IN VIVO AND IN VITRO METABOLISM OF FOREIGN CHEMICALS
IN VARIOUS ANIMAL SPECIES

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by

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TO MY WIFE AND MY PARENTS
"Those who refuse to go beyond fact rarely get as far as fact; and anyone who has studied the history of science knows that almost every step therein has been made by .... the invention of a hypothesis which, though verifiable, often had little foundation to start with ...."

T. H. Huxley

The essence of science 'lie not in discovering facts, but in discovering new ways of thinking about them'.

Sir Lawrence Bragg

"The History of Science"
I am greatly indebted to Dr. J.W. Bridges for his constant encouragement, interest and invaluable guidance during the course of this work. I would also like to express my gratitude to my colleagues, in particular Mr. R. Shirkey and Mr. S. Wood for many hours of valuable discussion and profitable collaborative research, and to Dr. J. Fray and the Cell Culture Unit for their help during the investigation of metabolism in isolated cell preparations. I would also like to thank Professor D.V. Parke and members of his departmental, academic, secretarial and technical staff, for their help over the last three years.

I am especially grateful to Dr. J.K. Faulkner for his interest and advice during this study and to members of his department for their help during my visits at Pfizer Research, Sandwich.

My thanks are also due to Mrs. Margaret Ho for the typing of this thesis, to Mr. Eric Leung for the photographs and to Jackie for her understanding and encouragement during the preparation of this thesis.

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ABSTRACT

The in vivo metabolism of some "model" compounds, i.e. benzoic acid, phenol and aniline was investigated in the sheep, the pig and the rat. Using anaesthetised ureter cannulated rats a preliminary examination was made of the time course of the urinary metabolic profile following intraduodenal administration. Some in vitro metabolism studies of these model compounds were also investigated using isolated cells from various organs (liver, kidney and intestine) from various laboratory animal species (rat, dog and ferret).

Following an oral dose of these "model" compounds urinary elimination in all three species was very rapid and some 80-100% of the dose was recovered in the urine during the first 24 hours. For aniline, the pig was an exception in that only 56% of the dose (50 mg kg\(^{-1}\)) was eliminated in the urine in 24 hours. Faecal excretion of these compounds in all three species was < 2% of the dose.

In vivo metabolism of benzoic acid (50 mg kg\(^{-1}\)) in all three species produced hippuric acid (> 90%) as the major 24 hour urinary metabolite and benzoylglucuronide as a minor metabolite (< 5%).

The major 8 hour urinary metabolites from phenol (25 mg kg\(^{-1}\)) metabolism were phenylglucuronide and phenylsulphate. In the sheep, the pig and the rat, the glucuronide was 49%, 83% and 42% of the total urinary metabolites respectively, while the sulphate represented about 32%, 1% and 55% respectively. O-Conjugates of quinol were minor urinary metabolites (< 7%) in all three species. In the sheep alone some 12% of the urinary metabolite was conjugated with phosphate.
**N**-Acetylated derivatives were identified as the major 24 hour urinary metabolites of aniline (50 mg kg\(^{-1}\)) representing 82%, 85% and 76% of the urinary metabolites from the sheep, the pig and the rat respectively. The double conjugate **N**-acetyl-\(\beta\)-aminophenylglucuronide was the major metabolite in the sheep and the pig (60% and 66% respectively) while **N**-acetyl-\(\beta\)-aminophenylsulphate was the major metabolite in the rat (56%). Minor urinary metabolites included O-conjugates of \(\alpha\)-, and \(\beta\)-aminophenol (\(\approx\) 20%), acetanilide (\(\approx\) 3%) and **N**-acetyl-\(\beta\)-aminophenol (\(\approx\) 10%).

First order kinetics were observed in the metabolism of these compounds in the anaesthetised ureter cannulated rat and some 70-80% of an intraduodenal dose of phenol (12.5 and 25 mg kg\(^{-1}\)) and benzoic acid (50 and 100 mg kg\(^{-1}\)) was eliminated in about 2 hours, whereas only about 50% of a dose of aniline (25 and 50 mg kg\(^{-1}\)) was eliminated in about 3 hours. Different rates of elimination of individual metabolites were observed and this was reflected in the changes in the ratio of metabolite with time.

Benzoic acid was conjugated extensively with glycine by isolated liver and kidney cells from the rat, whereas, the kidney cells but not the liver cells carried out this reaction in the dog and the ferret. Liver cells from these animals showed a limited ability to form benzoylglucuronide. Phenol was O-conjugated and aniline was **N**-acetylated by isolated intestinal mucosal cells from the rat. Isolated liver cells from the rat were shown to be able to metabolise phenol and aniline giving metabolic patterns similar to those obtained from in vivo metabolism studies.
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FOREWORD

The environment has always contained a wide variety of substances which are considered foreign to the animal body. These substances are usually consumed as a consequence of their presence in the normal diet and as man becomes more dependent on synthetic chemicals in the form of drugs, food additives, pesticides and herbicides, etc., for his well-being, other animal species, in particular, species of agricultural and economic importance will also be subjected to continuous increase in exposure to additional foreign compounds. To many chemicals lipophilic membranes present no barrier, thus it is apparent that large numbers of foreign compounds are being absorbed through ingestion in food and drink, in the inspired air and through the skin continuously and it is therefore important to know the fate of these foreign compounds in the animal body.

Probably all these foreign compounds are capable of evoking pharmacologic and toxicologic responses in animals which have been exposed to them and although animals in general are protected from the noxious effects of these foreign compounds by detoxication mechanisms present in the animal body, nevertheless, it is important to know and to understand the functional significance of these biochemical mechanisms, how they work and what are their limitations as protective systems. To this end the study of the biochemistry of foreign compounds has made tremendous advances in recent years and numerous scientific publications and treatise have been produced, so much so that it has become a basic scientific discipline of pharmacology, toxicology and pathology.

Detoxication is a biochemical defence mechanism of the body which limits the duration and intensity of a response evoked by
the exposure to foreign compounds and it is a complex dynamic function of the body. Metabolism is generally regarded as the most important process in detoxication, however, other processes such as absorption, distribution and excretion are also important in the detoxication and elimination of foreign compounds from the animal body. Thus to consider one process to the exclusion of another over simplifies the dynamic interactions involved so that an understanding of the mechanism of detoxication is necessarily only approximate. It is important to bear this in mind when considering the role of metabolism in detoxication.

The metabolic fate of many foreign compounds has been extensively investigated, but few studies have been conducted concerning the interaction of metabolism with other processes involved in detoxication. The role of metabolism in detoxication of drugs has been studied for nearly a century, and it is apparent that metabolism plays an important role in rendering an active foreign compound biologically inert or altering the properties of that compound so that other processes of detoxication such as renal or biliary excretion can remove that compound more readily from the body. The extent to which any one process plays a role in detoxication depends largely on the foreign compound, the animal species and route of administration. It is generally accepted that metabolism is mediated principally by the liver and relatively little significance has been attributed to metabolism by other organs even though it is known that they may be involved in the metabolism of foreign compounds. However, recent studies have shown that extrahepatic metabolism of foreign compounds does have functional significance in the detoxication of foreign compounds. Extrahepatic tissues which are being extensively studied include, the intestines, lungs, kidneys, blood, brain and placenta.
In the following thesis a comprehensive review on the metabolism of foreign compounds will not be attempted, but rather only those concepts pertinent to the understanding and discussions of the thesis will be considered.
CHAPTER I

INTRODUCTION
1.1 BIOTRANSFORMATION OF FOREIGN COMPOUNDS

1.1.1 Mechanisms of Biotransformation of Foreign Compounds

In the animal body foreign compounds undergo a wide range of biotransformations which in general are carried out in two phases and the reactions involved may be classified into oxidation, reduction, hydrolysis and synthesis or conjugation. Oxidation, reduction and hydrolysis constitute what are termed the phase I reactions while synthesis or conjugation constitute the phase II reactions (Williams 1959, Williams 1967, Parke 1968). In general most foreign compounds in the body undergo a phase I reaction and the products of the phase I reaction are metabolised further by a phase II reaction, to form more water soluble materials which are then eliminated from the body. Not all foreign compounds are metabolised by the two phase process, some compounds are metabolised mainly by just a phase I or a phase II reaction.

Most foreign compounds are metabolised in the liver of animals and the metabolising enzymes can occur in the soluble, mitochondrial or microsomal fractions of the hepatic tissues. A drug may be metabolised by more than one metabolic pathway, and indeed very often a foreign compound may be subjected to several competing pathways of metabolism simultaneously so that the extent of formation of the various metabolites will depend on the relative rates of the various pathways involved. However, enough information is available at present so that by analogy, the major route of metabolism of most foreign compounds may be anticipated, but it has not yet been possible to predict precisely the pathway of metabolism of a given compound in a given species.

This section will deal primarily with metabolic pathways of biotransformation of foreign compounds which are relevant to the
present investigation. However, for the purpose of completeness a list of characteristic drug metabolising reactions are given in tabulated form in the following subsections.

1.1.1.1 Phase I Reactions

Metabolic reactions which may be classified as phase I reactions are carried out by enzymes of the normal metabolic pathways of the body and by the so-called "drug metabolising enzymes" or "mixed function monooxygenases". Most compounds are metabolised by the latter which are enzymes associated with the endoplasmic reticulum of cells. Although the liver is the major site of metabolism of most foreign compounds, recent studies have shown that drug metabolising activities are also present in the microsomal fraction of cells of extrahepatic tissues. The subcellular location of typical phase I reactions is summarized in Table 1.1.

The phase I reactions which are most relevant to this thesis are aromatic hydroxylation and N-oxidation and it is perhaps significant that these are probably the most studied phase I oxidative reactions.

Most aromatic compounds are hydroxylated by the hepatic microsomal cytochrome \( P_{450} \) dependent enzymes to yield phenols. The hydroxylation of benzene to phenol is perhaps the classical example of aromatic hydroxylation affording a striking illustration of the reactivity of the monooxygenase system of enzymes. Numerous mechanisms of hydroxylation of aromatic compounds have been proposed and it is now well established that the insertion of molecular oxygen to give an epoxide (arene oxide) is the first step in the majority of aromatic hydroxylations. Figure 1.1 shows that an arene oxide intermediate can
Table 1.1

**Typical Phase I Reactions**

<table>
<thead>
<tr>
<th>Chemical Reactions</th>
<th>Location of Enzymes</th>
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<tbody>
<tr>
<td></td>
<td>Mic.</td>
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<tr>
<td><strong>OXIDATIONS:</strong></td>
<td></td>
</tr>
<tr>
<td>Hydroxylation</td>
<td></td>
</tr>
<tr>
<td>aliphatic</td>
<td>+</td>
</tr>
<tr>
<td>aromatic</td>
<td>+</td>
</tr>
<tr>
<td>Dealkylation</td>
<td></td>
</tr>
<tr>
<td>O-</td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td></td>
</tr>
<tr>
<td>S-</td>
<td></td>
</tr>
<tr>
<td>Epoxidation</td>
<td></td>
</tr>
<tr>
<td>Oxide formation</td>
<td></td>
</tr>
<tr>
<td>N-</td>
<td></td>
</tr>
<tr>
<td>S-</td>
<td></td>
</tr>
<tr>
<td>Aromatization</td>
<td></td>
</tr>
<tr>
<td>Alcohol oxidation</td>
<td></td>
</tr>
<tr>
<td>Aldehyde oxidation</td>
<td></td>
</tr>
<tr>
<td>Desulphuration</td>
<td></td>
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<tr>
<td>Dehalogenation</td>
<td></td>
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<tr>
<td>Oxidative deamination</td>
<td></td>
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<tr>
<td><strong>REDUCTION:</strong></td>
<td></td>
</tr>
<tr>
<td>Nitroreduction</td>
<td></td>
</tr>
<tr>
<td>Azoreduction</td>
<td></td>
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<tr>
<td>Aldehyde reduction</td>
<td></td>
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<tr>
<td>Oxide reduction</td>
<td></td>
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<tr>
<td><strong>HYDROLYSIS:</strong></td>
<td></td>
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<tr>
<td>Deesterification</td>
<td></td>
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<tr>
<td>Deamination</td>
<td></td>
</tr>
</tbody>
</table>

Mic. - Microsomes
Mit. - Mitochondria
Cyt. - Cytosol
FIGURE 1.1
MECHANISMS OF ENZYMIC AROMATIC HYDROXYLATION

R

TAUTOMERISM

O

mono-oxygenase

R

NIH shift

R

epoxide hydratase

O

GSH S-epoxide transferase

OH

conjugated and/or O-methylated

GSH = glutathione
give rise to a number of metabolites: phenols, dihydrodiols and mercapturic acids. The nature and distribution of these metabolites are dependent on a number of factors including the stability of arene oxides with regard to isomerization to the phenols, suitability as a substrate for epoxide hydrolase and its affinity to nucleophilic groups of tissue macromolecular components. The relative stability of arene oxides is a function of the number and type of substituents present in the oxirane ring, and electron donating substituents will tend to decrease while electron withdrawing substituents will tend to increase the stability of the arene oxide. Thus the lifetime of the arene oxides, which may leave the microsomal enzymes prior to further metabolism and have a high affinity for tissue macromolecular components in vivo, may have important significance in tissue response to the parent aromatic compounds. Other factors which are important include the susceptibility of arene oxides to epoxide hydratase and glutathione-S-epoxide transferase activity and the availability of glutathione.

The isomerization of arene oxides to phenols is a reaction which occurs nonenzymically and in neutral and basic environments it is a spontaneous rearrangement while at pH < 6, the arrangement is an acid catalysed isomerization. In both cases, the rate limiting step is believed to be the opening of the epoxide to form either the zwitterion or the protonated derivative. This isomerization is accompanied by intramolecular displacement of an aromatic substituent (e.g. isotopic hydrogen, halogen and methyl group) from the place of the final location of the phenolic group to a neighbouring carbon atom. This phenomenon is known as the "N.I.H. shift" (Guroff et al 1967) and has now been accepted as providing a priori evidence for the intermediacy of arene oxides in enzymic aromatic hydroxylation. The mechanism of isomerization to phenols
involving the "N.I.H. shift" has been considered by many authors and an authoritative review on the subject has been published recently (Daly et al 1972). Under physiological pH conditions, the proposed mechanism may be summarized in the following simplified scheme:

![Chemical structure](image)

The synthesis of trans-dihydrodiols is a reaction which is catalysed by the enzyme epoxide hydratase. It has been studied in great depth and a comprehensive review on the subject has been published recently (Oesch 1973). The enzyme is localised in the microsomal fraction and shows a close relationship with the microsomal mixed function mono-oxygenase system of enzymes. The liver is the main site of epoxide hydratase activity and low levels of epoxide hydratase have also been found in the kidney, lungs, intestines and skin, but apparently no activity was detectable in the brain, heart, spleen and muscle. The dihydrodiols formed by epoxide hydratase are either conjugated with glucuronic acid and/or converted to catechols by soluble hepatic hydrogenase and the catechols are subsequently metabolised by catechol-O-
methyltransferase or other conjugations. Formation of dihydrodiols is also evidence for the involvement of epoxides in enzymic aromatic hydroxylation.

Arene oxides are also readily opened by nucleophiles, especially those containing sulphur and the addition of the thiol group of glutathione to arene oxides can lead to glutathione conjugates. This conjugation proceeds either spontaneously or is catalysed by soluble hepatic glutathione-S-epoxide transferase(s). The glutathione conjugates formed are converted to premercapturic acids in vivo and subsequently to mercapturic acids under acid conditions. Arene oxides are also found covalently bound to tissues, probably through nucleophilic moieties in tissue macromolecules.

Other mechanisms for aromatic hydroxylations have also been postulated and it is likely that they may be involved in specific cases. The conversion of aniline to p-aminophenol and benzene to phenol has been shown to proceed via hydroxymethylation to give intermediates of p-aminobenzylalcohol (Sloane 1964, Sloane and Heinemann 1967) and benzylalcohol (Sloane 1965) respectively. This is a relatively minor pathway and the mechanism is unknown but a firmly bound form of an active folate derivative may be the carbon donor for the hydroxymethylation reaction (Sloane and Heinemann 1970).

Aromatic hydroxylation is generally catalysed by microsomal enzymes, but recently aniline hydroxylase activity in rat and human placenta was found in the cytosol and the catalytic activity was observed to be associated with haemoglobin (Juchau and Symms 1972). The o-hydroxylation of 2-acetyl-aminofluorene via an enzyme catalysed isomerization of the N-hydroxylated intermediate, N-hydroxy-2-acetylaminofluorene.
(Gutmann and Erickson 1969, 1970) offers another mechanism of aromatic hydroxylation. The reaction appears to be restricted to arylhydroxamic acids. It is catalysed by enzymes of the soluble fraction of the liver and it has been suggested that hydroxylamines may be intermediates in \( \alpha \)-hydroxylation of aromatic amines (Booth and Boyland 1964). It is usually only a minor pathway, but amines in which the \( \beta \)-position is blocked are more readily metabolised by this route (Von Jagow et al 1966, Kampffmeyer and Kiese 1964).

Because of the common occurrence of nitrogen in many drugs and foreign compounds, much interest has been directed towards the interrelationships of various biological oxidation reactions involving the nitrogen in organic molecules. There are at least two microsomal enzyme systems which are involved in the enzymic oxidation of organic nitrogen and a relationship has been suggested between the \( pK_a \)'s of the organic nitrogen and the enzyme system involved, namely that the basic amines with \( pK_a 8-11 \) are oxidised by a flavin-adenosine nucleotide (FAD) - dependent enzyme system while nonbasic nitrogen containing compounds such as those with adjacent carbonyl groups whose \( pK_a \)'s are 1 or less are oxidised by a cytochrome \( P_450 \) dependent system. Furthermore intermediate compounds with \( pK_a 1-7 \) are substrates of both enzyme system which would yield the same products but by different processes (Gorrod 1973). This concept has however been strongly challenged by Hlvica (Personal Communication). A mixed function amine oxidase has been solubilised from pig hepatic microsomal fraction with detergent, which is capable of oxidising some secondary and tertiary amines with "intermediate" and "basic" \( pK_a \) nitrogens (Zieglar and Mitchell 1972).
Oxidation of organic nitrogen gives rise to N-hydroxy or N-oxide metabolites depending on whether the nitrogen is primary, secondary or tertiary. Thus it has been shown that many aromatic primary amines, secondary amides and some carbamates are metabolised by N-oxidation to give N-hydroxy metabolites while tertiary amines are metabolised to N-oxides (Gorrod 1973). Typical examples of nitrogen oxidation include the N-hydroxylation of 2-acetyl-aminofluorene to give N-acetyl-N-2-fluorenylhydroxylamine (Cramer et al 1960), this reaction is probably essential for the carcinogenic properties of 2-acetylamino-fluorene, and the synthesis of phenylhydroxylamine from aniline (Kiese 1959). From the point of view of aromatic amines, N-oxidation to give arylhydroxylamine results in the synthesis of potentially toxic metabolites and arylhydroxylamines are implicated in ferrihaemoglobin formation in many species after administration of the parent amines (Kiese 1966).

1.1.1.2 Phase II Reactions

Phase II reactions or conjugations are biosynthetic reactions by which foreign compounds and their metabolites are conjugated to endogenous substrates. In general conjugation involves the transfer of a readily available endogenous substrate from a donor to the foreign compound containing functional groups (e.g. OH, SH, COOH, NH₂) which act as the acceptors.

A conjugation requires, amongst other things, an "active" intermediate, usually a nucleotide and a transferring enzyme and from the point of view of the mechanism, there are two kinds of conjugation reactions: one in which the conjugating agent forms part of the active intermediate (e.g. glucuronic acid conjugation) and the other in which
<table>
<thead>
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<th>Chemical Reactions</th>
<th>Donor</th>
<th>Major Acceptors</th>
<th>Enzyme Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucuronic acid conjugation</td>
<td>UDPGA</td>
<td>-OH, -COOH, -NH₂, NH, -SH</td>
<td>Mic. Mit. Cyt.</td>
</tr>
<tr>
<td>Sulphate conjugation</td>
<td>PAPS</td>
<td>ArOH, ArNH₂, ROH</td>
<td>+</td>
</tr>
<tr>
<td>Acetylation</td>
<td>Acetyl-CoA</td>
<td>ArNH₂, RNH₂H₂, RNH₂, ArSO₂NH₂</td>
<td>+</td>
</tr>
<tr>
<td>Amino acid conjugation</td>
<td>Glycine CoASH</td>
<td>ArCOOH, ArCH₂COOH</td>
<td>+</td>
</tr>
<tr>
<td>Mercapturic acid</td>
<td>Glutathione</td>
<td>-Cl, -Br, -NO₂, Epoxides, Aromatic hydrocarbons</td>
<td>+</td>
</tr>
<tr>
<td>Methylation</td>
<td>S-adenosyl-methionine</td>
<td>ArOH, -NH₂, NH, N, -SH</td>
<td>+</td>
</tr>
</tbody>
</table>

Mic. - Microsomes
Mit. - Mitochondria
Cyt. - Cytosol
the drug or its phase I metabolite forms part of the active intermediate (e.g. amino acid conjugation). Conjugation reactions are by no means restricted to foreign compounds and conjugates of endogenous compounds are also found. For example, they are important in the metabolism of bile pigments and bile salts and they are also involved in the synthesis, deactivation and transport of certain hormones. Typical conjugation reactions are summarized in Table 1.2.

