, show that most of the species examined except the cat and the pig excrete phenol conjugated with glucuronic acid and sulphate. The cat appeared to produce no glucuronide and the pig no sulphate. The cat and the pig were therefore examined in greater detail and the radiochromatograms of the urine of these animals were examined by scintillation counting after cutting the whole chromatogram into small pieces. The results of these experiments are shown in Table 3.

In the case of the cat the major urinary metabolite of phenol given orally or intraperitoneally at 5 or 25 mg/kg is phenylsulphate. There is, however, a small but definite excretion of phenylglucuronide which is less than about 1% of the ^{14}C in the urine. Quinol glucuronide was detected in the urine of one cat only in very small amounts (0·1% of ^{14}C in urine). The cat also excretes appreciable amounts of quinol sulphate, twice as much being excreted after intraperitoneal injection as after oral administration (see Table 3). The greater production of the quinol conjugate after intraperitoneal injection of phenol may be a reflection of the ability of the intestinal tissue of the cat to conjugate phenol given orally. If phenol is partly conjugated during absorption from the intestine, then less free phenol is available for oxidation to quinol.

The cat appears to have a defective glucuronic acid conjugation mechanism (see Dutton, 1966), but the conjugation is not entirely absent for it occurs at a very low level with phenol as indicated in Table 3. It is probable that for many compounds the capacity of the cat to conjugate them with glucuronic acid is at a low level. However, Mowat & Arias (1970) have shown that cat liver homogenates are able to conjugate *p*-nitrophenol but not *o*-aminophenol with glucuronic acid. It is thus possible that glucuronic acid conjugation in the cat may be at a low or negligible level for some compounds but not others.

The pig seems to be the reverse of the cat in that phenol is conjugated almost entirely with glucuronic acid. However, Table 3 shows that the sulphate conjugation is not entirely absent, but occurs at a very low level. Some preliminary experiments were carried out with pig liver homogenates and the results

Table 3. Urinary metabolites of [^{14}C] phenol in the cat and the pig

Four fully grown mongrel cats were used, A, B and D being females and C, a male. Five pigs (large White; 11–22 weeks old; 18–40 kg body wt.) were used. Nos. 1, 2, 3 and 5 being females and No. 4 a male. [^{14}C]Phenol was administered either orally mixed with food or in water by i.p. injection. Urine was collected for 24 h and chromatographed on Whatman No. 1 paper using solvent system C (see table 1) for cat urine and A for pig urine. The radiochromatograms were cut into suitable pieces which were then counted (see text). For cats, the average values are given with ranges in parentheses. In column 5, the ^{14}C output for each cat is given in the order found in column 1. The values for pigs are in the order given in column 1; – means < 0·1% of the ^{14}C in the urine.

Animals	Dose Phenol (mg/kg)	Dose ^{14}C (μCi/animal)	Route of administration†	^{14}C excreted in 24 h (%dose)	%^{14}C excreted in 24 h found as Phenyl sulphate	Quinol sulphate	Phenyl glucuronide	Quinol glucuronide
Cats*								
B, D, A	5	10	o	57(35,57,78)	93(89–95)	6·1(4·0–9)	1·4(1·0–2·0)	—
B, C, B	25	10	o	49(23,60,65)	88(81–98)	9·3(1·2–15)	0·2(<0·1–0·3)	0·1‡
B, A, A	5	10	i.p.	79(66,85,86)	85(81–89)	10(8·2–14)	0·3(<0·1–0·5)	—
B, B, A	25	10	i.p.	63(49,69,72)	67(64–69)	21(20–21)	0·2(<0·1–0·3)	—
Pigs								
5, 3	5	10	o	60,65	0·4,0·3	—	99,99	—
4, 3	25	10	o	76,95	3.6,1·2	—	96,99	—
1, 2	5	5	i.p.	89,97	3·3,0·2	—	93,96	3.8,3·4
5, 4	25	10	i.p.	79,115	4·3,8·3	—	96,92	—

*With cat urine some unidentified ^{14}C-containing material remains at the origin of the chromatograms. This accounted for 5 and 12% respectively of the ^{14}C excreted after intraperitoneal doses of 5 and 25 mg/kg and 0·2 and 2·5% for the same doses given orally.

† o = oral; i.p. = intraperitoneal

‡ [^{14}C]Quinol glucuronide was found in the urine of the male cat (C) only.

Table 4. Conjugation of [^{14}C]phenol with sulphate and glucuronic acid in rat and pig liver homogenates

Each incubate contained 201·9 μg (2·15 μmol) phenol and 0·5 μCi of ^{14}C, or 200 μg (1·84 μmol) *o*-aminophenol. The other components of the incubation mixtures and details of incubation and estimation are given in the text

Substrate	Species	Conversion to sulphate ester *		Conversion to glucuronide†	
		% added substrate	rate (μmol/g liver/h)	% added substrate	rate (μmol/g liver/h)
	Rat				
Phenol	1	80	8·6	—	—
	2	72	7·7	89	9·6
	3	67	7·2	91	9·8
o-Aminophenol	2	—	—	68	6·3
	3	—	—	64	5·9
	Pig				
Phenol	1	22	2·5	100	>10·8
	2	10	1·1	100	>10·8
	3	20	2·2	86	9·2
o-Aminophenol	2	—	—	61	5·6
	3	—	—	38	3·5

* Using the 100 000 ($g_{av.}$) supernatant (see text).
† Using the 10 000 ($g_{av.}$) supernatant (see text).

in Table 4 show that these preparations are not devoid of the ability to conjugate phenol with sulphate. The homogenates were able to conjugate phenol with glucuronic acid at a relatively fast rate but with sulphate at a slower rate. In similar experiments with rat liver the rate of conjugation of phenol with sulphate is almost equal to that with glucuronic acid. It would appear, therefore, that in the whole pig, glucuronic acid conjugation of phenol, administered at the level used in these experiments, is so facile that sulphate conjugation, which may be slow, has little opportunity of occurring. Rapid conjugation of phenol with glucuronic acid may also prevent the oxidation of phenol to quinol.

It has been reported by Roy (1963) that liver homogenates from the marsupial oppossum, *Trichosurus vulpecula*, had a low rate of sulphate conjugation of *p*-nitrophenol. Furthermore, sulphate esters were not detected in normal oppossum urine, and a dose of 1-naphthol was excreted mainly as glucuronides with only traces of the ester sulphate.

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