Glucuronic acid conjugation:

Conjugation reactions involving carbohydrates as the donor are very common and in mammals glucuronic acid conjugation is one of the more common routes of metabolism of foreign compounds in most species. Other carbohydrates such as glucose, N-acetylglucosamine and ribose have also been shown to be conjugated, but these are formed only in either specific species of animals or occurring with specific foreign compounds (Williams 1967).

The pathway of glucuronic acid conjugation involves a condensation reaction between "activated" glucuronic acid and the foreign compound or its biotransformation product and may be summarized in the following scheme:

\[
\text{Glucose-1-phosphate} + \text{UTP} \rightarrow \text{UDP-glucose} + \text{pyrophosphate} \\
\text{pyrophosphorylase}
\]

\[
\text{UDP-glucose} + 2\text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{UDP-glucuronic acid} + 2\text{NADH} + 2\text{H}^+ \\
\text{UDPG-dehydrogenase}
\]

\[
\text{UDP-glucuronic acid} + \text{RXH} \rightarrow \text{RX-glucuronic acid} + \text{UDP} \\
\text{UDP-glucuronyl transferase.}
\]

\[X = 0, \text{ C-O, NH or S.}\]
The biosynthesis of glucuronides has been the subject of a detailed monograph (Dutton 1966). It should be noted that as the result of the condensation reaction, the C-1 atom of glucuronic acid is changed from the \( \alpha \) configuration to a \( \beta \) configuration. The transfer of glucuronic acid from UDPGA to the acceptor drugs or foreign compounds is probably catalysed by a family of glucuronyltransferases (although the data could equally well be interpreted as one enzyme existing in a number of different microenvironments), which are microsomal enzymes found not only in the liver, but also widely distributed in a number of tissues including the lungs, small intestines, kidneys, brain placenta and skin (Miettinen and Leskinen 1970, Aitio 1973 a, b). Potentially compounds with OH, COOH, NH\(_2\) or SH groups are capable of acting as glucuronic acid acceptors in glucuronide formation and give rise to O-glucuronides (ether and ester types), N-glucuronides and S-glucuronides (Smith and Williams, 1966, Williams 1967).
Sulphate Conjugation:-

Conjugation with inorganic sulphate to give sulphoconjugates constitutes a very common biochemical reaction and sulphate conjugates are found widely distributed in the animal world. By far the most widely distributed sulphoconjugates are the sulphuric acid esters in which the C-O-SO$_3^-$ linkage is present. However, other conjugates exist in which sulphate is covalently attached to other linkages, e.g. P-O-SO$_3^-$, N-SO$_3^-$, N-O-SO$_3^-$ and S-SO$_3^-$. The subject of sulphoconjugation and sulphohydrolysis has been recently reviewed by Dodgson and Rose (1970).

The pathway of sulphate conjugation requires the activation of inorganic sulphate in a series of reactions involving ATP to form 3'-phosphoadenosine-5'-phosphosulphate (PAPS) which is the "active" sulphate donor. The sequence of events leading to sulphate conjugation is summarized in the following scheme:

\[
\begin{align*}
\text{SO}_4^{2-} + \text{ATP} &\rightarrow \text{Adenosine-5'-phosphosulphate (APS)} + \text{pyrophosphate} \\
\text{ATP-sulphateadenyltransferase} \\
\text{APS} + \text{ATP} &\rightarrow 3'-\text{phosphoadenosine-5'-phosphosulphate (PAPS)} + \text{ADP} \\
\text{APS-sulphokinase} \\
\text{PAPS} + \text{RXH} &\rightarrow \text{RX-SO}_3\text{H} + 3'-\text{phosphoadenosine 5'-phosphate (PAP)} \\
\text{sulphokinase.}
\end{align*}
\]

\(X = O\) or \(NH\).
These reactions occur in the soluble fraction of cells and the transfer of inorganic sulphate from PAPS to the acceptor molecule is catalysed by a family of enzymes known as sulphokinases (sulphotransferases). Several sulphokinases have been described and they are widely distributed in various tissues including the liver, kidneys and intestine (Dodgson and Rose 1970). Foreign compounds such as phenols, certain aliphatic alcohols and some aromatic amines are metabolised by sulphate conjugation and are excreted as ethereal sulphates and sulphamates.

**Acetylation:**

Acetylation of amino, hydroxyl and sulphydryl groups can occur in the intact animal, but only a few examples of acetylation of OH group (e.g. choline to acetylcholine) and SH groups (e.g. synthesis of acetyl-CoA) are known to occur in the body and no example of acetylation of these groups in drugs or other foreign compounds appears to be known.
Acetylation of amino groups on the other hand is comparatively common and the reaction involved may be illustrated by the acetylation of sulphanilamide to N⁴-acetylsulphanilamide.

There are at least 5 types of amino groups which may be acetylated and these include: aromatic amino groups (ArNH₂); aliphatic amino groups (RNH₂); α amino acids (R.CH.NH₂.COOH); hydrazino groups (R.NH.NH₂) and sulphonamido groups (Ar.SO₂.NH₂) (Williams 1967). The enzymes involved in these reactions are collectively known as N-acetyltransferases and evidence for the multiplicity of N-acetyltransferase is being accumulated. A review on the acetylation of drugs has appeared recently and it summarizes some of the available information regarding N-acetylation (Weber 1973).

N-Acetylation reactions occur in the soluble fraction of cells and it is believed that acetylation reactions take place in the reticulo endothelial rather than the parenchymal cells of the liver (Govier 1965). Acetylation of foreign compounds has been known to occur in tissues other than the liver and tissue distribution studies showed that acetylation reactions occur in most of the tissues of the body (Weber 1973).
Amino acid conjugation:

Conjugation with amino acids represents another pathway in phase II metabolism and the well known conjugations involving amino acids include the synthesis of hippuric acids (glycine conjugation) and synthesis of mercapturic acids (glutathione conjugation).

Glycine is probably the most important amino acid involved in phase II reactions with foreign compounds. It is a reaction which occurs usually with aromatic acids and also with some aliphatic acids (Williams 1963). The pathway of glycine conjugation may be summarized as follows:

\[
\begin{align*}
\text{Ar-COOH} + \text{ATP} & \rightarrow \text{Ar-CO-AMP} + \text{pyrophosphate} \\
\text{acyl synthetase} & \\
\text{Ar-CO-AMP} + \text{CoASH} & \rightarrow \text{Ar-CO-S-CoA} + \text{AMP} \\
\text{acyl kinase.} & \\
\text{Ar-CO-S-CoA} + \text{NH}_2\text{-CH}_2\text{-COOH} & \rightarrow \text{Ar-CO-NH-CH}_2\text{-COOH} + \text{CoASH} \\
\text{transacylase} &
\end{align*}
\]

e.g.

![Diagram of glycine conjugation with benzoic acid and glycine to form hippuric acid](image)
This reaction occurs in the mitochondrial fraction of cells and glycine is not the only amino acid capable of undergoing such a reaction, for in some species glycine may be replaced or supplemented by glutamine, ornithine, arginine or taurine (Williams, 1967, Smith 1968).

Mercapturic acid synthesis involves a conjugation reaction with glutathione catalysed by glutathione-S-transferase enzymes. The formation of mercapturic acids has been studied in considerable detail in animals and the pathway of biosynthesis of mercapturic acids and the nature and distribution of the enzymes catalysing the conjugation of glutathione with foreign compounds have been reviewed recently (Boyland and Chasseaud, 1969, Wood, 1970, Chasseaud, 1973). The biosynthesis of mercapturic acids which are derivatives of N-acetylcysteine is summarized in the following pathway:

\[
\begin{align*}
RX + HSCH_2CHCONHCH_2COOH & \xrightarrow{\text{glutathione-S-transferase}} R-SCH_2CHCONHCH_2COOH \\
R-SCH_2CHCONHCH_2COOH & \xrightarrow{\gamma\text{-glutamyl transferase}} R-SCH_2CHCONHCNH_2COOH \\
R-SCH_2CHCONHCNH_2COOH & \xrightarrow{\text{cysteinylglycinase}} R-SCH_2CHCOOH \\
R-SCH_2CHCOOH & \xrightarrow{\text{acetylase}} R-SCH_2CHCOOH
\end{align*}
\]
Unlike other conjugation mechanisms, glutathione conjugation does not appear to require an "activated" intermediate (Chasseaud 1973), although in the case of epoxide conjugates, the epoxide may be considered to be an activated intermediate. The enzymes involved in mercapturic acid synthesis are found to be widely distributed in the soluble fraction of many tissues including the liver and the kidneys, and foreign compounds which are conjugated with glutathione include aromatic hydrocarbons, hydrocarbons containing 'active' halogen and nitro groups, epoxides, and many others (Chasseaud 1973). Interestingly, aromatic amines have also been shown to be precursors of mercapturic acids (Boyland et al. 1962).

Another conjugation mechanism in which an amino acid participates is methylation. This involves the transfer of a methyl group from donor methionine via S-adenosylmethionine to an acceptor molecule catalysed by methyltransferase(s). This reaction is a very common pathway for methylation of many endogenous compounds, but represents a relatively minor pathway for foreign compounds and drugs. Some foreign polyhydric phenols are metabolised by this pathway to a very limited extent.

1.1.2 Factors Affecting Biotransformation of Foreign Compounds

It has already been stated that the metabolism of a foreign compound may be carried out by several different pathways of biotransformation simultaneously resulting in many different metabolites. The rate at which these biotransformations proceed and their relative importance in determining the metabolic profile and biological activities of foreign compounds are affected by a number of factors. These factors
may be genetic, physiological and environmental in origin (Parke 1968, Vesell et al 1976, Max Lang and Vesell 1976) and in the study of foreign compound metabolism these factors must be taken into consideration, particularly in areas where comparisons between species of animals including man are concerned, as in the testing and safety evaluation of foreign compounds for use in man. In this section, a brief summary of some of the factors affecting metabolism of foreign compounds in animals together with the implications of these factors will be discussed in terms of the present investigation.

1.1.2.1 Genetic Factors

Genetic factors which affect the biotransformation of foreign compounds may be classified as those affording species differences and strain differences, i.e. genetic polymorphism and the study of intra-species differences has given rise to the relatively new science of pharmacogenetics.

In all species the metabolism of foreign compounds follows a common biphasic pattern of reaction as discussed in the preceding section. These reactions are mediated by enzymes and the nature and distribution of these enzymes which are subject to genetic controls are the main contributory factors in the inter- and intra-species differences in foreign compound metabolism (Kalow, 1965, Williams, 1967, Hathway, 1970, Hucker, 1970, La Du, 1972, Vesell, 1972, 1973, Nebert and Felton, 1976). The enzymes which are involved in the metabolism of foreign compounds may vary qualitatively and quantitatively so that species differences may arise as the result of qualitative or quantitative differences in the actual pathways of metabolism. Differences may arise due to the presence or absence of particular enzymes in different
species, the amount and localization of drug metabolising enzymes, the presence of natural inhibitors of these enzymes and the possible presence of enzymes which compete for the same foreign compounds. Furthermore, the presence of enzymes which are capable of reversing the reaction of biotransformation of a particular foreign compound and its metabolites may have a significant role in the observed differences in foreign compound metabolism between species.

Thorough investigations on the species variation in the actual enzymes involved in foreign compound metabolism have yet to be carried out, but based on our knowledge of pathways of drug metabolism and the observed metabolic profile, some insight into the factors contributing towards species variation has been obtained. Thus aromatic hydroxylation, one of the most studied reactions in phase I metabolism has been shown to occur in most species studied including insects, fish, birds, reptiles and mammals, but the extent to which this reaction occurs in the metabolism of a given compound and the orientation of the resultant hydroxylated derivatives varies considerably from species to species so that in the case of aniline, which is hydroxylated both at the o- and p- positions, the ratio of the p-hydroxylated to the o-hydroxylated urinary metabolites shows considerable species variation (Parke 1960). This has been interpreted to be due to the presence of two aniline hydroxylases in different amounts in different species so that the activity of o-aniline hydroxylase is greater in species with low p:o ratios while species with high p:o ratios may have very active p-aniline hydroxylase.

Many observations of species differences have been made with regards to phase I and phase II metabolism, but systematic
investigations on species variation have generally been restricted to phase II reactions. For example, glucuronic acid conjugation is found to be present in most animal species, but is absent in insects where it is replaced by β-glucoside synthesis (Smith, 1968) and in the cat and the Gunn rat there appear to be some deficiencies in glucuronyl-transferase activities. Deficiencies in other conjugating mechanisms are also observed in some "common" species. Thus the pig has been shown to have a deficiency in sulphate conjugation with respect to some phenols while the dog and hen are believed to be deficient in acetylation of aromatic amines and glycine conjugation of aromatic acids respectively (Williams 1967).

In the animal body there are several mutually antagonistic enzyme systems, e.g. glucuronyltransferase-β-glucuronidase (Miettinen and Leskinnen, 1970), sulphotransferase-sulphotase (Dodgson and Rose, 1970) and acetylase-deacetylase (Weber, 1973), but unfortunately the functional significance of these enzyme systems are as yet unexplained. However, their presence suggests that they may contribute to species variation in foreign compound metabolism in that the excretion of a particular conjugate may be an expression of the relative activities of conjugation and deconjugation enzymes.

1.1.2.2 Physiological Factors

Factors which may affect the biotransformation of foreign compounds from a physiological standpoint are numerous and they may alter the metabolism of foreign compounds both qualitatively and quantitatively. Age is an important factor for consideration (Wilson, 1972, Yaffe and Juchau, 1973, Neims, et al., 1976), since it affects not only the
development of enzyme systems involved in metabolism as in the neonatal
development of glucuronyltransferase (Dutton 1966), but also the hormonal
state of the animal which plays an important part in the maintenance of
the homeostatic regulation of the animal. Thus in the rat the marked
sex differences in drug metabolism observed are apparently controlled
by the production of androgens and although such differences are also
observed in some other species (e.g. the mouse), sex differences in the
metabolism of foreign compounds are generally small or absent in most

Changes in the availability of endogenous constituents
such as co-factors in foreign compound metabolism will also alter the
metabolic profile and perhaps the toxicity of the administered foreign
compound. Thus the availability of endogenous conjugating substrates
such as inorganic sulphate and amino acids, which depends on the nutritional
status of the animal may be altered by changes in the diet of the animal
(Arnstein and Neuberger, 1951, Bray, et al., 1952b, Miettinen and Leskinen,
1970, Campbell and Hayes, 1974). Indeed the dose level and the frequency
of the exposure of an animal to foreign compounds will also have important
implications on their metabolism since metabolic reactions may be
saturated by high levels of foreign compounds either because of the
limiting capacity of the metabolising enzyme or because of the readily
exhaustible supply of co-factors. Where metabolism is mediated by more
than one pathway, such factors will have important significance in
the competition between pathways of metabolism, resulting in changes
in metabolic profile with changes in the dose level of the foreign
chemical.

When a foreign compound is administered to an animal,
numerous factors are involved in the transport of the compound and its
metabolites to and from its site of metabolism. These factors will have important roles in the rate of metabolism of the compound, the duration of the compound in the animal and hence the potential hazards of the compound in the animal. Such factors may include absorption, plasma protein binding, tissue distribution, biliary and urinary excretion and enterohepatic circulation. Furthermore, these factors are influenced by the nature of the foreign compound, blood flow to and from the site of metabolism and the activities of the drug metabolising enzymes (Gillette, 1971a, Vesell, et al, 1976). The route of administration also serves as an important factor for consideration (Dollery, et al, 1971, Conolly et al, 1972, George, et al, 1974), since compounds administered orally may be subjected to the actions of the gastrointestinal microorganisms, intestinal secretions, enzymes of the intestinal wall, all of which are by passed by an intraperitoneal injection and the absorption of the compounds may be influenced by factors such as gastric emptying and intestinal mobility (Dent, 1975). Other physiological factors which may influence metabolism of foreign compounds include seasonal variation and circadian rhythm (Moore Ede, 1973, Romero, 1976).

The disease state of the animal will also influence the metabolism of foreign compounds. It is well known that human patients with severe liver damage show an increased sensitivity to a wide variety of drugs. This phenomenon has been attributed to the impairment of the detoxicating function of the liver (Parke, 1968). However, other organs are also involved in the metabolism of foreign compounds so that physiological abnormalities due to diseases, which result in tissue redistribution of administered foreign compounds and functional alteration of organs with specific roles in the metabolism of foreign compounds will also affect the metabolic profiles of the foreign compounds.
1.1.2.3 Environmental Factors

Environmental factors which may affect the metabolism of administered foreign compounds include stress due to adverse conditions, changes in the atmospheric conditions and exposure to ionising radiation, but perhaps the most studied environmental factor is the interaction with other foreign compounds either administered deliberately or accidentally. These interactions may result in alteration in the metabolism of not only foreign compounds, but also normal body constituents (Parke, 1968, Conney, 1971, Brown, 1970; 1972; 1975, Fouts, 1976).

The administration to animals of numerous drugs and environmental chemicals can inhibit or potentiate the pharmacological and toxicological action of the drugs and this may be achieved by a variety of mechanisms apart from direct effects on drug metabolising enzymes, including the alteration in drug distribution as in the competition for receptor sites and the displacement of protein bound drugs by other administered drugs (Dayton and Perel, 1971). However, alteration in the activities of the drug metabolising enzyme system is probably the most important medium by which affects of environmental factors are manifested (Gillette, 1976) and furthermore, steroid hormones are substrates of the same liver microsomal enzyme system that metabolises foreign compounds so that environmental factors which affect drug metabolism may also affect the hormonal status of the animals concerned.

The activation of the metabolism of foreign compounds by the chronic administration of other foreign compounds such as drugs, pesticides and polycyclic hydrocarbons is well known and has been studied extensively because of their possible association with carcinogenesis and drug tolerance (Conney, 1967, 1971). This activation of the enzymes
involved in metabolism of foreign compounds is due to an increase in the amount of drug metabolising enzymes resulting from enzyme induction which probably requires the de novo synthesis of enzyme protein (Gelboin 1971).

The inhibition of metabolism of foreign compounds by other foreign compounds has also been observed and unlike induction, inhibition does not require repeated administration of the effecting agent. The mechanism of inhibition is not fully understood and several inhibitory mechanisms may exist, including competitive inhibition for the enzyme systems by acting as alternative substrates, non-competitive inhibition by "allosteric" modification, uncoupling of the oxidative mechanisms involved in hepatic microsomal drug metabolism and alteration of the properties of the hepatic microsomal enzymes (Anders 1971, Leibman and Ortiz 1973).

Many foreign compounds and environmental contaminants are inducers and inhibitors of drug metabolism and interestingly administration of sub-optimal levels of different inducers concurrently gives rise to additive effects of induction. The classical inducers of drug metabolising enzymes are phenobarbital and 3-methylcholanthrene while SKF525A (α-diethylaminoethylphenylpropylacetate) and metyrapone are classic inhibitors of drug metabolism in animals.

Inducers and inhibitors may have considerable effects on the metabolism of other foreign compounds leading to changes in the toxicity and the intensities of pharmacological activities of these compounds. Thus in the presence of an inhibitor the therapeutic action of a drug may be potentiated because the inhibitor functions as a synergist, but this will also prolong any possible toxic effect of the
parent drug, if detoxication of the drug is mediated by metabolism. On the other hand the presence of inducers may accelerate the metabolism of the drug and hence reduce its therapeutic value. Moreover, many drugs such as phenobarbital will also induce their own metabolism so that repeated dosage of the drug will lead to the development of tolerance as a result of increase in detoxication of the drug due to increase in metabolism.

1.1.3 Consequences of Biotransformation of Foreign Compounds

The biological activities of foreign compounds in an animal are usually governed by the rate at which these compounds are eliminated from the body, and although there are numerous routes by which foreign compounds may be removed from the body, by far the most important are the renal and biliary excretory routes. Other routes of excretion include pulmonary excretion, secretions in the saliva, milk, sweat and gastrointestinal tract (Stowe and Plaa, 1968, Werner 1976, Chasseaud, 1971, Plaa, 1971).

In general, the renal elimination of foreign compounds is governed by a number of factors including those which affect the rate of glomerular filtration and tubular transport of foreign compounds (Cafruny, 1971, Rennick, 1972), but by far the most important factor is the polarity of the foreign compound. Thus the elimination of lipid soluble substances by the kidneys is slow because such substances are likely to diffuse from the glomerular filtrate back into the plasma until the concentrations in the plasma and the urine are virtually identical, whereas in general, polar and ionized compounds are not reabsorbed in the renal tubules. The renal excretion of some polar
metabolites may be enhanced by active transport mechanisms, thus many acidic and basic foreign compounds are actively secreted in the proximal tubules of mammalian kidneys. It can be seen then, that detoxication occurs as a consequence of the change in polarity of the foreign compound following biotransformation thus permitting a subsequent improvement in its renal excretion rate.

The increased polarity and hence water solubility of an administered foreign compound, although involving both phase I and phase II metabolism is normally primarily due to the conjugation reactions (Parke, 1968). The two most important properties of a bioactive molecule are its biochemical activity and its lipid solubility. The former often shows a high degree of molecular structural dependency so that subsequent to conjugation reactions considerable structural alteration results and this may be expected to result in the loss of bioactivity. The lipid solubility on the other hand gives a measure of the ability to penetrate biomembranes to potential sites of action and as a consequence of conjugation reactions, the lipophilicity of the compound may be altered so as to result in limited bioavailability and hence detoxication. Conjugations, however, do not necessarily increase the water solubility of foreign compounds and their metabolites, thus acetyl metabolites of many sulphonamides are very insoluble in water and cause kidney damage as a result of their intratubular crystallization (Mandel, 1971).

Although most foreign compound metabolites are less biologically active than their parents, in quite a few instances, metabolism is capable of increasing the pharmacological or toxicological activities of the parent compounds. Classic examples of metabolic activation of pharmacologically inactive compounds include the reaction...
of prontosil to sulphanilamide (antibacterial agent) and the deethylation of phenacetin to N-acetyl-p-aminophenol (analgesic). Numerous examples of metabolic activation of foreign compounds to toxic metabolites are known. Thus bioactivation may be involved in the carcinogenesis of some polycyclic hydrocarbons, hepatotoxicity of some commonly used drugs and methaemoglobinemia produced by some aromatic amines. Such activations generally result in the formation of "active" intermediates which may undergo nonenzymic irreversible reactions with tissue macromolecular constituents leading to toxic responses. Other foreign compounds are sufficiently similar to normal endogenous compounds that they participate in the pathways of intermediary metabolism causing interference with their functioning in what has been termed "lethal synthesis". Indeed such selective mechanisms of lethal synthesis are often utilised in the action of insecticides and pesticides.

From this very brief discussion it can be seen that metabolism generally affords increased polarity and water solubility of foreign compounds so as to facilitate their elimination from the body and hence detoxication. However, metabolism may also give rise to active intermediates which may have toxicological and pharmacological activities. The understanding of these reactions will give some insight into the biochemical basis of toxicology and pharmacology.

1.2 TECHNIQUES USED FOR STUDYING BIOTRANSFORMATION OF FOREIGN COMPOUNDS

The fate of foreign compounds has been studied by both in vivo and in vitro techniques. The choice of techniques depends largely on the scope and objective of the studies and the level of cellular
organization at which the studies are designed. Thus foreign compound metabolism may be investigated at a molecular level to study enzyme mechanisms, at cellular and tissue levels and at the level of the intact animal for studying metabolism and disposition of foreign compounds. In this section a brief comment on the various techniques used in drug metabolism from the standpoint of "level of organization" will be considered in terms of the advantages and limitations of each technique in the overall understanding of metabolism of foreign compounds.

1.2.1 In Vivo Investigations

Since the products of metabolism of foreign compounds are finally excreted by the animal, the classic method for investigating metabolism of foreign compounds involves the most unequivocal, but exacting techniques of feeding experiments. Living animals are dosed with foreign compounds and the metabolites and their conjugates are isolated from the animal excreta which is followed by chemical characterization of the excreted metabolites by various standard analytical techniques.

In vivo experiments also allow for investigations into disposition of foreign compounds within living animals and summarize a complex sequence of events in which an administered foreign compound is handled by the body. Disposition includes not only metabolism, but also absorption, distribution and excretion of foreign compounds. Thus studies on the disposition of foreign compounds in the living animal provides information on the kinetics of absorption and excretion, the length of time a foreign compound remains in the body, route of elimination from the body, the pharmacological and toxicological activities
of the foreign compound in relation to its site of localization and the concentration of the foreign compound and its metabolites in various body fluids such as blood, bile and urine and tissues such as liver, kidney, spleen, lung, heart, muscle, brain and adipose tissues, which reflect the bioactivity of the foreign compound. Furthermore from the point of view of interspecies differences in foreign compound metabolism, in vivo studies give an overall picture of the factors affording species variation such as the disposition of administered foreign compounds which may be related to differences in the role of gastrointestinal microorganisms, differences in diet between carnivorous and herbivorous animals or genetic deficiencies in pathways of metabolism and many other genetic and physiological factors as described in Section 1.1.2. In vivo experiments will also give important information regarding changes in metabolic profile due to route of administration of the compound or changes in dose levels. Enteral and parenteral routes of administration may give rise to differences in metabolic profile particularly if metabolism of the foreign compounds is carried out extensively by other organs as well as the liver (Dollery, et al., 1971). Differences in the route of administration may illicit differences in the pharmacological responses due to metabolism and possibly also the concentration of the active compound at the site of action. In vivo dose response studies may also indicate changes in metabolic profile due to saturation of mechanisms involved in metabolism. They may give information regarding the therapeutic and toxic levels of the foreign compound and the effect of chronic dosage on metabolism and metabolic profiles of the foreign compounds.

Many other factors affecting the metabolism of foreign compounds are studied by in vivo techniques and from this short discussion it can be seen that following the identification and characterization of metabolites of a particular foreign compound, it is possible to elucidate
the pathways of metabolism of that compound and the factors which control its metabolism in the living animal by means of in vivo investigations.

1.2.2 **In Vitro Investigations**

In the study of foreign compound metabolism, in vitro investigations are frequently undertaken to supplement in vivo investigations. The advantages offered by in vitro investigations are numerous. They provide a more controlled means whereby the mechanism of biotransformation may be investigated so that information regarding the site of metabolism, pathways in the formation of metabolites, the enzyme systems that catalyse the various steps in the production of metabolites, the components of the enzyme systems involved and the biochemical properties of these components together with mechanisms which control the enzymic reactions involved in biotransformation of foreign compounds may be obtained and correlated with those observed in the intact animal.

Correlation of in vitro results with in vivo observations can only be made provided the limitations of the in vitro techniques are realised and taken into account. There are a variety of in vitro techniques and tissue preparations which may be used for the investigation of foreign compound metabolism and each will have inherent advantages and disadvantages. However, they are able to provide some information regarding factors involved in metabolism of foreign compounds at different cellular levels of organization. Understanding of these various factors will provide an insight into the overall metabolism of foreign compounds in the intact animal.

In the intact organ, metabolism of foreign compounds may be studied by perfusion of drugs and other foreign chemicals through the
organ in question under conditions closely resembling those which occur in the living animal (Evans et al. 1963). Indeed such organ perfusion experiments may be carried out in situ in the intact animal. Organ perfusion experiments provide information concerning the function of that organ in the metabolism of foreign chemicals and include the rates and pathways of metabolism which occur in the organ being perfused, the possibility of the formation of stable intermediate metabolites which are subsequently metabolised by other organs and the limiting factors in metabolism which may be the activities of the enzyme systems or the rate at which foreign compounds are conveyed from the blood to the site of metabolism. Unfortunately organ perfusion provides little or no information regarding the mechanism of formation of metabolites or properties of the enzyme systems involved. Indeed for the identification of principle metabolites it frequently offers no advantages over studies of urinary and/or biliary metabolites, but for evaluating specific organ function, organ perfusion offers considerable advantages and provides a means for studying the contribution of various organs in the overall metabolism of a foreign compound (Gillette, 1971b).

Because of the relatively complex methodology involved in organ perfusion experiments, tissue slices have been used as an alternative for studying foreign compound metabolism in different tissues. The methodology of tissue slice experiments is relatively simple, but because the transfer of foreign compounds and nutritional material from the incubation medium depends solely on passive diffusion, the concentration of substances between the inner and outer areas of the slices may not be identical so that very thin slices must be used and for this reason tissue slices may not provide the best technique for obtaining information representative of drug metabolism within the cells of the tissue in
question, in particular the transport of foreign compounds to and its metabolites from, the cells. Nevertheless some knowledge regarding the transport of foreign compounds into tissues and pathways of metabolism may be obtained from tissue slice experiments.

With the currently improving methodology in the isolation and culturing of large numbers of functional and viable cells from various tissues, such in vitro preparations are gaining ascendancy as a tool for the study of foreign compound metabolism in its various aspects (Schindler, 1969, Fry and Bridges, 1977). Among the obvious advantages of this type of preparation are that cells are constantly bathed in the medium containing the nutrients so that the diffusion barrier to oxygen, nutrients and foreign compound substrates is minimal. Moreover isolated cells provide a preparation in which various mechanisms involved in the intracellular homeostasis are intact, so that study of the metabolism of foreign compounds which are metabolised by more than one pathway may be investigated concurrently and understanding of the functional significance and the factors affecting the various metabolic pathways may be achieved. Indeed such isolated cell preparations may have widespread application as a mid point in the various in vivo and in vitro comparative studies.

Other in vitro techniques which are currently employed in the study of drug metabolism include the use of tissue homogenates, subcellular fractions of tissues and solubilised and purified preparations of enzymes involved in foreign compound metabolism. Such techniques that are used are directed towards the obtaining of information regarding the enzymes involved in biotransformation of foreign compounds and its relationship to the metabolism of various endogenous materials, the mechanism of the enzymic reaction, the components of the enzyme systems
and factors affecting the properties of these components.

In the use of tissue homogenates and subcellular fractions it must be realised that once cells are broken, intracellular control mechanisms, compartmentalization and interrelationship between various intracellular organelles are lost and intracellular components may be damaged so that steady state levels of intracellular substrates and co-factors will not correlate with those observed in vivo. Furthermore, the process of homogenization of tissues often causes apparent inactivation of certain enzymic activities, frequently as a result of inactivation of co-factors involved in the enzyme system. Thus a great deal of care must be taken before correlation between such in vitro observations and in vivo observations can be made (Gillette, 1971b). However, the use of tissue subcellular fractions has demonstrated that many subcellular particles contain enzyme systems which are important in the metabolism of foreign compounds. Thus hepatic microsomal fractions have been demonstrated to carry out many oxidative metabolic reactions involving a wide variety of lipid soluble foreign compounds and steroids which require molecular oxygen and NADPH as co-factor, glucuronic acid conjugation reactions and the reduction of many azo- and nitro- compounds. The mitochondrial fraction, on the other hand has been shown to catalyse the oxidation of amines, formation of amino acid derivatives and the oxidation of fatty acids. The soluble subcellular fraction also contains many enzymes capable of metabolising foreign compounds and indeed it can be demonstrated that microsomal enzymes convert many foreign compounds to intermediates which in turn are converted to other metabolites by enzymes in the soluble fraction as in the synthesis of sulphates, mercapturates and acetyl derivatives.
Although much has been learnt regarding the enzyme systems involved in the metabolism of foreign compounds, the actual mechanisms of enzymic reaction remain elusive and in order to elucidate the mechanisms of enzymic reaction, attempts are being made to solubilise and purify these enzymes. Some investigations using partially purified enzymes have been carried out and although information ranging from the sequences of the catalysed reactions, nature of the enzyme and number of enzymes catalysing similar reactions has been obtained, the significance of these findings is limited in their correlation with the mechanism of reaction of these enzymes in the intact animal. Solubilization and purification of membrane bound multienzymes invariably alters the interrelationship of the components of the enzymes and the environment in which the enzyme system normally functions so that reactions which occur with enzymes purified from subcellular particles may not have a direct bearing on the mechanism of the reaction in the living animal. Nevertheless, studies on partially purified enzyme preparations have provided valuable information on possible mechanisms of foreign compound metabolism, the kinetics of the reaction and factors affecting the metabolism of these compounds at the molecular level.

Thus it can be seen that study of foreign compound metabolism requires the complementary use of both in vivo and in vitro techniques and the information obtained can then be correlated so as to lead to a better overall understanding of the biotransformation of foreign compounds and their consequences in the living animal.

1.3 OBJECTIVE OF THIS THESIS

A brief survey of the current literature on the comparative metabolism of foreign compounds in various animal species showed that
relatively few systematic studies have been carried out in this area. Some investigations on species variation in the metabolism of organic compounds which include benzoic acid (Baldwin, et al., 1960, Bridges, et al. 1970), phenylacetic acid (James, et al., 1972), indoleacetic acid (Bridges, et al., 1974), aniline (Parke, 1960), quinic acid (Adamson, et al., 1970a), amphetamine (Dring, et al., 1970), sulphadimethoxine (Adamson, et al., 1970b), phenol (Capel, et al., 1972), disodium cromoglycate (Ashton, et al., 1973) and phenacetin (Smith and Timbrell, 1974) have been reported. However, although these compounds were examined in a broad range of vertebrate species including fish, amphibians, mammals and birds, small animals have generally been employed and detailed metabolic studies on large animals have been very limited. Apart from work in the monkey, some very preliminary foreign compound metabolism studies have been conducted in the pig (Bridges, et al., 1970, Capel, et al., 1972) and the use of the sheep as an animal model for the study of drug detoxication in vivo has been the subject of a PhD thesis (Maylin, 1971).

There are a number of reasons for studying metabolism of foreign compounds in farm animal species. They are important food producing species and in view of the economic importance of the development of drugs and therapeutic agents for the maintenance and growth promotion of these animals which are used for the production of food and other products of economic value, comparative study on the metabolic capabilities of these species becomes an important aspect of the development of veterinary products. In this context the problem of drug residues in the edible tissue of these species is a key factor for consideration and since the identification of drug residues requires a knowledge of the metabolic fate of the drug in the animal species in question, a study of the major pathways of drug detoxication and elimination in these animals
is therefore desirable. Furthermore, the role of gut microorganisms in the metabolism of orally administered drugs in ruminants such as the sheep may give rise to new and unidentified pathways of metabolism and metabolites.

Although various aspects of species variation in foreign compound metabolism have been considered by many authors, and considerable amounts of data exist in the literature on the major pathways of drug detoxication and elimination in laboratory animals, very little is known about the metabolism of drugs in farm animals. In fact, very few systematic studies on drug metabolism have been carried out in these species. However, the pharmacology and pharmacokinetics of a number of drugs have been studied in animals of agricultural interest and some of these are summarized in Table 1.3.

It is a general belief that large animals tend to metabolise drugs slower than small laboratory animals, this view however lacks good experimental verification and a study on this aspect of drug metabolism may prove to be significant. From the point of view of the pig, a knowledge of its metabolic capabilities is valuable in view of the potential use of this species in toxicological studies.

In order to elucidate the principle pathways of metabolism in these animals, it is necessary to obtain background information on the metabolism of some "model" organic molecules by these animals and compare it with that observed in laboratory animals. In the present investigation, the metabolism of benzoic acid, phenol and aniline was investigated in the sheep and the pig and the results compared with those obtained in the laboratory rat. Benzoic acid was chosen for the study of amino acid conjugation, in particular glycine conjugation,
### Table 1.3

**Some Investigations Involving Animal Species of Agricultural Interest**

<table>
<thead>
<tr>
<th>Foreign Compounds</th>
<th>Species</th>
<th>Nature of Studies</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol</td>
<td>Horse</td>
<td>Metabolism</td>
<td>Plapp (1973)</td>
</tr>
<tr>
<td>Aflatoxins</td>
<td>Pig Sheep Goat</td>
<td>Metabolism</td>
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</tr>
<tr>
<td>Amphetamine</td>
<td>Goat Horse</td>
<td>Pharmacokinetics</td>
<td>Baggot et al (1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metabolism</td>
<td>Chapman &amp; Marcroft (1973)</td>
</tr>
<tr>
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<td>Domestic animals</td>
<td>Pharmacology</td>
<td>Dunlop (1967)</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>Pig Sheep</td>
<td>Metabolism</td>
<td>Bridges et al (1970)</td>
</tr>
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<td>Metabolism</td>
<td>Matin (1966, 1975)</td>
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<td>Douch (1973)</td>
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<td>Sisodia et al (1973a, b)</td>
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<td></td>
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<td>Davies et al (1972)</td>
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<td>Goat</td>
<td>Mammary excretion</td>
<td>Rasmussen et al (1975)</td>
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<td>Histamine</td>
<td>Pig Goat Cow Sheep</td>
<td>Metabolism</td>
<td>Eliaseen (1973a, b, c)</td>
</tr>
<tr>
<td>2-Methyl-4,6-dinitrophenol</td>
<td>Sheep</td>
<td>Pharmacology</td>
<td>Froslie (1973, 1974)</td>
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<tr>
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<td>Pig</td>
<td>Metabolism</td>
<td>Capel et al 1974a)</td>
</tr>
<tr>
<td>Foreign Compounds</td>
<td>Species</td>
<td>Nature of Studies</td>
<td>References</td>
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<td>Phenol</td>
<td>Sheep</td>
<td>Metabolism &amp; Pharmacokinetics</td>
<td>Maylin (1971)</td>
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<td>Pig</td>
<td>Metabolism</td>
<td>Capel et al (1972, 1974b)</td>
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<td>Pharmacokinetics</td>
<td>Hvidberg &amp; Rasmussen (1975)</td>
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<td>Various species</td>
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<td>Neff et al (1972)</td>
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<td>Goat</td>
<td>Metabolism</td>
<td>Atef &amp; Nielsen (1975)</td>
</tr>
<tr>
<td>Sulphadoxin</td>
<td>Cow</td>
<td>Pharmacokinetics</td>
<td>Davitiyananda &amp; Rasmussen (1974)</td>
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<td>Sulphonamides</td>
<td>Ruminants</td>
<td>Disposition</td>
<td>Austin (1976)</td>
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<td></td>
<td></td>
<td>Metabolism</td>
<td>Nielsen (1973a, b)</td>
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<td>Tetracycline analogues</td>
<td>Cow, Sheep</td>
<td>Pharmacokinetics</td>
<td>Ziv &amp; Sulman (1974)</td>
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<td>Trimethoprim</td>
<td>Pig, Goat, Cow</td>
<td>Urinary elimination</td>
<td>Nielsen &amp; Rasmussen (1972, 1975a)</td>
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<td>Pharmacokinetics</td>
<td>Davitiyananda &amp; Rasmussen (1974)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Distribution</td>
<td>Nielsen &amp; Rasmussen (1975b)</td>
</tr>
</tbody>
</table>
phenol for sulphate and glucuronide conjugation and aniline for aromatic hydroxylation and conjugation reactions.

An examination of the literature revealed that although some aspects of metabolism of these "model" compounds have been investigated in the rat, nevertheless studies regarding the site of metabolism of these "model" compounds and the kinetics of in vivo metabolism which may reveal information regarding possible influences of some factors in the metabolism and disposition of these compounds such as species variation have not been investigated satisfactorily. In an attempt to investigate such problems, ureter cannulated rats were used in some metabolism studies and the metabolism of these "model" compounds was also investigated in isolated cells prepared from rat liver, kidney and small intestine in an attempt to establish, not only the site of metabolism of these compounds, but also the ability of these cell preparations to metabolise foreign compounds and hence the ability of these various organs to metabolise foreign compounds and their consequences in the intact animal.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 Radiochemicals

[U-\textsuperscript{14}C]-Aniline hydrogen sulphate, 60 mCi mmol\textsuperscript{-1}; \(\text{[\text{Ring-U-14}C]}\)-Benzoic acid, 36.3 mCi mmol\textsuperscript{-1} and \(\text{[U-14}C]\)-Phenol, 34 mCi mmol\textsuperscript{-1} were obtained from The Radiochemical Centre (Amersham, Bucks.).

The radiochemical purity of these compounds was checked by standard thin-layer chromatographic methods and found to be at least 98% in all cases.

2.1.2 Chemicals

Chemicals used in liquid scintillation counting were obtained from Packard Instruments Ltd. (Caversham, Berks.). \(p\)-Bis (\(p\)-methylstyryl)-benzene (bis-MSB) was from Koch-Light Laboratories (Colnbrook, Bucks.). 1:4 Dioxan was purchased in bulk from Maybridge Chemicals (Tintagel, Cornwall). The toluene used for scintillation counting was low in sulphur and was supplied by Fisons (Loughborough, Leics.).

Chemicals used in metabolic studies were of analar grade from British Drug Houses (Poole, Dorset). Saccharic acid 1:4 lactone and \(N\)-acetyl-\(p\)-aminophenol were from Sigma (London). Phenyl-\(B\)-D-glucuronide monohydrate was from Koch Light Laboratories and \(N\)-phenylsulphamic acid was from ICN Pharmaceuticals (Horsham, Surrey).

All other solvents and chemicals were from BDH and were of analar or laboratory reagent grades.
Benzoylglucuronide was obtained from the excretion products of a turkey dosed with benzoic acid. The isolation of benzoylglucuronide was essentially as described by Baldwin, et al., (1960).

A turkey (6 Kg) was starved for 12 hours before the oral administration of benzoic acid (5 g). The excreta were collected in galvanized metal trays placed below the cage. Benzoic acid was administered as a solution of its sodium salt by means of a stomach tube. The bird was allowed free access to drinking water which contained glucose (1% w/v).

After 24 hours the excreta was collected and acidified with a few drops of conc. HCl. It was homogenised, centrifuged to remove solid debris and continuously extracted with ether for 2-3 days. A small amount of isoamyl alcohol was added to break up emulsions.

The ether extract was evaporated in vacuo and the residue was dried overnight in a vacuum desiccator containing P₂O₅ and anhydrous Na₂CO₃. The dried residue was taken up in a minimum amount of ethanol and 10 vols of chloroform added. On keeping at approximately -4°C a white material (benzoylglucuronide) precipitated out.

The precipitate gave a positive naphthoresorcinol test. It ran as a single spot on thin layer chromatography. The spot was located under UV light and identified using naphthoresorcinal spray. The spot did not correspond to benzoic acid, hippuric acid or glucuronic acid, which were co-chromatographed. The dried precipitate had a melting point of 179°C-181°C and it was hydrolysed by β-glucuronidase (ketodase) to give benzoic acid. The benzoylglucuronide so prepared was used as a chromatographic standard without further purification.
Phenylsulphate was prepared according to the method of Hawkins and Young (1954).

Carbon disulphide (70 ml) and dimethylaniline (14.5 ml) were stirred and cooled in ice water. Chlorosulphonic acid (2.0 ml) was added followed by phenol (2.8 g) with stirring. The mixture was stirred for 1 hour and allowed to stand overnight. Aqueous KOH (50% w/v, 12 ml) was added slowly, the separated dimethylaniline was poured off and removed by washing with ether (3 x 100 ml).

The resultant potassium salt was dissolved in a minimum quantity of water at 70-80°C and the hot solution filtered. The filtrate was cooled and the white crystalline material formed was dried and washed with ether.

Thin layer chromatography of an aqueous solution of the crystalline material ran as a single spot and it did not correspond to phenol or phenylglucuronide which were co-chromatographed. It was hydrolysed by arylsulphatase to give phenol. This potassium salt of phenylsulphate was used as a chromatographic standard without further purification.

2.2 METHODS

2.2.1 Measurement of Radioactivity

Nuclear Chicargo MK.II, LKB Wallac 1210 and Packard Tricarb 3320 scintillation counters were used throughout this work.

Counting efficiencies were determined using external standard channel ratio techniques from quench curves prepared using CCl₄.
as the quenching agent and \( n-(1^{14}\text{C}) \) - hexadecane (The Radiochemical Centre, Amersham) as the standard. Different curves were prepared for the different scintillant and counting conditions used and in all cases the validity of the external standard channel ratio results were checked by internal standardization of a randomly selected range of samples: Agreement between the two methods of counting was in the order of ±2%. Counting efficiencies were in the order of 70%-90%.

Several scintillant mixtures were used, they included:

1. Instagel (Packard Instruments Ltd.)

2. Dioxan based scintillant

   - Naphthalene 60 g
   - 2.5 Diphenyloxazole (PPO) 4 g
   - p-Bis- 2-(4-methyl-5-phenyloxazdyl) benzene (dimethyl POPPO) 0.2 g
   - Methanol (analar) 100 ml
   - 1:2 ethandiol 40 ml
   - and 1:4 dioxan to give 1000 ml

3. Toluene based scintillant

   - PPO 5 g
   - Bis MSB 0.25 g
   - Toluene 1000 ml

4. Dioxan/Toluene based scintillant

   - Naphthalene 100 g
   - PPO 5 g
   - Dimethyl POPOP 0.3 g
   - Dioxan 800 ml
   - Toluene 200 ml

For the counting of urine samples (0.1-0.5 ml), Instagel and dioxan based scintillants were used while for the counting of homogenised faecal samples (1% w/v, 0.5 ml) a dioxan based scintillant containing a thixotropic gel powder (5% w/v), Cab-o-sil (Packard Instruments Ltd.) was used.
Freeze dried faecal samples (50 mg) were counted in the Dioxan/Toluene based scintillant after combustion in a Packard Tricarb oxidizer to determine the radioactivity of the resultant $^{14}$CO$_2$ which was trapped in a solution of ethanolamine: ethyleneglycol monomethylether: toluene (1:8:10 v/v) (Jaffay and Alvarez, 1961).

Sections of paper chromatograms were counted in the toluene based scintillant while sections of silica gel thin layer chromatograms were counted in the toluene based scintillant containing Cab-o-sil.

Aliquots (0.5 ml) of hydrolysates, ether extracts, and aqueous residues from enzymic hydrolysis experiments were counted in the dioxan based scintillant while crystalline material from reverse isotope dilution analysis experiments were first dissolved in dimethylformamide (0.5 ml) before being counted in the dioxan based scintillant.

In all cases 10 ml scintillant was used and counting was carried out at 4°C after storage at 4°C in the dark to avoid possible errors due to chemiluminescence.

2.2.2 **In vivo Investigations**

2.2.2.1 **Animal and Animal Dosing Procedures**

Details of animals used throughout this research project are summarized below:-

<table>
<thead>
<tr>
<th>Species</th>
<th>Sheep (mixed breed)</th>
<th>Pig (large white)</th>
<th>Rat (Wistar albino)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>male</td>
<td>male</td>
<td>male</td>
</tr>
<tr>
<td>Age</td>
<td>2-3 months</td>
<td>2-3 months</td>
<td>5-6 weeks</td>
</tr>
<tr>
<td>Weight</td>
<td>30-40 kg</td>
<td>15-20 kg</td>
<td>0.2-0.3 kg</td>
</tr>
<tr>
<td>Diet</td>
<td>Ewe and Lamb Diet</td>
<td>Commercial Pig Weaner Rations</td>
<td>Spillers No.1</td>
</tr>
<tr>
<td></td>
<td>(Pauls Feeds Ltd.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>and hay</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TEXT CUT OFF IN ORIGINAL
The animals were kept in metabolism cages appropriate to their size. The sheep and the pig were kept in metal metabolism cages at Pfizer (Central Research) Ltd., Sandwich, Kent, while the rats were kept in all glass small animal metabolism cages - "Metabowl" (Jencons, Scientific Supplies, London). The animals were allowed free access to food and water at all times throughout the experiments.

In all cases radiolabelled compounds under investigation were administered orally. The pig was dosed by means of a stomach tube while the rat and the sheep were dosed by a syringe.

The radiolabelled compounds were administered as an aqueous solution of their salts. (For the sheep and the pig 15-20 ml, for the rat 1 ml). In the case of the sheep and the pig, small aliquots of the dosing solutions were taken for the determination of its $^{14}\text{C}$ content. The actual amount of radioactivity received by the animals was calculated as the difference between the $^{14}\text{C}$ level in the dosing solution and the residual washings after administration. In the case of the rat, a stock dosing solution of known specific radioactivity was prepared and 1 ml aliquots were taken for oral administration.

2.2.2.2 Collection and Treatment of Excreta

Urine from the animals was collected in suitable receptacles, at regular intervals during the first 24 hours after administration of the compound. In the case of the sheep and the pig, the urine was collected almost as soon as it was voided.

All urine samples were kept frozen after collection until needed, but were assayed for $^{14}\text{C}$ immediately after collection.
Faeces from the large animals were collected in bags worn as a harness by the animals. These samples were homogenised, freeze dried and analysed for \( ^{14}\)C after combustion. Faeces from the rat were homogenised, diluted and small aliquots were taken for \( ^{14}\)C determination.

The radioactivity of all urine samples (0.5 ml for the sheep and pig, 0.1 ml for the rat) was counted in duplicate at 4°C after storage at 4°C in the dark to avoid possible errors due to chemiluminescence as described previously. Triplicate samples (\(\approx 50\) mg) of freeze dried faeces were counted as described after combustion to determine the radioactivity of the resultant \( ^{14}\)CO\(_2\). Quadruple samples of homogenised rat faeces (0.5 ml) were counted as described.

2.2.2.3 Identification of Urinary Metabolites

General scheme used for metabolite identification:

\[
\text{Urine} \quad \text{TLC and PC}
\]

enzyme hydrolysis (overnight)

\[
^{14}\text{C determination} \quad \text{Hydrolysate} \quad \text{TLC}
\]

ether extraction

\[
^{14}\text{C determination} \quad \text{Aqueous residue} \quad \text{ether extract} \quad \text{TLC and reverse isotope dilution analysis.}
\]
Studies on Urine Samples

a) Chromatography of Urine Samples

Both descending paper chromatography and thin layer chromatography were employed. Whatman No.1 paper was used for the descending method while silica gel 60 F<sub>254</sub> precoated thin layer plates, 0.25 mm (Merck, Darmstadt, West Germany) were used for thin layer chromatography.

The following solvent systems were used in the chromatographic investigation of urine: -

For descending paper chromatography: -

A  Butan-1-ol : Acetic acid (glacial) : Water (4:1:2 v/v)
B  Butan-1-ol : Ethanol : Acetic acid (glacial) : Water (3:1:0.1:1 v/v)
C  Butan-1-ol saturated with equal volumes of 1.5 M ammonia and 1.5 M ammonium carbonate.
D  Propan-1-ol : Ammonia (880 s.g.) (7:3 v/v)
E  Butan-1-ol : Ammonia (880 s.g.) : Water (10:1:1 v/v)
F  Butan-1-ol : Benzene : Pyridine : Water (5:1:3:2 v/v)

For thin layer chromatography: -

G  Butan-1-ol : Acetic acid (glacial) : Water (4:1:1 v/v)
B  Butan-1-ol : Ethanol : Acetic acid (glacial) : Water (3:1:0.1:1 v/v)
H  Propan-1-ol : Butan-1-ol : Ammonia (0.1N) (2:1:1 v/v)

Urine from the experimental animals was applied as a band (2 cm for TLC and 4 cm for PC) and the chromatograms were developed in the above solvent systems. Where possible authentic metabolites were co-chromatographed with the urine samples.

Several methods were employed in the location and detection of the radiolabelled metabolites on the radiochromatograms and they are summarized below: -
1. Quenching of background fluorescence under UV light.

2. Preparation of radiochromatogram scans on a Berthold Camlab Radiochromatogram Scanner.

3. Thin layer radiochromatogram autoradiography using Kodak blue brand medical X-ray films. Autoradiography was carried out for 5-10 days and the films were developed using Kodak universal developer and fixed using "Kodafix" in the manner recommended by the manufacturer. Areas corresponding to the dark spots on the autoradiographs were marked on the chromatograms and these areas were cut out, placed into scintillation vials and counted as described.

4. "Strip counting".
Radiochromatograms were cut into sections (0.5 cm) and the associated $^{14}$C was determined by liquid scintillation counting.

5. Colour reaction with the following spray reagents

a) Naphthoresorcinol in acetone (1% w/v) to which phosphoric acid (10% v/v) was added just before use (4:1 v/v) followed by heating the chromatogram to 140°C (Bridges et al 1965).

b) p-Dimethylaminobenzaldehyde in acetic anhydride (4% w/v) containing a little sodium acetate followed by gentle heating of the chromatogram. (Bridges et al 1970).

c) p-Dimethylaminocinnamaldehyde in acetic anhydride (0.05% w/v) followed by heating the chromatogram
to 120°C (Bridges et al 1970).

d) Freshly diazotized p-nitroaniline followed by 0.5M potassium hydroxide in ethanol (Copius-Peerboom 1960).

e) Equal volumes of freshly prepared potassium ferricyanide (1% w/v) and ferric chloride (2% w/v) followed by 2N HCl (Barton et al 1952).

f) Ninhydrin in acetone (0.2% w/v) followed by heating the chromatogram at 95°C for 10-15 minutes.

g) 0.1M Potassium dichromate in acetic acid (0.1M) followed by 0.1M silver nitrate after 5 minutes (Knight and Young 1958).

h) p-Dimethylaminobenzaldehyde in ethanol (0.5% w/v) containing concentrated HCl (1% v/v).

i) Sodium nitrite in 0.1N HCl (0.2% w/v) followed by ammonium sulphamate (5% w/v) after 3 minutes and after a further 3 minutes by an aqueous solution of N-1-naphthylethlenediamine hydrochloride (1% w/v) (modified Bratton and Marshall 1937).

j) 0.5N HCl followed by heating to 100°C for 10-20 minutes and the chromatogram sprayed with reagents d, h and i.

b) Enzymic Hydrolysis and Ether Extraction

Enzymes

Enzymic hydrolysis of urine samples (3-5 ml) was carried out and the following hydrolyses were conducted:-
ß-glucuronidase hydrolysis:

The enzyme used was Ketodase (15,000-20,000 Fishman units) ox liver ß-glucuronidase (Warner and Chilcopt) and overnight hydrolysis was carried out at pH 5.0 in 0.1M acetate buffer at 37°C in a shaking water bath. Incubations carried out under the same conditions but in the absence of enzyme were taken as controls. Ketodase is essentially free of sulphatase activity.

Arylsulphatase hydrolysis:

The enzyme used was arylsulphatase (3,000-5,000 units), a partially purified powder from Helix pomatia (Sigma Type 1) and overnight hydrolysis was carried out at pH 5.0 in a 0.5M acetate buffer at 37°C in a shaking water bath. Saccharic acid 1:4 lactone (Sigma) 20 mM was used to inhibit the ß-glucuronidase activity present in the enzyme preparation. Incubations carried out under the same conditions but in the absence of enzyme were taken as controls.

Acid phosphatase hydrolysis:

The enzyme used was a crude preparation of acid phosphatase (15-20 units) from wheat germ (Sigma type 1). It also contains lipase activity. Overnight hydrolysis was carried out at pH 4.8 in a 0.1M citrate buffer at 37°C in a shaking water bath. Saccharic acid 1:4 lactone was used to inhibit the ß-glucuronidase activity present in the enzyme preparation. Incubations carried out under the same conditions, but in the absence of enzyme were taken as controls.

The definitions of the enzyme activity units mentioned above are summarized below:-
Ketodase - one Fishman unit will liberate 1.0 µg phenolphthalein from phenolphthalein glucuronide per hour at 37°C at pH 5.0.

Arylsulphatase - one unit will hydrolyse 1.0 µmole p-nitrocatechol sulphate per hour at pH 5.0 at 37°C.

Acid phosphatase - one unit will hydrolyse 1.0 µmole p-nitrophenyl phosphate per minute at pH 4.8 at 37°C.

The specificity of the enzymes used were checked using p-nitrophenylglucuronide (25mM); p-nitrophenylsulphate (25 mM) and p-nitrophenylphosphate (25 mM) as substrates for the three enzymes used.

It was found that Ketodase shows essentially only β-glucuronidase activity while arylsulphatase, besides causing the hydrolysis of p-nitrophenylsulphate will also hydrolyse p-nitrophenylphosphate. Acid phosphatase, on the other hand, hydrolysed p-nitrophenylphosphate, showed little or no sulphatase activity, but caused some hydrolysis of p-nitrophenylglucuronide. However, the β-glucuronidase activity present in acid phosphatase from wheat germ was found to be inhibited by saccharic acid 1:4 lactone (20 mM) and saccharic acid 1:4 lactone does not appear to affect the hydrolysis of p-nitrophenyl phosphate by acid phosphatase.

After overnight incubation, aliquots of the hydrolysate were taken and chromatographed on Silica gel 60 F_{254} pre-coated thin layer plates in solvent systems B and G.

Ether Extraction

After overnight incubation, the pH of the hydrolysates from enzyme hydrolysis were suitably adjusted and aliquots (5-10 ml) taken
and extracted with diethyl ether (10-15 ml) two or three times. Isoamyl alcohol (1% w/v) was added to prevent emulsion formation during extraction and in some cases extraction was facilitated by saturation of the hydrolysate with sodium chloride. Following ether extraction, aliquots (0.1-0.5 ml) of the different fractions, i.e. hydrolysate, ether extract and aqueous residue were suitably diluted and taken for \(^{14}\text{C}\) determination as previously described.

The efficiency of the ether extraction after enzymic hydrolysis was also investigated. In the case of phenol and phase I metabolites of phenol, ether extraction was carried out at pH 5.0 and it was found that under these conditions phenol was quantitatively extracted into ether. Quinol (pKa 9.9 and 11.4) which has pKas similar to that of phenol (pKa 10) was assumed to have similar extraction properties. For aniline and phase I metabolites of aniline, ether extraction was carried out at pH 7-8 after saturation of the hydrolysate with sodium chloride. Aniline and acetanilide were extracted quantitatively while p-aminophenol (pKa 10.3) was extracted to about 90%. \(\sigma\)-Aminophenol and \(m\)-aminophenol with pKas of 9.9 and 9.8 respectively were assumed to be extracted similarly. \(N\)-Acetyl-p-aminophenol on the other hand was extracted with an efficiency of about 80%.

A range of concentrations of authentic materials were used in these extraction experiments. Authentic metabolites were dissolved in control urine to give a range of concentration (0.1-2.0 mM) and extracted with ether as described above. The amount of material extracted into the ether fraction was determined colourimetrically or by liquid scintillation counting and compared with standard curves prepared by dissolving authentic metabolites in ether. Thus the efficiency of the extraction procedure was determined. The amount of metabolites found in
the urine of the experimental animals were within the range of concentrations used.

Studies on Ether Extracts of Hydrolysed Urine Samples

a) Chromatography

Thin layer chromatography was carried out on silica gel 60 F<sub>254</sub> pre-coated thin layer plates. (Merck, Darmstadt, West Germany) and Silica gel G1500/LS254 pre-coated thin layer plates (Schleicher and Schüll, Dassel, West Germany).

The following solvent systems were used in the chromatographic investigation of the ether extracts:

- I Benzene : Dioxan : Acetic acid (glacial) (90:25:4 v/v)
- J Benzene : Methanol : Acetic acid (glacial) (90:16:8 v/v)
- K Chloroform : Methanol : Acetic acid (glacial) (80:20:1 v/v)
- L Chloroform : Methanol : Ammonia (880 s.g.) (80:20:1 v/v)
- M Chloroform : Ethylacetate : Formic acid (50:40:10 v/v)
- N Chloroform : Ethylacetate : Acetic acid (glacial) (50:40:10 v/v)
- O Toluene : Methanol (90:10 v/v)
- P Toluene : Methanol : Acetic acid (glacial) (90:10:1 v/v)

The ether extracts were dried over anhydrous sodium sulphate and concentrated either in vacuo or over a stream of nitrogen. The concentrated extracts were applied as a band (1.5-2.0 cm) and chromatograms developed in the above solvent systems. Authentic metabolites were co-chromatographed with the ether extracts.

Methods for the location and detection of the radiolabelled metabolites were as described for the chromatographic investigation of urine samples.
b) Reverse Isotope Dilution Analysis

The basic principle of reverse isotope dilution analysis depends on the fact that if a radioactive compound is added to the corresponding unlabelled compound and mixed homogeneously, the amount of radioactivity per unit of mass of the substance (i.e. specific radioactivity) will be reduced. That is, the radioactive material will be diluted by the nonradioactive material. If the reduction in the specific radioactivity can be measured, the amount of diluting non-radioactive material added can be calculated. Thus it can be seen that if isolation and purification procedures are incorporated with the radioactive isotope dilution analysis, this method may be used to determine the amount of one component in the presence of several others, by the addition of a radioactive tracer, of known specific radioactivity, of the component in question.

The isolation and purification can be carried out by any suitable procedures such as recrystallization, distillation and chromatography. Since accurate specific radioactivity determination depends on the purity of the sample and is independent of the amount of material used, the yield is relatively unimportant and rapid, but wasteful methods of purification can be used. Also, derivatives of the compound can be prepared, isolated and measured if this assists in the purification of the material; the principle of the method remains unchanged.

In reverse isotope dilution analysis the principle is essentially the same, except that a known weight of non-radioactive material is added to a mixture of radioactive materials. Hence, reverse isotope dilution.
The principles of reverse isotope dilution may be summarized as follows:

If $A_n$ is the total radioactivity in a mixture of several radioactive compounds whose radioactivities are $A_a$, $A_b$, $A_c$..... and whose mass are $W_a$, $W_b$, $W_c$..... respectively.

If the corresponding non-radioactive compounds are $a^0$, $b^0$, $c^0$..... are added to fractions of the mixture so that the mass of the radioactive compounds is negligible compared with the mass $W_a^0$, $W_b^0$, $W_c^0$..... of the non-radioactive compounds, i.e. $W_a^0 = W_a^0 + W_a$, etc.

If compounds $a$, $b$, $c$ are now isolated and purified from the various fractions of the mixture to constant specific radioactivities $S_a$, $S_b$, $S_c$..... respective; where

$$S_a = \frac{A_a}{W_a^0}, \quad S_b = \frac{A_b}{W_b^0}, \quad S_c = \frac{A_c}{W_c^0} \quad \ldots$$

Thus knowing $S_a$, $S_b$, $S_c$..... and $W_a^0$, $W_b^0$, $W_c^0$..... and $A_n$, then $A_a$, $A_b$, $A_c$..... may be calculated and hence the amounts of $a$, $b$, $c$..... in the mixture may be determined.

In the identification of urinary metabolites by reverse isotope dilution, authentic metabolites (200-500 mg) were added to separate portions of the ether extracts (5-10 ml), dissolved or refluxed for a few minutes and evaporated to dryness in vacuo on a rotary evaporator. The residues were either recrystallised to constant specific radioactivity and constant melting point or in some cases, they were derivatized and the derivatives were recrystallized to constant specific radioactivity and constant melting point.

In the study of the metabolism of phenol, toluene-4-sulphonyl derivatives of phenol and quinol were prepared during the reverse isotope
dilution experiments for the identification of metabolites. Catechol was recrystallized successfully from hot water to constant specific radioactivity and melting point (105° C).

Toluene-4-sulphonyl derivatives were prepared by dissolving the phenol and quinol residues in NaOH (10%, 2.5 ml), toluene-4-sulphonyl chloride (1.0 g for phenol, 2.0 g for quinol) in acetone (4 ml) was added and stirred vigorously for 15-20 minutes. The mixture was cooled and the crystalline precipitate formed was poured into water (25 ml), stirred and filtered at the pump. The precipitate was washed with water and the resultant crystalline material was recrystallized from industrial methylated spirit successively to constant specific radioactivity and constant melting point. The melting point of toluene-4-sulphonyl derivatives of phenol and quinol were 96° C and 159° C respectively.

In the study of the metabolism of aniline, ON-dibenzoyl derivatives of aminophenols were prepared during the reverse isotope dilution experiments for the identification of metabolites. Acetanilide and N-acetyl-p-aminophenol were recrystallized successively from water containing industrial methylated spirit (1% v/v) and hot water respectively, to constant specific radioactivity and constant melting point. The melting points of acetanilide and N-acetyl-p-aminophenol were 114° C and 168° C respectively.

The ON-dibenzoyl derivatives of aminophenols were prepared by the Schotten and Baumann reaction. The aminophenol residues were dissolved in NaOH (10%, 25 ml) and benzoyl chloride (2 ml) was added drop by drop with stirring. Stirring was continued for 15 minutes and the resultant ON-dibenzoyl derivatives were filtered and washed with 0.1M HCl, water and recrystallized.
QN-dibenzoyl-o-aminophenol was recrystallized successively from ethanol, ethylacetate and ethanol to constant specific radioactivity and constant melting point ($183^\circ C$). The ON-dibenzoyl derivative of m-aminophenol was recrystallized successively from ethanol, ethylacetate and toluene to constant specific radioactivity and melting point ($153^\circ C$) while the derivative of p-aminophenol was recrystallized from methanol, ethylacetate and methanol to constant specific radioactivity and melting point ($234^\circ C$).

The radioactivity associated with the crystalline material was monitored during reverse isotope dilution analysis at each stage of the recrystallization. Crystals (15-50 mg) were dissolved in dimethylformamide (0.5 ml) and the radioactivity was determined by liquid scintillation counting in a dioxan based scintillant (10 ml) as described.

2.2.3 **Ureter Cannulation Experiments**

2.2.3.1 **Surgical Procedure**

Male, Wistar albino rats weighing about 250 g were used throughout the ureter cannulation experiments.

Animals were anaesthetised with sodium pentobarbitone by intraperitoneal injection of Nembutal (0.25-0.3 ml per animal) (Abbott Laboratories Ltd. Kent).

The animals were laid ventral side uppermost and a mid-ventral line incision made through the muscle of the abdominal wall between the xiphisternum and the pubic region. Care was taken to avoid cutting the underlying organs.
The abdominal viscera were exposed and the bulk of the intestine was displaced to the left of the animal. The right ureter was located by its pulsing movement caused by urine passing down the ureter to the bladder.

The right ureter was ligated at a position just anterior to the bladder and the bulk of the intestine was displaced to the right of the animal and the left ureter was located and ligated in a similar manner.

Ligation of the ureter allows urine to be retained in the ureters, causing a slight distension which facilitates the introduction of the ureter cannulae (polyethylene cannula PP10, Portex Ltd.), and delinearates the ureters.

Fatty tissue around the ureters was removed using forceps, a small incision was made anterior to the ligature and the cannula introduced through the incision and directed towards the kidney. Care was taken not to sever the ureter when making the incision. The cannula was gently eased along the ureter towards the kidneys. Extreme care was taken to avoid damaging the ureters and urine in the ureters appears to facilitate this process by acting as a lubricant. The final position of the tips of the cannulae was several millimeters posterior to the kidneys and the cannulae were sutured into place.

It was found that the initial flow of urine along the polyethylene cannulae was facilitated if the cannulae were partially filled with isotonic saline before being introduced into the ureters. This also helped in preventing blockage of the cannulae during the operation.
The cannulae from both ureters were exteriorized, cut to appropriate lengths and the intestine replaced. Isotonic saline (5-10 ml) at 37°C was introduced into the abdominal cavity to facilitate the production of urine and urine was collected from the cannulae at timed intervals.

Throughout the surgical procedure care was taken to avoid damaging blood vessels and major organs and disturbance of the abdominal viscera was kept to a minimum.

The animals were kept anaesthetised throughout the surgical procedure and the subsequent experiments by periodic administration of Nembutal (0.05-0.1 ml) into the opened abdominal cavity. It was found that the rats may be kept in this anaesthetised state for as long as 4-6 hours and urine flow along the cannulae was maintained throughout this time. At the end of the experiments the animals were killed by cervical dislocation. No experiments were conducted on conscious, post-operative animals.

2.2.3.2 Dosing Procedure for Anaesthetised and Ureter Cannulated Rats

In all cases the administration of radiolabelled compounds was by intraduodenal injection and the compounds were administered as a solution of their salts in isotonic saline (3-5 ml) at 37°C.

The dosing procedure was as follows:-

The duodenum of the experimental animal was located and the bile duct identified. Intraduodenal injections were made at a point distal to the junction between the bile duct and the duodenum. The
needle of the syringe was introduced directly into the intestinal lumen through the gut wall and the duodenum was ligated just behind the needle point but in front of the point of entry of the needle. The dose solution was injected into the lumen of the intestine and the ligature was secured as the needle was withdrawn so that leakage of the dose solution was prevented. After securing the ligature, the duodenum was carefully replaced into the abdominal cavity.

Throughout this procedure, extreme care was taken to avoid damage to the intestine distal to the point of injection and the intestine was kept moist during the procedure by periodic wetting with isotonic saline at 37°C.

2.2.3.3 Collection and Assay of Urine and Identification of Urinary Metabolites from Ureter Cannulated Rats

Urine (100-200 μl) was collected from the ureter cannulae at timed intervals (usually 10 minutes) after intraduodenal injection of radiolabelled compounds. Urine was assayed for $^{14}$C in the dioxan based scintillant as described.

Qualitative and quantitative estimation of urinary metabolites was by thin layer chromatography of urine samples followed by radiochromatogram autoradiography and scintillation counting. The methods used were as described previously for the identification of urinary metabolites.

2.2.4 Isolated Cell Preparation Experiments

2.2.4.1 Isolated Rat Hepatocytes

Suspensions of isolated rat hepatocytes were supplied by
Dr. J. Fry and the method for the isolation of rat hepatocytes was according to Fry et al. (1976).

Essentially the method consists of washing rat liver slices in Dulbecco's Ca\(^{2+}\) and Mg\(^{2+}\) free phosphate buffer saline at 37°C, followed by incubation with ethylglycol-bis-\((\beta\)-amino-ethylether\)N,N\'\(-\)tetraacetic acid (EGTA). After treatment with EGTA, the liver slices are digested with collagenase/hyaluronidase (0.05%/0.01% w/v Sigma type I and II) in Hank's Mg\(^{2+}\) free balanced salt solution containing CaCl\(_2\) (5mM) at 37°C in a shaking water bath.

The resultant enzymic digest was filtered through a layer of Boulting Cloth (150 \(\mu\)m pore size Henry Simon Ltd., Stockport) and the filtrate centrifuged at approximately 200g\(_{av}\) for 2 minutes. The cell pellet was washed and resuspended in Hank's balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\). The total yield of cells was determined by counting in an improved Neubauer counting chamber and the viability of the cells was determined on the basis of the ability of the cells to exclude the dye trypan blue.

The cells were finally resuspended in L15 Leibovitz medium with glutamine (Gibco, Scotland) containing tryptose phosphate broth (10% w/v) and foetal calf serum (10% w/v). The cell density was 6-8 \(\times\) 10\(^6\) viable cells ml\(^{-1}\). Viability of cells was in the order of 80-90%.

Protein content of the hepatocytes was estimated by the following procedure. The cell suspension was centrifuged and the resultant cell pellet disrupted in a solution of Triton X-100 (1% v/v) and digested in 0.5M NaOH. The protein was estimated according to the method of Lowry et al. (1951).
2.2.4.2 Isolated Rat Intestinal Mucosal Cells

Suspensions of isolated rat intestinal mucosal cells were supplied by R. Shirkey and the method for the isolation of rat intestinal mucosal cells was according to Shirkey (1976).

Intestinal mucosal cells were isolated from the upper third of the small intestine. This section of the small intestine was flushed with Kreb's Ca$^{2+}$ and Mg$^{2+}$ free phosphate buffer pH 7.4 containing 0.1% glucose (Kreb's CMF buffer), everted and filled with Kreb's CMF buffer containing albumin (0.5% w/v bovine serum albumin fraction V, Sigma). The everted gut was incubated in Kreb's CMF buffer containing 0.5% w/v ethylene diamine tetraacetic acid (EDTA 1mM) with a bacterial protease (1% w/v Sigma type VII) for 45 minutes at 37°C in a shaking water bath.

The resultant enzymic digest was filtered through a layer of Boulting cloth (150μm pore size, Henry Simon Ltd., Stockport) and the filtrate centrifuged at approximately 200g$_{av}$ for 1 minute. The cell pellet was washed with Kreb's phosphate buffer pH 7.4 containing glucose (0.1% v/w) and albumin (0.5% w/v) and resuspended in L15 Leibovitz medium with glutamine (Gibco, Scotland) containing foetal calf serum (10% w/v). Total cell count and cell viability were calculated as described for rat hepatocytes. The cell density was 7-9 × 10$^6$ viable cells ml$^{-1}$. Viability of cells was in the order of 90-95%.

2.2.4.3 Isolated Rat Kidney Cells

Suspensions of isolated kidney cells were supplied by Dr. J. Fry and the method for their isolation was essentially as described for rat hepatocytes.
Kidney slices were prepared from rat kidneys from which surrounding fatty tissue and the medulla had been excised and the slices were washed, incubated and enzymically digested as described for rat hepatocytes.

The cells thus obtained, which also contained nephrons and tubules, were suspended in Hank's balanced salt solution without Ca$^{2+}$ and Mg$^{2+}$. The viability of the cells was estimated to be 80-90% by the exclusion of the dye trypan blue. The kidney cells were finally resuspended in L15 Leibovitz medium with glutamine containing tryptose-phosphate broth (10% w/v) and foetal calf serum (10% w/v) for metabolic studies.

Protein content of the kidney cell suspension was estimated as described for hepatocytes.

2.2.4.4 Methods Used in the Study of the Metabolism by Isolated Cell Preparations

Radiochemical compounds (5-10 $\mu$Ci, 100 $\mu$M) were incubated with the cell suspension (5 ml) in a conical flask (50 ml) in a shaking water bath at 37°C. In the case of the rat hepatocytes and the kidney cells, aliquots (100 $\mu$L) of the incubate were taken at timed intervals and added to ice-cold acetone (20 $\mu$L) to stop the reaction, while in the case of intestinal mucosa cells, the incubations were stopped by the addition of ice-cold acetone (1 ml) to the total incubate (5 ml). Ice-cold acetone used at the concentration described caused as immediate uptake of the dye trypan blue by the cells, indicating cell damage.

The incubation were centrifuged and the supernatents (20-30 $\mu$L) were chromatographed on silica gel 60 F$_{254}$ pre-coated thin
layer plates (Merck). Qualitative and quantitative estimation of metabolites were by radiochromatogram autoradiography, liquid scintillation counting and reverse isotope dilution. The methods used were as described previously for the identification of urinary metabolites.
CHAPTER 3

BENZOIC ACID
3.1 INTRODUCTION

Benzoic acid (C6H5COOH) is a colourless, odourless, light feathery crystalline material of M.W. 122.1 and m.p. 122°C. It has a pKa of 4.17 and is readily soluble in several organic solvents including ethanol, ether and chloroform. It is only sparingly soluble in cold water, however, it is soluble in hot water from which it may be recrystallized. It also sublimes at 100°C.

It occurs naturally both in the free and combined form. It is found generally as esters in plants, as essential oils, gums and resins. For example, most berries contain approximately 0.05% benzoic acid and in gum benzoin, from which benzoic acid was first isolated, there may be as much as 20%.

It has anti-microbial properties and at concentrations of 0.1% it acts as an effective preservative for pharmaceutical preparations. It is also employed as a preservative in the food industry where the permitted level is not more than 1 part in 1000. The antibacterial and antifungal properties of benzoic acid are due to the undissociated acid and its preservative effect is exhibited in slightly acid media. Anti-microbial properties are apparently not exhibited at pH above 5, although some undissociated material must still be present.

Medically benzoic acid has been used as a urinary antiseptic and it is used in the treatment of fungal infection of the skin, e.g. as Compound Benzoic Acid Ointment which contains 6% benzoic acid and 3% salicylic acid. Also, benzoic acid has been used as the basis of a liver function test.
Benzoic acid shows little toxicity in man, although mild irritation of the skin and mucous membrane may be experienced on exposure to large doses. The LD$_{50}$ of benzoic acid in the rat has been shown to be 4.1g kg$^{-1}$ while the lethal dose in dogs is 2g kg$^{-1}$.

### 3.2 METABOLISM OF BENZOIC ACID

Since the discovery more than 120 years ago (Keller 1842) that benzoic acid is conjugated with glycine in the mammalian body, the fate of benzoic acid in the animal body has received much attention.

The metabolites of benzoic acid in most species are hippuric acid (benzoylglycine) and benzoylglucuronide, although some species may also produce ornithuric acid ($N^2 N^5$ dibenzoylornithine).

There is apparently no evidence to show that benzoic acid forms sulphate conjugates (Bray et al 1946), furthermore, it is not decarboxylated neither by mammalian tissues nor by gastrointestinal organisms. The administration of carboxyl-$^{14}$C benzoic acid to rats does not give rise to $^{14}$CO$_2$ in the expired air (Bernhard et al 1955) and in vitro incubation of rat caecal contents with ring-U-$^{14}$C benzoic acid for 4 days gives 99% recovery of benzoic acid by reverse isotope dilution (Brewster 1976).

However, benzoic acid has been reported to be hydroxylated in the rat and the guinea pig and trace amounts of 2-, 3- and 4-hydroxybenzoic acids have been detected in the hydrolysed urine of these animals dosed with carboxyl-$^{14}$C benzoic acid (Acheson and Gibbard 1962). Also, it appears that these hydroxylated benzoic acids may be present as sulphate conjugates, since it has been observed that incubating benzoic
acid with rat liver slices in the presence of $^{35}$S sulphate, followed by chromatogram autoradiography showed a faint, but clear radioactive spot corresponding to 4-sulphato-oxybenzoic acid (Sato et al 1956).

The conjugation of benzoic acid with glycine and glucuronic acid to form hippuric acid and benzoylglucuronide respectively is subject to species variation and is dose dependent, while the conjugation with ornithine to form ornithuric acid appears to be restricted to birds and reptiles.

In a study of the fate of benzoic acid in various species (Bridges et al 1970) it was found that when given orally at 50mg kg$^{-1}$, benzoic acid is excreted almost entirely (90-100%) as hippuric acid in most of the herbivorous and omnivorous animals investigated. The animals studied included man and several species of monkeys, such as rhesus monkey, squirrel monkey and capuchin monkey, and several species of rodents including the rat, mouse, guinea pig, lemming, hamster and gerbil. The pig and the rabbit were also included in the study. In these species benzoylglucuronide was excreted as a metabolite, but only in trace amounts.

On the other hand, appreciable amounts of benzoylglucuronide were excreted by the carnivorous species, the dog and the ferret ($\approx 20\%$), but not by the cat, which is not unexpected since the cat has a defective glucuronic acid conjugation mechanism (Robinson and Williams 1958).

The differences in the amount of benzoylglucuronide and hippuric acid excreted by various species following benzoic acid administration may be explained in terms of the rate of mobilization of glycine for conjugation and by the dose level of benzoic acid. For example, the ferret excreted some 22% of the 24 hour excretion as
benzoylglucuronide when benzoic acid was administered at 50 mg kg\(^{-1}\), but when the dose was increased to 198 mg kg\(^{-1}\) glycine and glucuronic acid conjugation was about equal and at 400 mg kg\(^{-1}\) glucuronic acid conjugation predominated with 49% benzoylglucuronide and 30% hippuric acid being excreted. The ferret also excreted appreciable amounts of free benzoic acid but it is suggested that this may well have arisen from breakdown of benzoylglucuronide, which is relatively labile. (Bridges et al 1970).

The rabbit, on the other hand, shows no such changes in the amount of hippuric acid and benzoylglucuronide excreted. Thus at 50 mg kg\(^{-1}\) the rabbit excreted benzoic acid entirely as hippuric acid. When the dose level was increased to 200 mg kg\(^{-1}\) glycine conjugation of benzoic acid was still nearly complete (97-100%) and only trace amounts of benzoylglucuronide were detectable in the urine (Bridges et al 1970).

It is suggested that the rate of glycine mobilization is an important factor in the synthesis of hippuric acid from benzoic acid and may account for the differences in the ratio of benzoylglucuronide to hippuric acid excreted by various species. For example, the ratio of glucuronide to hippuric acid is about 2:1 in the dog and 1:4 in the rabbit when the dose was 450 mg kg\(^{-1}\) (Williams 1959) and it has been calculated that the rate of glycine mobilization in the dog is about 3.5 mg kg\(^{-1}\) hr\(^{-1}\) while in the rabbit it is about 24 mg kg\(^{-1}\) hr\(^{-1}\) (Quick 1931). Furthermore, it has been shown that if the availability of glycine is increased by feeding exogenous glycine, hippuric acid excretion is increased and glucuronide formation depressed (Hainline and Lewis 1953). Indeed when no glycine was fed, the rate of glycine mobilization in the rabbit was calculated to be about 21 mg kg\(^{-1}\) hr\(^{-1}\), but when glycine was fed with the benzoate, the rate of glycine mobilization was calculated to be about
It has been shown that only free glycine may be utilized in hippuric acid synthesis and any precursors must first be converted to glycine before utilization. However, some free glycine is present in mammalian tissues which is immediately available for conjugation with small doses of benzoic acid. For example, in the rat this amounts to 10 mg 100 g⁻¹ and is referred to as the "first glycine pool" (Arnstein and Neuberger 1951). This "first glycine pool" is in a relatively slow equilibrium with a larger "pool" of glycine and glycine precursors and the larger "pool" only participates in hippuric acid synthesis when larger doses of benzoic acid are administered. Indeed it has been stated that the rate of hippuric acid synthesis is independent of the dose except for doses of benzoic acid of 25 mg kg⁻¹ or less, whereas that of glucuronide synthesis is dependent upon the dose (Bray et al 1951). Thus it can be seen that both the dose level of benzoic acid and the rate of glycine mobilization play important parts in species variation in benzoic acid metabolism. At small doses all species tend to excrete benzoic acid as hippuric acid, but with larger doses, species with relatively high rates of glycine mobilization will excrete predominantly hippuric acid (e.g. rat), whereas in species where glycine mobilization is relatively slow, saturation of the glycine pathway may occur resulting in an increase in glucuronic acid conjugation.

In the 20 or so species investigated by Bridges et al (1970), the cat and the Indian fruit bat are of particular interest in that they are apparently deficient in glucuronic acid and glycine conjugation mechanisms respectively. Thus the cat excretes benzoic acid entirely as hippuric acid while the Indian fruit bat excretes benzoic acid predominantly as benzoylglucuronide. The deficiency of glucuronic acid conjugation mechanism in the cat is well known. Indeed, it appears that
the cat like animals such as the lion, civet and genet show a similar pattern of conjugation mechanism as the domestic cat (French, et al 1974).

Conjugation with glycine or some other amino acid (e.g. ornithine) is almost universal among animals ranging from insects to man. The absence of such conjugation is unusual, but it has been reported that the carrion crow and grey African parrot did not excrete hippuric or ornithuric acid when dosed with benzoic acid (Baldwin, et al 1960). The absence of hippuric acid synthesis by the Indian fruit bat has been confirmed both in vivo and in vitro experiments (Bababunmi, et al 1973), but deficiency in glycine conjugation is apparently substrate dependent since the Indian fruit bat excretes the glycine conjugate phenaceturic acid as the major metabolite of phenylacetic acid (Ette, et al 1974). The metabolites of benzoic acid and phenylacetic acid are also different in man, they are hippuric acid and phenylglutamine respectively (Williams, 1959), however, both acids are conjugated with glycine in the rat.

The excretion of ornithuric acid ($N^2N^5$ dibenzoylornithine) as a metabolite of benzoic acid was discovered by Jaffe (1877) in hens and it has been shown that in several species of birds studied, ornithuric acid is the major metabolite of benzoic acid. However, not all birds form ornithine conjugates. For example, chicken, turkey, goose and duck excrete ornithuric acid after benzoic acid administration whereas pigeon and dove appear to form only hippuric acid (Baldwin et al 1960, Bridges, et al, 1970). All species of birds examined also form benzoylglucuronide.

Ornithuric acid is also excreted as a major metabolite of benzoic acid by several species of reptiles including lizard, snake, tortoise and crocodile (Smith, 1957) and the gecko (Bridges, et al, 1970).
The excretion of ornithuric acid does not preclude the excretion of hippuric acid from benzoic acid since species such as the hen excrete some 5-20% of the dose of benzoic acid as hippuric acid (Bridges et al, 1970). Indeed glycine conjugation occurs in the hen to a minor extent as reported by Suga, (1919) and Chang and Johnson, (1956). Also the formation of hippuric acid from ingested aromatic acids has been reported (Komori et al, 1926) and hippuric acid is a normal urinary metabolite of turtles (Schiff, 1859, Khalil, 1947). It appears that in all species studied so far ornithine conjugates are always accompanied by small amounts of glycine conjugates (Smith, 1957).

The tissue distribution in the ability to metabolise benzoic acid has been investigated using tissue slices and homogenates and it has been shown that benzoic acid is metabolised not only by the liver, but also by the kidneys and the small intestine. Indeed, in the dog glycine conjugation of benzoic acid has been shown to occur only in the kidneys (Friedmann and Tachan, 1911, Quick, 1932, Snapper et al, 1923, Borsook and Dubnoff, 1940). Metabolism of benzoic acid by other tissues has not been demonstrated.

The ability of the kidneys to conjugate benzoic acid with glycine has been demonstrated by several workers (Schmiedeberg and Burge, 1877, Quick, 1932) and in a pharmacokinetic investigation in the rabbit it was shown that the enzyme system involved may be different from that of the liver but there was considerable individual variation (Wan and Rigelman, 1972).

The involvement of the kidney in the in vivo metabolism of benzoic acid has been considered and in an attempt to investigate the
fractional metabolism of benzoic acid by specific organs, namely the kidneys and the liver (Wan and Rigelman, 1972), it was found that there was considerable variation and that fractional metabolism of benzoic acid in the kidneys ranged from 21-88%. Clearly the kidneys make an appreciable contribution to the in vivo metabolism of benzoic acid in the rabbit, but unfortunately it was not possible to determine the relative contribution of the liver and the kidney in the in vivo metabolism of benzoic acid and it is likely that dose level and availability of glycine are important factors for consideration.

Using liver and kidney slices and homogenates Irjala, (1972) was able to demonstrate hippuric acid synthesis in these tissues in several species including the rat, guinea pig, dog and cat. Similar rates of hippuric acid synthesis were observed in liver slices and kidney slices in all species investigated except the dog and the cat, where the rate of synthesis of hippuric acid in kidney slices was greater than that found in liver slices. On the other hand, liver homogenates showed considerably more glycine conjugating activity than kidney homogenates in all species studied except the dog and the cat, where activity in the kidney homogenates predominated. It was suggested that differences between the observations from the tissue slices and homogenates may have been due to factors such as the penetration of substrate into tissue slices and the greater exposure of the potential enzyme mechanism for glycine conjugation in homogenates. Kidney slices have been shown to actively accumulate aryl substrates whereas liver slices do not (Cross and Taggart, 1950, Scharchter, et al. 1955), and this may be significant in the in vivo metabolism of benzoic acid. Such observations illustrate the difficulties involved in investigations using relatively crude biological preparations.
The ability of the intestine of the rat to conjugate benzoic acid with glycine has also been demonstrated (Strahl and Barr 1971), and the synthesis of hippuric acid in everted gut sacs was found to be low (≤1%). There was apparently no hydrolysis of hippuric acid by the gastrointestinal mucosa or by intestinal microorganisms, but there was some accumulation of benzoic acid and hippuric acid in the intestinal tissues. Interestingly an extramitochondrial thermolabile factor was found to be present in the intestinal mucosa which apparently inhibited the in vitro glycine conjugation of benzoic acid in both the liver and the kidneys and it is possible that this inhibitory factor may be involved in the low level of synthesis of hippuric acid in the intestine. The nature of the inhibition was not known and it is suggested that since glycine conjugation occurs in the mitochondria, extramitochondrial inhibition may not occur in vivo (Irjala, 1972).

The tissue distribution of glycine conjugation of benzoic acid has also been investigated in various species and in most species studied the major site of glycine conjugation was the liver. However, glycine conjugation of benzoic acid in the dog occurring only in the kidneys has already been described, but recently, using a sensitive radiochemical technique, hippuric acid synthesis has been demonstrated in dog liver slices and homogenates, although this was insignificant compared with the synthetic activity of the dog kidneys (Irjala, 1972).

It was also shown that synthesis of hippuric acid by cat kidney homogenates was greater than cat liver homogenates (Irjala, 1972). In man hippuric acid synthesis is markedly affected by liver damage and indeed formation of hippuric acid from ingested sodium benzoate has been used in liver function tests. This suggests that glycine
FIGURE 3

COMPOUNDS IDENTIFIED AS METABOLITES OF BENZOIC ACID

HIPPURIC ACID

BENZOIC ACID

BENZOYLGLUCURONIDE

HYDROXYBENZOIC ACID CONJUGATES

(TRACE METABOLITES)

$N^2 \, N^5$ DIBENZOYLORNITHINE
conjugation of benzoic acid in man occurs predominantly in the liver. Interestingly the rhesus monkey was found to metabolise benzoic acid only in the liver (Wan and Rigelman, 1972).

The synthesis of ornithuric acid in species which excrete this compound as a metabolite of benzoic acid also shows some tissue distribution. For example, ornithine conjugation of benzoic acid in the chicken was found to occur in the kidneys, the liver apparently shows no such activity (McGilvery and Cohen, 1950). It is probable that the mechanism of synthesis of ornithuric acid and hippuric acid is similar.

3.3 IN VIVO INVESTIGATION OF BENZOIC ACID METABOLISM

\[ \text{Ring-U-}^{14}\text{C} \text{-Benzoic acid (50 mg kg}^{-1} \text{) was administered to the animals as described in Chapter 2. Urine and faeces were collected and assayed as described.} \]

The \( R_f \) values and methods of detection of benzoic acid and its metabolites are given in Table 3.1.

The results of orally administered \( ^{14}\text{C}-\text{benzoic acid} \) in the sheep, the pig and the rat are summarized in Tables 3.2 and 3.3.

It is significant that a major proportion of the administered dose was excreted by these animals relatively rapidly after oral administration. Thus in the case of the pig 77% of the administered dose was excreted within 5 hours after oral administration of \( ^{14}\text{C}-\text{benzoic acid} \) while in the sheep 57% was excreted within 4 hours after administration. 24 hours after oral administration the radioactivity excreted in the urine was 91, 82 and 92% of the administered dose in the sheep, the pig and the rat respectively.
Negligible amounts of radioactivity (<1%) were excreted in the faeces, indicating that the major pathway for the elimination of orally administered benzoic acid was by urinary excretion.

The major metabolite in all these species was hippuric acid. Benzoylglucuronide and benzoic acid were also detected in the urine. In the rat they were 4.8% and 2.8% of the dose respectively, but in the sheep and the pig only traces of these metabolites were present (less than 1%). Free benzoic acid found in the urine may arise from the bacterial decomposition of benzoylglucuronide which is relatively labile (Bridges et al 1970).

It has been shown that the amount of benzoylglucuronide and hippuric acid excreted following oral administration of benzoic acid depends upon the dose and that in some species such as the ferret, increase in the dose of benzoic acid results in an increase in benzoylglucuronide excretion with a concomitant decrease in hippuric acid excretion, while in other species, e.g. rabbit, only hippuric acid was excreted irrespective of the dose of benzoic acid although traces of benzoylglucuronide may be detected at higher doses of benzoic acid (Bridges et al, 1970). Thus, following on from these reports, $^{14}$C benzoic acid at various dose levels (10-400 mg kg$^{-1}$) was administered orally to a group of rats. The excreta were collected and analysed as described and the results are summarized in Table 3.4.

The results showed that at all dose levels studied some 80% of the dose was excreted in the urine within 24 hours. Negligible amounts of radioactivity were detected in the faeces implying that urinary excretion is the major route of elimination of orally administered $^{14}$C-benzoic acid in the rat.
### Table 3.1

**R<sub>f</sub> Values and Colour Reactions of Benzoic acid and its Metabolites**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; Values in Solvent Systems</th>
<th>Colour Reactions with Spray Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paper Chromatography</td>
<td>Thin Layer Chromatography</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>0.92-0.94</td>
<td>0.90-0.92</td>
<td>0.48-0.50</td>
</tr>
<tr>
<td>Benzoylglucuronide</td>
<td>0.50-0.57</td>
<td>0.69-0.70</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>0.84-0.88</td>
<td>0.80-0.83</td>
</tr>
</tbody>
</table>

PC on Whatman No. 1 Paper.  TLC on Silica gel 60 (Merck)

### Table 3.2

**Urine Excretion of 14C-Benzoic acid by Sheep and Pig**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (mg Kg&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Time (hr)</th>
<th>Volume of Urine (ml)</th>
<th>% of Administered Dose Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>50</td>
<td>0- 4</td>
<td>184</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0- 9</td>
<td>236</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-24</td>
<td>390</td>
<td>12</td>
</tr>
<tr>
<td>Pig</td>
<td>50</td>
<td>0- 3</td>
<td>53</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0- 5</td>
<td>184</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0- 8</td>
<td>202</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-24</td>
<td>240</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 3.3

**Urinary Metabolites of **\(^{14}\)C-Benzoic acid at 50 mg Kg\(^{-1}\)**

<table>
<thead>
<tr>
<th>Species</th>
<th>% Dose in 24 hr. Urine</th>
<th>% of 24 Hour Excretion as</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Benzoic acid</td>
</tr>
<tr>
<td>Sheep*</td>
<td>91</td>
<td>3.0</td>
</tr>
<tr>
<td>Pig*</td>
<td>82</td>
<td>0.0</td>
</tr>
<tr>
<td>Rat*</td>
<td>92</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\* - Results are obtained from 1 animal
\* - Results are obtained as the mean of 2 animals

Table 3.4

**Urinary Metabolic Profiles of Rats Dosed with **\(^{14}\)C-Benzoic acid**

<table>
<thead>
<tr>
<th>Dose (mg Kg(^{-1}))</th>
<th>% Dose in 24 hr. Urine (μCi Animal(^{-1}))</th>
<th>% of 24 Hour Excretion as</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Benzoic acid</td>
</tr>
<tr>
<td>10</td>
<td>1.0</td>
<td>86.8, 78.4</td>
</tr>
<tr>
<td>50</td>
<td>1.0</td>
<td>105.6, 78.5</td>
</tr>
<tr>
<td>100</td>
<td>1.0 - 2.5</td>
<td>90.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(94.0 - 85.1)</td>
</tr>
<tr>
<td>200</td>
<td>1.0 - 2.5</td>
<td>87.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(93.6 - 84.4)</td>
</tr>
<tr>
<td>250</td>
<td>1.0</td>
<td>68.0, 36.3</td>
</tr>
<tr>
<td>300</td>
<td>2.5</td>
<td>88.4, 69.2</td>
</tr>
<tr>
<td>400</td>
<td>2.5</td>
<td>78.1, 40.2</td>
</tr>
</tbody>
</table>

Where 3 or more animals were used, the results are expressed as the mean with the range in parentheses and where only 1 or 2 animals were used, the individual results are given.
A study of the metabolic profile showed that at all dose levels studied, hippuric acid was the major urinary metabolite. A small amount of free benzoic acid was also detected, but this may have arisen from bacterial hydrolysis of benzoylglucuronide. Benzoylglucuronide was found to be present in all urine samples. Also there appears to be a trend in that there was a slight increase in the proportion of benzoylglucuronide and a slight decrease in hippuric acid when the dose level of benzoic acid was increased.

3.4 METABOLISM OF BENZOIC ACID IN URETER CANNULATED RATS

The ureters of anaesthetised male rats (250 g) were cannulated as described, they were dosed with \[^{14}C\] benzoic acid at 50 mg kg\(^{-1}\) and 100 mg kg\(^{-1}\) by intraduodenal injection as described. After intraduodenal injection urine was collected at intervals of 10 minutes for a period of 2 hours and urine samples were assayed for radioactivity as described. Metabolic profiles of the 10 minute samples were determined by thin layer chromatography of aliquots (20-40 µl) of urine on silica gel 60 pre-coated plates (Merck) in solvent system G followed by radiochromatogram autoradiography. The areas of the chromatograms corresponding to benzoic acid (R\(_f\) 0.95), hippuric acid (R\(_f\) 0.71) and benzoylglucuronide (R\(_f\) 0.37) were located and compared with the corresponding dark spots on the autoradiograms. These areas were marked on the chromatograms, cut out and placed into scintillation vials and counted as described. The radioactivity associated with each of the areas was expressed as a percentage of the radioactivity recovered from the radiochromatogram.
The rate of urinary elimination of radioactivity after intraduodenal administration is summarized in Figure 3.1. Metabolic profiles of the 10 minute samples are illustrated in Figure 3.2 and the rate of excretion of hippuric acid and benzoylglucuronide are shown in Figures 3.3, 3.4 and 3.5.

The results obtained from anaesthetised animals (Figure 3.1) showed that at the dose levels studied some 2% of the dose was recovered in the urine within 10 minutes after administration and that at 50 mg kg\(^{-1}\) about 80% of the dose was excreted within 2 hours while at 100 mg kg\(^{-1}\) about 70% of the dose was recovered in the urine in 2 hours.

The rate of elimination of radioactivity, which may be calculated from the linear portion of the curve showed that at 50 mg kg\(^{-1}\) the rate was in the order of 8.5% of the dose per 10 minutes while at 100 mg kg\(^{-1}\) it was in the order of 7.7% of the dose per 10 minutes. The linear portion of the graph represents the maximal rate of elimination of metabolites of benzoic acid at the particular dose studied.

At both dose levels the major metabolite was hippuric acid and it can be calculated that the rate of elimination of hippuric acid was 1.47 \(\mu\)mole min\(^{-1}\) (0.26 mg min\(^{-1}\)) and 0.85 \(\mu\)mole min\(^{-1}\) (0.15 mg min\(^{-1}\)) when 205 \(\mu\)moles (100 mg kg\(^{-1}\)) and 102.5 \(\mu\)moles (50 mg kg\(^{-1}\)) of benzoic acid was administered to the rats (250 g) respectively. The rate of elimination of benzoylglucuronide was calculated to be 0.2 \(\mu\)mole min\(^{-1}\) (59.6 \(\mu\)g min\(^{-1}\)) when the animals were dosed with 205 \(\mu\)moles of benzoic acid and 0.14 \(\mu\)mole min\(^{-1}\) (41.7 \(\mu\)g min\(^{-1}\)) when dosed with 102.5 \(\mu\)mole of benzoic acid (Figures 3.4 and 3.5).

These figures showed that the metabolism of benzoic acid followed the kinetics of a first order reaction. The rate of elimination
RESULTS ARE PLOTTED AS THE MEAN FROM 3-4 ANIMALS AND TE THE RANGE
FIGURE 3.2

% $^{14}$C EXCRETED AS HIPPURIC ACID AND BENZOYLGLUCURONIDE AT TIMED INTERVALS

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
FIGURE 3.3

URINARY ELIMINATION OF HIPPURIC ACID AND BENZOYLGLUCURONIDE

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
FIGURE 3.4

THE RATE OF URINARY ELIMINATION OF HIPPURIC ACID

O BENZOIC ACID at 50 mg kg\(^{-1}\) (102.5 μmole animal\(^{-1}\))

● BENZOIC ACID at 100 mg kg\(^{-1}\) (205 μmole animal\(^{-1}\))

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
FIGURE 3.5

THE RATE OF URINARY ELIMINATION OF BENZOYLGLUCURONIDE

○ BENZOIC ACID at 50 mg kg\(^{-1}\) (102.5 \(\mu\) mole animal\(^{-1}\))

● BENZOIC ACID at 100 mg kg\(^{-1}\) (205 \(\mu\) mole animal\(^{-1}\))

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
of both metabolites is dependent upon the dose and increases with increasing doses of benzoic acid. Saturation of the systems involved in the metabolism of benzoic acid was not observed at the dose levels used and it is likely that a maximum rate of elimination would be reached when saturating levels of benzoic acid were administered.

The ratio of hippuric acid to benzoylglucuronide at 2 hours after intraduodenal administration of benzoic acid was calculated to be 6.6:1 at 50 mg kg⁻¹ and 8:1 at 100 mg kg⁻¹. This suggests that relatively more benzoylglucuronide was being excreted at a dose of 50 mg kg⁻¹ that at 100 mg kg⁻¹, although it can be demonstrated that there is a trend towards a slight increase in the relative amounts of benzoylglucuronide with increasing doses of benzoic acid. However, it should be noted that at 2 hours after administration benzoylglucuronide found in the urine has reached a maximum and plateaued when the dose was 50 mg kg⁻¹, but at 100 mg kg⁻¹ this has yet to be achieved (Figure 3.3).

When the ratio of hippuric acid to benzoylglucuronide found in the urine at different times after intraduodenal administration of benzoic acid was plotted against time (Figure 3.6), it can be seen that more hippuric acid than benzoylglucuronide was excreted throughout the 2 hour period, but the relative amounts of hippuric acid to benzoylglucuronide found in the urine appears to change with time. Initially the ratio of hippuric acid to benzoylglucuronide in the urine was high, but it drops rapidly to a constant level and then gradually rises again as the administered benzoic acid is metabolised and excreted.

Figure 3.2 shows the percentage of the dose excreted at each time interval as hippuric acid and benzoylglucuronide and it can be
FIGURE 3.6

RATIO OF HIPPURIC ACID TO BENZOYLGLUCURONIDE IN THE URINE AFTER ADMINISTRATION OF BENZOIC ACID IN RATS

RESULTS ARE THE MEAN FROM 2 ANIMALS
seen that maximum excretion occurs between 50-60 minutes and 40-50 minutes for doses of 50 mg kg\(^{-1}\) and 100 mg kg\(^{-1}\) respectively.

3.5 METABOLISM OF BENZOIC ACID IN ISOLATED CELL PREPARATIONS

Isolated cell preparations were prepared as described in Chapter 2 and \(\text{[ring-}^{14}\text{C}]\text{benzoic acid (100 }\mu\text{M 5 }\mu\text{Ci/10 }\mu\text{Ci})\) was incubated with the cell suspension (5 ml) as described.

Samples of the incubates obtained at 5 minute intervals from rat kidney cells and liver cells (100 }\mu\text{L}), and from intestinal mucosal cells (5 ml) at 15 minute intervals were assayed by thin layer chromatography after centrifugation to remove denatured protein. Aliquots of the supernatents (20-40 }\mu\text{L}) were chromatographed on silica gel 60 plates (Merck) in solvent system G. The areas corresponding to benzoic acid \((R_f 0.93)\), hippuric acid \((R_f 0.71)\) and benzoylglucuronide \((R_f 0.37)\) were located and compared with the corresponding dark spots on the autoradiograms. These areas were marked on the radiochromatogram and were cut out and placed into scintillation vials and counted as described. The radioactivity associated with each area was expressed as a percentage of the radioactivity recovered from the radiochromatograms.

Autoradiograms from isolated rat intestinal mucosal cells, kidney cells and hepatocytes are shown in Figures 3.7; 3.8; 3.9; and it can be seen that essentially no metabolism of benzoic acid by isolated intestinal mucosal cells was detected during the period of 1 hour. On the other hand, isolated rat liver and kidney cells show progressive metabolism of benzoic acid to hippuric acid and benzoylglucuronide.
FIGURE 3.7

AUTORADIOGRAM OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-BENZOIC ACID IN ISOLATED RAT INTESTINAL MUCOSAL CELLS

B - Benzoic acid

TLC on Silica gel 60 (Merck), in Solvent System G.
FIGURE 3.8

AUTORADIOGRAMS OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}\text{C}-\text{BENZOIC ACID}$ IN ISOLATED RAT KIDNEY CELLS

B – Benzoic acid
H – Hippuric acid
BG – Benzoylglucuronide
x – "Hydroxybenzoic acid" Metabolites

TLC on Silica gel 60 (Merck), in Solvent System G.
AUTORADIOGRAMS OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-BENZOIC ACID IN ISOLATED RAT HEPATOCYTES

B - Benzoic acid
H - Hippuric acid
BG - Benzoylglucuronide

TLC on Silica gel 60 (Mreck), in Solvent System G.
Quantitative results for benzoic acid metabolism by rat kidney cells and hepatocytes are given in figures 3.10 and 3.11 and they show an increase of hippuric acid and benzoylglucuronide with time accompanied by a concomitant decrease in benzoic acid.

The rate of synthesis of hippuric acid by rat hepatocytes was calculated to be $0.69 \text{ nmole} \ 10^6 \text{ cells}^{-1} \text{ min}^{-1}$ while the rate of metabolism of benzoic acid was calculated to be $0.75 \text{ nmole} \ 10^6 \text{ cells}^{-1} \text{ min}^{-1}$. At the end of the incubation, the ratio of hippuric acid to benzoylglucuronide to benzoic acid was found to be 18:1:1.

The rate of synthesis of hippuric acid by isolated kidney cells was calculated to be $0.25 \text{ nmole} \ \text{mg protein}^{-1} \text{ min}^{-1}$, while the rate of benzoic acid metabolism was calculated to be $0.26 \text{ nmole} \ \text{mg protein}^{-1} \text{ min}^{-1}$. Some synthesis of benzoylglucuronide was also observed and at the end of a one hour incubation only about 1% (5 nmole) of benzoic acid was converted to benzoylglucuronide. Interestingly, the autoradiogram from kidney cells also showed the presence of two extra radioactive spots which were not seen in the autoradiogram from liver cells. The nature of these spots has not been investigated, but their chromatographic behaviour compared to benzoic acid and benzoylglucuronide suggest that they may be hydroxy-benzoic acids and their conjugates and at the end of the incubation they accounted for about 2-3% (10-15 nmoles) of the benzoic acid substrate.

The presence of nephrons and tubules in the isolated kidney cell preparations presented a problem in the counting of cells as described. Consequently, quantitative results in metabolism studies using kidney cells are expressed in terms of the protein content of the kidney cells in the cell suspension.
METABOLISM OF $^{14}$C-BENZOIC ACID BY RAT HEPATOCYTES

FIGURE 3.11
METABOLISM OF $^{14}$C-BENZOIC ACID BY RAT KIDNEY CELLS

RESULTS ARE PLOTTED AS THE MEAN OF 2 EXPERIMENTS
In comparing the rate of metabolism of benzoic acid and hippuric acid synthesis by liver and kidney cells it can be calculated that the rates of hippuric acid synthesis were 0.46 and 0.25 \(\text{n mole mg protein}^{-1} \text{min}^{-1}\) for liver cells and kidney cells respectively while the rate of metabolism of benzoic acid was 0.5 \(\text{n mole mg protein}^{-1} \text{min}^{-1}\) for liver cells and 0.26 \(\text{n mole mg protein}^{-1} \text{min}^{-1}\) for kidney cells.

The results show that under the conditions of the isolation and metabolism studies as described, both liver cells and kidney cells are capable of metabolising benzoic acid by glycine conjugation and to a lesser extent by glucuronic acid conjugation, liver cells being twice as active as kidney cells. Also it is speculated that kidney cells may be capable of hydroxylating benzoic acid to form hydroxybenzoic acid. Intestinal mucosal cells do not appear to metabolise benzoic acid under the conditions of the experiment.

3.6 DISCUSSION

The results of the investigations involving benzoic acid were summarized in the previous sections and in the in vivo studies a significant result was that 80-90% of the \(^{14}\text{C}\) administered to the animals was excreted in the urine within 24 hours of dosing. Indeed a majority of the dose of benzoic acid (50 mg kg\(^{-1}\)) was excreted within about 8-9 hours by both the sheep (79% in 9 hours) and the pig (80% in 8 hours). However, previous reports have shown that the pig excreted only about 50% of the dose in 24 hours after oral administration of 50 mg kg\(^{-1}\) of benzoic acid (Bridges, et al., 1970).
This rapid excretion of metabolites of benzoic acid appears to be common in most animal species and some 50-100% of the dose was recovered in the urine within 24 hours and in most laboratory animals, including the rat, this 24 hour urinary excretion is in the order of 80-100% (Bridges et al., 1970). Unfortunately, little is known about the rates of excretion and 50-100% excretion in 24 hours represents a wide variation. It is likely that the rate of excretion of metabolites may show considerable species variation and may reflect the importance of different metabolic pathways in different species.

Little or no metabolites of benzoic acid were excreted in the faeces of these animals indicating that urinary excretion is the major pathway for the elimination of benzoic acid. Furthermore, it suggests that there is little or no biliary excretion of benzoic acid and its metabolites. Indeed it has been shown that biliary excretion of metabolites of benzoic acid is in the order of about 2% in several laboratory animals after intraperitoneal and intravenous injection (Abou-el-Makarem et al., 1967a).

Like the majority of mammalian species, the major metabolite of benzoic acid in the sheep and the pig was hippuric acid and traces of some benzoylglucuronide were also detected. This confirms previous observations that in the sheep (Martin, 1966) and the pig (Bridges et al., 1970) benzoic acid is converted mainly to hippuric acid and some benzoylglucuronide. However, the present investigation shows only traces of benzoylglucuronide synthesis and little or no free benzoic acid was detected in the urine, although previous investigations have shown the presence of considerable amounts of free benzoic acid (~10%) in the urine of the sheep and the pig, and benzoylglucuronide (2-15%) in the
urine of sheep, but only traces in the pig.

In the investigation of benzoic acid metabolism by the sheep (Martin 1966) several grams of benzoic acid together with glycine were administered to the sheep by infusion into the rumen or abomasum via permanent cannulae. A range of dose levels was used ranging from 0.2 g kg\(^{-1}\) to 1 g kg\(^{-1}\), and it was found that the amount of benzoyl-glucuronide increased with increasing dose of benzoic acid. Also it was believed that free benzoic acid found in the urine may have arisen from hydrolysis of benzoylglucuronide and hippuric acid since free glucuronic acid and glycine were also found in the urine of the sheep.

Bacterial decomposition of benzoylglucuronide, which is relatively labile was also assumed to be the major source of free benzoic acid in the urine of pig and several other species studied (Bridges, et al, 1970). Furthermore, it is possible that hydrolysis of hippuric acid may also give rise to free benzoic acid in the urine since the enzyme hippuricase which hydrolysates glycine conjugates is found to be present in kidneys of several species including the rabbit, pig, cow and chicken (Schachter, et al, 1955). Faecal microorganisms capable of hydrolysing hippurates have also been reported (Norman and Grupp 1955).

In the investigation of metabolism of benzoic acid in the sheep, a ruminant, it should be noted that benzoic acid is a normal constituent of sheep urine. It is the principle aromatic compound excreted in the urine of sheep (Martin, 1975). Its origin is unknown, but is believed to be dietary in nature.

The amount of benzoic acid excreted varies with the variety of food intake and has been determined to be 2.7-13.3 g kg\(^{-1}\) of food and it has been suggested that the urinary benzoic acid excreted
by ruminants may be derived from rumen microbial metabolism of 3-hydroxy derivatives of 3-phenyl-propionic or cinnamic acid. The latter being widely distributed in plants (Martin, 1975). Indeed, because of the ability of intestinal bacteria and mammalian tissues to synthesise benzoic acid from dietary precursors, hippuric acid is often found as a normal constituent of urine in many species.

It has been pointed out previously that in some species, increase in the dose of benzoic acid was accompanied by an increase in the relative amounts of benzoyleglucuronide excreted (Williams, 1959c, Bridges, et al, 1970). The rat appears to follow the same trend when dose levels ranged from 10 mg kg\(^{-1}\) to 400 mg kg\(^{-1}\), but the change in the relative amounts of hippuric acid and benzoyleglucuronide was not very significant. However, it is possible that more marked changes would be observed if higher dose levels were used in the investigation. This phenomenon is believed to be due to a combination of factors including the rate of glycine mobilization (Bray, et al, 1951) and availability of free glycine (Arnstein and Neuberger, 1951).

The dose level used in the present in vivo investigation was 50 mg kg\(^{-1}\). This is well below the toxic level of benzoic acid in these species. In the rat, LD\(_{50}\) of benzoic acid was 4.1 g kg\(^{-1}\) and in the sheep the lethal dose was 1.1-1.8 g kg\(^{-1}\) (Martin, 1966). Toxic symptoms in the sheep included severe muscular weakness accompanied by muscular tremors and anorexia. The lethal dose of benzoic acid in the pig is not known, however, no undue symptoms of stress were observed in the pig dosed with 50 mg kg\(^{-1}\) of benzoic acid and it was assumed that this was well below the toxic level.
The metabolism of benzoic acid was also studied in ureter cannulated rats in an attempt to investigate the kinetics of in vivo metabolism and the changes in the urinary metabolic profile with time.

The kinetics of benzoic acid metabolism have been studied previously in the rabbit (Bray, et al. 1951). The dose of benzoic acid administered was in the order of 1-2 g kg\(^{-1}\) and it was found that hippuric acid formation took place at a constant rate and that the acid was excreted immediately up to a limiting rate which appears to vary considerably between rabbits, but the average rate of formation and excretion of hippuric acid was calculated to be 147 mg hr\(^{-1}\). It was also found that the rate of hippuric acid synthesis may be increased when glycine was administered together with benzoic acid. On the other hand, synthesis of benzoylglucuronide was a first order process and the percentage of the dose of benzoic acid excreted as benzoylglucuronide is dependent not only upon the dose, but also upon whether or not glycine was administered. Indeed it has been suggested that the rate of hippuric acid formation is independent of the amount of benzoic acid in the body probably for amounts down to 25 mg kg\(^{-1}\), whereas, conjugation with glucuronic acid is dependent on the dose of benzoic acid.

For comparison with in vivo studies, the dose levels used in the present investigation were 50 mg kg\(^{-1}\) and 100 mg kg\(^{-1}\) and it was found that in the rat, the metabolism of benzoic acid approximates to a first order process. The synthesis and elimination of both hippuric acid and benzoylglucuronide were dose dependent and they were eliminated in the urine at a constant rate after an apparent initial lag phase. At the dose levels studied constant elimination of metabolites occurred for a period of about 1 hour and as the amount of metabolites eliminated in the urine increased, the rate of elimination decreased so that elimination
plateaued when the majority of the dose had been excreted. The constant rate of elimination of hippuric acid and benzoylglucuronide by the rat at 50 mg kg\(^{-1}\) was calculated to be 150 \(\mu g\) min\(^{-1}\) and 41.7 \(\mu g\) min\(^{-1}\) respectively; at 100 mg kg\(^{-1}\) the rates were 260 \(\mu g\) min\(^{-1}\) and 59.6 \(\mu g\) min\(^{-1}\) respectively.

Benzoic acid has a pKa of 4.17. It is a weak electrolyte and is equally well absorbed from the stomach or the intestine of rats (Schanker, et al, 1957, 1958). Thus it is interesting to note that within 10 minutes after the administration of benzoic acid into the duodenum, conjugated metabolites in the order of 2% of the dose were detected in the urine and after 2 hours some 70-80% of the dose was excreted in the urine.

This suggests that at the dose levels studied, absorption from the intestine, distribution, metabolism and excretion of benzoic acid were very rapid and not rate limiting. Indeed a limiting rate of excretion of metabolites of benzoic acid was not observed and it is likely that this would occur only when saturating levels of benzoic acid were administered. The limiting factor in the synthesis of hippuric acid is apparently the availability of free glycine (Arnstein and Neuberger, 1951, Hainline and Lewis, 1953). It has also been shown that the rate of hippuric acid synthesis is increased if glycine was added exogenously (Hainline and Lewis, 1953, Arnstein and Neuberger, 1951, Bray, et al 1953) and this was accompanied by a simultaneous decrease in glucuronide excretion.

The species difference in the degree of conjugation of benzoic acid with glucuronic acid has also been explained in terms of the availability of free glycine and the rate of glycine mobilization (Bray
et al. 1951, Hainline and Lewis 1953). Thus if glycine mobilization is relatively fast, large doses of benzoic acid will tend to be predominately excreted as hippuric acid, but if glycine mobilization is relatively slow, then glucuronide formation becomes an important aspect in the metabolism of benzoic acid.

In the present investigation the results suggest that the rate of glycine mobilization in the rat, sheep and pig may be relatively rapid, but unfortunately, the dose levels used may not be high enough to demonstrate benzoylglucuronide formation. At a dose of 500 mg kg\(^{-1}\) it has been reported that 31% of the dose of benzoic acid is excreted as benzoylglucuronide by the pig (Cronka 1924). The sheep also excretes significant amounts of benzoylglucuronide when high doses of benzoic acid are administered (Martin, 1966).

An interesting observation in the ureter cannulated rat experiments was that the ratio of hippuric acid to benzoylglucuronide found in the urine at different times after intraduodenal administration varies with time. Initially, considerably more hippuric acid was excreted than benzoylglucuronide, but as metabolism and excretion proceeds, the ratio of hippuric acid to benzoylglucuronide reaches a constant which is maintained for some time, and as the amount of benzoic acid in the body is reduced due to metabolism and elimination, the relative amount of hippuric acid to benzoylglucuronide excreted increases indicating that hippuric acid synthesis is very significant in the metabolism of benzoic acid and that benzoylglucuronide synthesis depends on the level of benzoic acid in the body.

The ratio of hippuric acid to benzoylglucuronide is always in favour of hippuric acid, that is hippuric acid excretion in the rat...
predominates. However, this variation in the ratio may reflect the importance of various pathways of metabolism of benzoic acid in vivo. Interestingly, the period of time that the ratio of hippuric acid to benzoyleglucuronide was constant also corresponded with the maximal excretion of metabolites of benzoic acid at the dose levels studied.

Studies on the kinetics of urinary elimination and the urinary metabolic profile of benzoic acid gives little information on the ability of various tissues to metabolise benzoic acid and the contribution of various tissues towards its overall metabolism.

In an attempt to investigate the tissue distribution in the ability to metabolise benzoic acid, isolated cell preparations from the rat were used. It was found that both the liver and the kidneys but not the intestine were capable of metabolising benzoic acid. Hippuric acid was the major metabolite in both cases and on the basis of protein content of the cell preparations, it can be demonstrated that rat liver cells are about twice as active as rat kidney cells in conjugating benzoic acid with glycine. The rate of hippuric acid synthesis in liver cells was calculated to be 0.46 \( \text{n mole mg}^{-1} \text{ min}^{-1} \) and for kidney cells 0.25 \( \text{n mole mg}^{-1} \text{ min}^{-1} \). Interestingly both liver cells and kidney cells from the rat show some ability to synthesise benzoyleglucuronide.

Previously, several workers, using crude tissue preparations were able to demonstrate that besides the liver, both the kidneys and the intestine were capable of metabolising benzoic acid to hippuric acid (see Section 3.2), but unfortunately no information was available on the ability of extrahepatic tissues to form benzoyleglucuronide.

The ability of the intestine of the rat to conjugate benzoic acid with glycine was demonstrated using everted gut sacs
(Strahl and Barr 1971) and intestinal slices and homogenates (Irjala, 1972). The synthesis of hippuric acid by the intestine was extremely low and is negligible compared with the liver and the kidneys. In the present investigation using isolated cells from the intestinal mucosa, no metabolism of benzoic acid was detected and it is likely that other factors may be involved in the metabolism of benzoic acid by the intestine and further investigation on these factors may be necessary in order to elucidate the involvement of the intestine in benzoic acid metabolism.

It is worth pointing out that an advantage of using isolated cell preparations rather than tissue slices and homogenate in the study of the metabolism of benzoic acid is that both metabolic pathways of glycine and glucuronic acid conjugation are demonstrated concurrently by the isolated cells and information on the relative importance of each pathway in the different tissues may be determined.

In the rat liver and kidneys, benzoylglucuronide formation is detectable and it is a minor pathway in the overall metabolism of benzoic acid.

The tissue distribution of glycine conjugation of benzoic acid found in various species studied suggest that it may have a significant role in the species difference in the relative amounts of hippuric acid and benzoylglucuronide excreted in the urine.

The dog, and ferret excrete a considerable amount of benzoylglucuronide following a single dose of benzoic acid compared with other mammalian species studied which excrete only a small amount of benzoylglucuronide. Indeed, the ferret shows an increase in benzoylglucuronide excretion with increase in dose of benzoic acid (Bridges, et al, 1970).
The cat and the dog have also been shown to exhibit greater hippuric acid synthetic activity in the kidneys than in the liver (Irjala, 1972). Although information on tissue distribution of glycine conjugation in the ferret is not available, our preliminary investigations on benzoic acid metabolism by isolated cells from the ferret have indicated that glycine conjugation in ferret liver is very low, but there was a significant level of benzoylglucuronide synthesis, and it is likely that ferret kidneys may show considerable glycine conjugation of benzoic acid.

These observations suggest that a supplementary explanation for species differences in the amount of hippuric acid and benzoylglucuronide excreted in the urine may be proposed.

After a single dose of benzoic acid, the liver is the major organ of metabolism first encountered and species with active hepatic glycine conjugating mechanisms will tend to metabolise benzoic acid to hippuric acid, while benzoylglucuronide may be the major hepatic metabolite of benzoic acid in species such as the dog, the cat and perhaps the ferret where glycine conjugation of benzoic acid in the liver is absent or low. Benzoic acid is a relatively polar material, therefore, it is likely that unmetabolised benzoic acid may be cleared from the liver and passed to the kidneys where further metabolism by glycine conjugation may take place prior to elimination from the body. Indeed fractional metabolism of benzoic acid by the liver and the kidneys has been demonstrated in the rabbit (Wan and Rigelman, 1972). Glycine conjugation of benzoic acid in tissues other than the liver and the kidneys has not been demonstrated except in the intestine where hippuric acid synthesis was very low (Strahl and Barr, 1971, Irjala, 1972) so that fractional metabolism of benzoic acid by the liver and the kidneys...
together with the availability of glycine and the rates of glycine mobilization may be involved in the species difference in the metabolism of benzoic acid.

Interestingly, the fruit bat which excretes only benzoyl-glucuronide shows an apparent absence of hippuric acid synthesis in both its liver and kidneys (Babunm et al 1973).
ADDENDUM

Subsequent to the completion of Chapter 3, some preliminary investigations on the metabolism of benzoic acid by isolated cells from the dog and the ferret were performed. The methodology was essentially as described in Section 3.5 and quantitative results for the metabolism of benzoic acid by ferret and dog kidney cells and hepatocytes are summarized in Figure 3.12 and 3.13 respectively.

In the kidney cells from the dog the rate of formation of hippuric acid was calculated to be 0.56 \( \text{nmol mg protein}^{-1} \text{ min}^{-1} \) while in the ferret kidney cells this was calculated to be 0.29 \( \text{nmol mg protein}^{-1} \text{ min}^{-1} \). The protein concentration of the kidney cell suspensions from the dog and the ferret were 5.7 mg ml\(^{-1}\) and 11.5 mg ml\(^{-1}\) respectively. Synthesis of benzoylglucuronide from kidney cells was found to be negligible and after 1 hour of incubation benzoylglucuronide accounted for \(<2\%\ (\leq 10 \text{ nmol})\) and \(<1\%\ (\leq 5 \text{ nmol})\) of the benzoic acid substrate from the dog and the ferret respectively. Interestingly the kidney cells from both species gave three minor metabolites (\(\approx 2\%\)) of benzoic acid which have yet to be identified, but their chromatographic behaviour compared to hippuric acid and benzoylglucuronide suggests that they may be hydroxybenzoic acid conjugates.

Compared to the kidney cells, metabolism of benzoic acid by liver cells is very low. In the dog liver cells only about 5\% (25 \( \text{nmol} \)) of the benzoic acid substrate was metabolised after 1 hour of incubation, and the metabolites were benzoylglucuronide and hippuric acid and they were found to be in the ratio of about 4:1. The rate of benzoylglucuronide synthesis by dog liver cells was calculated to be 1.0 \( \text{nmole mg protein}^{-1} \text{ hr}^{-1} \). In the ferret liver cells about 10\%
METABOLISM OF $^{14}$C-BENZOIC ACID BY ISOLATED CELLS FROM THE FERRET

LIVER CELLS

KIDNEY CELLS

$\mu$ mole

BENZOIC ACID
BENZOYLGLUCURONIDE

BENZOIC ACID
HIPPURIC ACID

10 20 30 40 50 60 mins

10 20 30 40 50 60 mins
METABOLISM OF $^{14}$C-BENZOIC ACID BY ISOLATED CELLS FROM THE DOG

LIVER CELLS

KIDNEY CELLS

- BENZOIC ACID
- BENZOYLGLUCURONIDE

- BENZOIC ACID
- HIPPURIC ACID

µ mole

mins
(50 n mole) of the benzoic acid substrate was converted to benzoylglucuronide in 1 hour while hippuric acid formation was negligible, accounting for \(< 0.5% (\approx 2.5 \text{n mole}). The rate of benzoylglucuronide synthesis in ferret liver cells was calculated to be \(0.54 \text{n mole mg protein}^{-1} \text{hr}^{-1}\).

The hepatic cellular protein concentration in the dog liver cell suspension was 3.9 mg ml\(^{-1}\) while that of the ferret liver cell suspension was 10.7 mg ml\(^{-1}\).

These results showed that in both species the kidneys are the major organ involved in the metabolism of benzoic acid by glycine conjugation and these observations may be presented as additional support for the hypothesis that a contributory factor in the species variation of metabolism of benzoic acid is in the tissue distribution of glycine conjugation of benzoic acid in different species as discussed in Section 3.6.
CHAPTER 4

PHENOL
4.1 INTRODUCTION

Phenol (C₆H₅O) is a colourless, volatile, crystalline material, or sometimes a white crystalline mass, with a characteristic odour. It tends to redden on exposure to air and light, particularly in an alkaline atmosphere. It has a M.W. of 94.11 and melts at 35-40°C depending on the purity. Commercial preparations of phenol generally contain water and some traces of cresols.

It has a flash point at 79°C and is easily liquified in the presence of water (about 8%). It's pKa at 25°C is 10.0 and it is soluble in water. The pH of an aqueous solution of phenol is about 6.0. It is soluble in several organic solvents including alcohol, chloroform, ether, benzene and carbon disulphide, but is almost insoluble in petroleum ether.

It occurs naturally and is found as a normal constituent of animal urine, e.g. in man about 10 mg day⁻¹ is excreted in conjugated forms (Parke 1968). The diet is the main source of phenols in animal urine and results from microbial decomposition of food residues in the digestive tract, e.g. microbial decomposition of tyrosine gives rise to phenol.

Phenol has many uses and in industry it is often the starting material for the manufacture of a great many products such as dyes, plastics, resins, antiseptics and therapeutic agents.

It has been used as a general disinfectant either as a solution or mixed with shaled lime. Medically phenol has been used as a topical anaesthetic and antiseptic. In veterinary medicine it has been prescribed as an intestinal antiseptic.
Phenol has an oral LD$_{50}$ of 530 mg kg$^{-1}$ in rats. In man, phenol is extremely toxic and ingestion of phenol even in small amounts causes intense burning of the mouth and throat, followed by marked abdominal pains and distress. Other symptoms may include nausea, vomiting, circulatory collapse, paralysis, convulsion and coma. Death from phenol ingestion is generally due to respiratory failure and sometimes cardiac arrest.

Poisoning by absorption through the skin is also possible and if exposure is sufficiently severe, local contact with skin or mucous membrane may induce gangrene or corrosion. Chronic poisoning with renal and hepatic damage may occur from industrial contact. The fatal oral doses of phenol for adults have ranged from 1 g - 10 g, for infants from 50 mg - 500 mg.

It has been stated that phenol is extremely toxic and Oehme (1969) conducted toxicity studies in mice with phenylsulphate, phenylglucuronide and phenol. It was found that the toxicity of phenylglucuronide is about one-fifth that of phenol while phenylsulphate is approximately one-eighth as toxic as phenol. It is apparent from this work that conjugation greatly reduced the toxicity of phenol and is an example of conjugation as a true detoxication mechanism.

4.2 METABOLISM OF PHENOL

The metabolic fate of phenol has been studied for over a century and the biological reactions involved in the urinary excretion of phenol were initially reported by Baumann, (1876a, 1876b, 1876c). In this series of papers it was reported that phenol was excreted as a sulphuric acid conjugate in both man and dog. Oxidation of phenol to
catechol and quinol was also reported a few years later (Baumann and Preusse 1879a and 1879b). Indeed they suggested that the dark colour of urine excreted by dogs following phenol administration may be a result of further oxidation of catechol and quinol. Kulz (1890) reported the presence of phenylglucuronide in rabbit urine following the injection of phenol.

The metabolites of phenol in most species are now known to be phenylglucuronide, phenylsulphate, some conjugated quinol and trace amounts of conjugated catechol.

The synthesis of phenylglucuronide and phenylsulphate is influenced by a number of factors including the dose, the diet and the route of administration.

For example, in the rabbit it appears that glucuronic and conjugation of phenol is considerably higher than sulphate conjugation when the dose of phenol is relatively large, so that for doses of about 125 mg - 250 mg kg⁻¹ the percentage of phenol excreted as sulphate is about 20% of the dose while glucuronic acid conjugation accounts for about 70% in a 24 hour urine. However, at doses lower than 100 mg kg⁻¹ the amount of sulphate conjugate rises with falling dose level (Williams, 1938). Indeed it appears that at 50-60 mg kg⁻¹ the amount of glucuronide and sulphate conjugates of phenol excreted by the rabbit is about equal (Parke and Williams, 1953), whereas at 25 mg kg⁻¹ sulphate conjugation exceeds that of glucuronic acid conjugation (Capel, et al, 1972). This dose dependency in the elimination of phenol conjugates has also been demonstrated in the sheep. In a Kinetic study of metabolism of phenol by the sheep (Maylin, 1971) it was shown that phenol was metabolised and eliminated in the urine as phenylglucuronide and phenylsulphate and it was observed that
the relative amounts of these conjugates was dose dependent. Thus at low doses phenylsulphate was the major urinary metabolite, whereas at higher doses phenylglucuronide became the major metabolite. Free phenol, in the order of about 5%, was also found in the urine.

The nature of the diet can play a major role in the metabolism of foreign compounds. In the case of phenol it has been shown that glucuronic acid conjugation is a first order process and is proportional to the body level of phenol, whereas sulphate conjugation is independent of the body level of phenol, but dependent upon the availability of sulphate (Bray, et al., 1952). Thus if sulphate precursors such as L-cysteine and sodium sulphate are added simultaneously to the diet of animals dosed with phenol, sulphate conjugation will be increased while glucuronic acid conjugation is correspondingly decreased. For example, at a dose of 250 mg kg\(^{-1}\) phenylsulphate excreted by rabbit in the 24 hour urine, following administration of phenol in the absence and the presence of sulphate precursors in the diet is 15% and 30% of the dose respectively (Bray, et al., 1952b).

It is well known that the route of administration of a compound may play an important part in the metabolic profile of that compound. Indeed Palkan and Wipple, (1922) have pointed out that the metabolism of phenol after oral administration is different from that after intravenous administration. They found both free and conjugated phenol following intravenous doses, but only conjugated phenol was detected in blood following the equivalent dose administered orally. Relatively large oral doses were required to produce significant concentrations of free phenol. Further investigations concerning this aspect of phenol metabolism have not been reported. However, studies describing the influence of the route of administration on metabolism have been reported
for other compounds.

Interestingly, when Garton and Williams (1949) investigated the metabolic fate of phenylsulphate and phenylglucuronide, they found that phenylsulphate was excreted rapidly following oral and intravenous administration. Approximately 90% of the dose was recovered in the urine within 24 hours. However, rabbit when injected with phenylglucuronide excreted the dose quantitatively within 24 hours, but following oral administration only 50% of the dose was recovered. A considerable increase in ethereal sulphate output occurred concurrently with the decreased excretion of phenylglucuronide. Garton and Williams (1949) postulated that the phenylglucuronide is poorly absorbed in the intestine and consequently hydrolysis of the glucuronide by gut microorganisms gives rise to phenol which is then absorbed and metabolised by conjugation with both glucuronic acid and sulphate.

In a study of the fate of phenol in various species (Capel, et al., 1972), it was found that when given orally at 25 mg kg\(^{-1}\), the majority of species studied excreted four metabolites, namely, the sulphates and glucuronides of phenol and quinol. Species which excreted four metabolites included man and several rodent species such as the rat, mouse, hamster, guinea pig, lemming, jerboa and gerbil. In all cases phenylsulphate and phenylglucuronide were the major metabolites while conjugates of quinol occurred only in small amounts.

In man, the total quinol excreted in 24 hours after administration is about 1% of the phenol metabolites in the urine. In the rat and guinea pig, the amount of quinol excreted was in the order of 3-5% while in other rodent species, the ability to oxidise phenol to quinol appears to be quite significant and is in the order of 20-30%.
In some species, only three metabolites were observed, for example, in the squirrel monkey and Capuchin monkey, quinol sulphate is apparently absent while quinol glucuronide appears to be absent from the ferret, dog, hedgehog and rabbit. However, it is likely that these metabolites were present, but the levels were too low for detection by the methods used.

No quinol conjugates were detected in the rhesus monkey, fruit bat and chicken and it is likely that these species do not oxidize phenol to quinol.

Capel et al., (1972) were able to demonstrate the species variation in the amount of sulphate and glucuronide conjugates of phenol excreted. Thus at 25 mg kg\(^{-1}\), it appears that sulphate conjugation is more effective than glucuronic acid conjugation in the rat, mouse, jerboa, gerbil, chicken, hedgehog, dog, rhesus monkey and man (0.01 mg kg\(^{-1}\)), while the reverse appears to be the case with the guinea pig, lemming, hamster, fruit bat, capuchin monkey and squirrel monkey. The two conjugation mechanisms appear to be equally effective in the ferret and the rabbit.

The cat and the pig are of special interest in that the cat appears to excrete only sulphate conjugates while the pig appears to form only phenylglucuronide. Thus it appears that they have defective conjugation mechanisms with respect to orally administered phenol. The cat being defective in glucuronic acid conjugation while the pig is defective in sulphate conjugation. However, activities of these conjugation mechanisms are not entirely absent since trace amounts of glucuronide and sulphate conjugates of phenol are detectable in cat and pig urine respectively.
The metabolism of phenol was also investigated in cat-like animals by French et al. (1974) and it was shown that the lion, civet and gannet excreted orally administered phenol at 25 mg kg\(^{-1}\) almost entirely as phenylsulphate indicating that they may have a defective glucuronic acid conjugation mechanism similar to that of the domestic cat. Interestingly, a phosphate conjugate has been reported as a urinary metabolite in cats dosed with phenol (Capel et al., 1974b).

The tissue distribution in the ability to metabolise phenol has been investigated and earlier work suggested that sulphate conjugation of phenol occurs both in the liver and in the intestine (Williams, 1959).

That the liver is the major organ in the metabolism of foreign compounds is well known, but the ability of the intestine to conjugate phenols has been demonstrated. For example, intestinal sulphate conjugation of isoprenaline has been shown to be the major route of sulphate conjugation of orally administered isoprenaline (George et al., 1974). Indeed this represents an example of differences in metabolism due to route of administration. When given intravenously isoprenaline is excreted mainly as free isoprenaline, whereas after oral dosing excretion is largely as an etheral sulphate conjugate (Conolly et al., 1972).

Glucuronic acid conjugation of 1-naphthol by the intestine has also been demonstrated recently by means of an in situ intestinal loop technique where blood draining from the venous supply of the perfused loop is collected and assayed for 1-naphthylglucuronide (Bock and Winne, 1974). By means of exteriorized cannulae, phenol and phenol conjugates in the blood plasma from the portal vein and the hepatic vein together with the bile may be monitored and Maylin (1971) was able to demonstrate that in the sheep, both the liver and the intestine were involved in the metabolism of phenol. Thus phenylglucuronide, phenylsulphate
and some free phenol were detected in the hepatic venous plasma following infusion of phenol into the cannulated mesenteric vein, while free phenol and phenol conjugates were detected in the portal vein as well as the hepatic vein following administration of phenol via intestinal cannulae. No biliary excretion was observed, so that enterohepatic circulation is not significant in the metabolism of phenol by the sheep. Interestingly, it was noted that after administration of phenol, the relative proportions of phenol, phenylglucuronide and phenylsulphate found in the plasma of both hepatic and portal venous blood changes with time.

The ability of the intestine to conjugate phenols may be of considerable importance in the detoxication of phenols. Indeed, recently it has been proposed that the gastrointestinal tract plays an important part in the detoxication of phenol, so much so that the liver is not essential in the detoxication of phenol (Powell et al. 1974). Evidence for this proposition included the observation that whole body autoradiograms of animals which had received $^{14}$C phenol orally or intraperitoneally showed that the level of isotope in the liver did not at any time exceed that of the blood suggesting that the liver has no ability to concentrate either phenol or its metabolites and this was due either to the limiting uptake of phenol by the liver or a rapid turnover of phenol in the liver. Furthermore, it was demonstrated that in vitro metabolism of phenol by isolated gut preparations resulted in the synthesis of phenylsulphate (5%) and phenylglucuronide (95%). No unchanged phenol was detected. In situ intestinal loop experiments on wholly anaesthetised rats showed that only conjugates of phenol were detected in the portal blood after administration of phenol in the intestinal loop by a continuous perfusion technique. Phenol conjugates were also detected in the intestinal perfusate and after 2 hours, no unchanged phenol was detected and phenylsulphate (72%) and phenylglucuronide (28%)
FIGURE 4

COMPOUNDS IDENTIFIED AS METABOLITES OF PHENOL

PHENOLSULPHATE

PHENYLGLUCURONIDE

CATECHOL CONJUGATES
(TRACE METABOLITES)

PHENOL

QUINOLSULPHATE

QUINOLGLUCURONIDE
were the metabolites present. Thus it was suggested that free phenol in the intestine is transported as conjugates so that the role of the liver is minimal in the detoxication of orally ingested phenol. However, when the gastrointestinal tract was by-passed as in intravenous administration of phenol, detoxication of phenol by the liver assumes its major importance.

Some information is also available on the ability of tissues other than the liver and the intestine to metabolise phenols. For example, phenol sulphotransferase activity towards p-nitrophenol and 4-methylumbelliferone in rabbit lung soluble fraction has been demonstrated (Hook and Bend, 1976) and sulphate conjugation of a pulmonary metabolite of benzpyrene has also been identified recently (Cohen, et al, 1976). It is likely that where sulphate and glucuronic acid conjugation mechanisms are present, conjugation of phenol may take place.

4.3 IN VIVO INVESTIGATIONS OF PHENOL METABOLISM

\[ [U-^{14}C] \text{phenol, } 25 \text{ mg kg}^{-1} \] was administered to the animal as described in Chapter 2. Urine and faeces were collected and assayed as described.

The \( R_f \) values and methods of detection of phenol and some of its metabolites are given in Table 4.1 and the results of orally administered \( ^{14}C \)-phenol metabolism in the sheep, the pig and the rat are summarized in Tables 4.2, 4.3, 4.4.

The tables show that after oral administration of \( ^{14}C \)-phenol a significant proportion of the dose was excreted by these animals.
Table 4.1

**R_f-Values and Colour Reactions of Phenol and Some of its Possible Metabolites**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R_f Values in Solvent Systems</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paper Chromatography</td>
</tr>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Phenol</td>
<td>0.92-0.96</td>
</tr>
<tr>
<td>Catechol</td>
<td>st.</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>st.</td>
</tr>
<tr>
<td>Quinol</td>
<td>st.</td>
</tr>
<tr>
<td>Phenylglucuronide</td>
<td>0.36-0.40</td>
</tr>
<tr>
<td>Phenylsulphate</td>
<td>0.65-0.67</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Colour Reactions with Spray Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>Phenol</td>
<td>-</td>
</tr>
<tr>
<td>Catechol</td>
<td>-</td>
</tr>
<tr>
<td>Resorcinol</td>
<td>-</td>
</tr>
<tr>
<td>Quinol</td>
<td>-</td>
</tr>
<tr>
<td>Phenylglucuronide</td>
<td>Blue</td>
</tr>
<tr>
<td>Phenylsulphate</td>
<td>-</td>
</tr>
</tbody>
</table>

PC on Whatman No. 1 Paper
TLC on Silica gel 60 (Merck)

* Yellow after a few minutes
st. Streak
Table 4.2

Urinary excretion of $^{14}$C-Phenol by Sheep and Pig

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose mg Kg$^{-1}$</th>
<th>Time (hr.)</th>
<th>Volume of Urine (ml)</th>
<th>% of Administered Dose Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep 1</td>
<td>25</td>
<td>0-3</td>
<td>178</td>
<td>46.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-5</td>
<td>228</td>
<td>33.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-8</td>
<td>235</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-24</td>
<td>1060</td>
<td>2.4</td>
</tr>
<tr>
<td>Sheep 2</td>
<td>25</td>
<td>0-2</td>
<td>90</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-4</td>
<td>36</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-6</td>
<td>215</td>
<td>79.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-8</td>
<td>105</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-24</td>
<td>300</td>
<td>1.5</td>
</tr>
<tr>
<td>Pig 1</td>
<td>25</td>
<td>0-3</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-5</td>
<td>222</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-8</td>
<td>170</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-24</td>
<td>315</td>
<td>3.0</td>
</tr>
<tr>
<td>Pig 2</td>
<td>25</td>
<td>0-2</td>
<td>90</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2-4</td>
<td>60</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-8</td>
<td>120</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-19</td>
<td>410</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19-21</td>
<td>60</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21-24</td>
<td>112</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Table 4.3

**Urinary Excretion of Metabolites of $^{14}$C-Phenol**

<table>
<thead>
<tr>
<th>Species</th>
<th>% Dose in Urine at 24 hr.</th>
<th>% of 8 Hour Excretion as</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 hr.</td>
<td>Glucuronide</td>
</tr>
<tr>
<td>Sheep 1</td>
<td>87.7</td>
<td>49.0</td>
</tr>
<tr>
<td>2</td>
<td>85.9</td>
<td>53.2</td>
</tr>
<tr>
<td>Pig 1</td>
<td>86.1</td>
<td>99.2</td>
</tr>
<tr>
<td>2</td>
<td>85.2</td>
<td>81.8</td>
</tr>
<tr>
<td>Rat*</td>
<td>96.5</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>(93.9-103.0)</td>
<td>(35.5-56.5)</td>
</tr>
</tbody>
</table>

* - Results are obtained as the mean from 4 animals and the range is given in parentheses
An unidentified sulphate conjugate (0.9%) was found in the urine of Pig 2, probably catechol sulphate
nd. - Not detected

Table 4.4

**Urinary Metabolites of $^{14}$C-Phenol at 25 mg Kg$^{-1}$**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Sheep</th>
<th>Pig</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Phenylglucuronide</td>
<td>47.5</td>
<td>50.3</td>
<td>89.0</td>
</tr>
<tr>
<td>Quinol glucuronide</td>
<td>1.5</td>
<td>2.9</td>
<td>10.2</td>
</tr>
<tr>
<td>Phenylsulphate</td>
<td>36.5</td>
<td>29.5</td>
<td>nd.</td>
</tr>
<tr>
<td>Quinol sulphate</td>
<td>3.7</td>
<td>4.2</td>
<td>nd.</td>
</tr>
<tr>
<td>Phenylphosphate</td>
<td>8.0</td>
<td>13.4</td>
<td>nd.</td>
</tr>
<tr>
<td>Quinol phosphate</td>
<td>nd.</td>
<td>2.9</td>
<td>nd.</td>
</tr>
</tbody>
</table>

Results are expressed as % of 8hr. urinary excretion. In the Rat the results are given as the mean of 4 aminals and the range is given in parentheses.
nd. - Not detected
relatively rapidly. At 8 hours after administration some 85% of the
dose was recovered from sheep urine, 84% from pig urine and 90% from
rat urine, and at 24 hours the elimination of radioactivity was 87%,
86% and 97% for the sheep, pig and rat respectively. Negligible amounts
of radioactivity (less than 0.5% of the administered dose) were excreted
in the faeces suggesting that urinary elimination is the major route
for the excretion of orally administered phenol.

Identification of conjugates of phase I metabolites of
phenol was based on differential enzymic hydrolysis and solvent extraction,
while identification of phase I metabolites was by thin layer chromatography
and reverse isotope dilution analysis. The methods used were as described
in Chapter 2.

Chromatography, followed by radiochromatogram scanning
and "strip counting" of urine samples from the species studied showed
the presence of two major peaks (I and II) for the sheep and the rat,
which corresponded to phenylglucuronide and phenylsulphate respectively.
In the pig essentially only one peak (I) was observed which corresponded
to phenylglucuronide (see Figures 4.1, 4.2). Colour reactions with spray
reagent (a) showed that I gave a positive naphthoresorcinol reaction
indicating the presence of glucuronides. Spray reagent (d), a coupling
reagent for phenols and (e) a typical spray reagent for reducing centres
such as free aromatic hydroxyl groups, gave positive reactions at the
areas of the chromatograms associated with I and II, indicating that
components of I and II may also consist of hydroxy-metabolites of phenol
such as quinol and/or catechol conjugates. Indeed when pig urine was
chromatographed in solvent system G a radioactive area on the thin layer
chromatogram which had an Rf value of 0.34-0.38 (Ia) gave a positive
reaction with spray reagents (a), (d) and (e). When sheep urine was
PAPER CHROMATOGRAM OF URINE FROM ANIMALS DOSED WITH $^{14}$C-PHENOL

**Sheep**

I = GLUCURONIDES
II = SULPHATES

"STRIP COUNTING" - SEE TEXT
THIN LAYER RADIOCHROMATOGRAM SCAN OF URINE FROM ANIMALS DOSED WITH $^{14}$C-PHENOL

RAT

SHEEP

PIG

I = GLUCURONIDES
II = SULPHATES
similarly chromatographed an additional radioactive area with $R_f$ 0.66-0.68 (IIa) was observed and gave faint but positive reactions with spray reagents (d) and (e). Ia and IIa were subsequently determined to be quinolglucuronide and quinolsulphate respectively.

Following enzymic hydrolysis, thin layer chromatography of urine samples and the hydrolysates, together with radiochromatogram autoradiography revealed that the dark spots on the autoradiogram corresponding to peak I were lost after hydrolysis with $\beta$-glucuronidase, while the dark spots corresponding to peak II were hydrolysed by arylsulphatase (Figures 4.3, 4.4 and 4.5). It should be noted that after enzymic hydrolysis, phenol which is extremely volatile is the major radioactive component in the hydrolysate. Consequently detection of phenol by chromatography and autoradiography as described, gives poor results. Indeed Figures 4.3, 4.4 and 4.5 which are autoradiograms of thin layer chromatograms developed in solvent system G of urine which has been hydrolysed by $\beta$-glucuronidase and arylsulphatase showed little or no phenol ($R_f$ 0.96-1.0) at the solvent front, radiolabelled phenol being lost from the thin layer plate during the drying of the developed chromatogram and during the autoradiography procedure. Also, it should be noted that in some cases (see Figures 4.4 and 4.5), complete hydrolysis of glucuronic acid conjugates of metabolites of phenol with ketodase was not observed. This was believed to be due to the presence of an inhibitor in the urine samples, however, if additional enzyme was added to the hydrolysate and reincubated as described, complete hydrolysis of the glucuronides may be achieved.

Quantitative estimation of radioactivity in etheral extracts showed that after $\beta$-glucuronidase hydrolysis of 8 hour urine samples some 50%, 90% and 46% of the radioactivity of the hydrolysates from
FIGURE 4.3

ENZYMIC HYDROLYSIS OF URINE FROM THE SHEEP DOSED WITH $^{14}$C-PHENOL

A - Untreated Urine
B - "Control"
C - β-Glucuronidase Hydrolysis
D - Arylsulphatase Hydrolysis

I - Glucuronides
II - Sulphates
FIGURE 4.4

ENZYMIC HYDROLYSIS OF URINE FROM THE PIG DOSED WITH $^{14}$C-PHENOL

A - Untreated Urine
B - "Control"
C - β-Glucuronidase Hydrolysis *Incomplete hydrolysis of I
D - Arylsulphatase Hydrolysis

I - Glucuronides
II - Sulphates
P - Phenol
ENZYMIC HYDROLYSIS OF URINE FROM THE RAT DOSED WITH $^{14}$C-PHENOL

A - Untreated Urine
B - "Control"
C - β-Glucuronidase Hydrolysis
D - Arylsulphatase Hydrolysis

*Incomplete hydrolysis of I

I - Glucuronides
II - Sulphates
sheep, pig and rat urine respectively was extracted into the ether phase, while the amount extracted after arylsulphatase hydrolysis was 38%, 2% and 56% from the sheep, pig and rat respectively. When the 8 hour urine samples were incubated with acid phosphatase at pH 4.5 and the hydrolysates were solvent extracted as described, the amount of radioactivity extracted into the ether phase was found to be 12% from the sheep urine, while extraction from the pig and rat urine hydrolysates gave essentially control levels of radioactivity. Control values ranged from 0-4% for all enzymic hydrolysis experiments and were taken into account when results from hydrolysis experiments were calculated.

Negligible amounts of radioactivity (<1%) were extracted into the ether phase when untreated urine from the animals was solvent extracted, indicating that essentially no unconjugated metabolites were present in the urine.

From the enzyme hydrolysis experiments it can be deduced that orally administered phenol at 25 mg kg$^{-1}$ is excreted as glucuronides and sulphates. In the rat 46% of the 8 hour urinary metabolites was found to be glucuronides and 56% to be sulphates, while in the sheep, the corresponding figures are 50% and 38%. Very low levels of sulphate conjugates of phenol metabolites were observed in the pig and at 25 mg kg$^{-1}$ only 2% of the urinary metabolites was identified as sulphate conjugates, while glucuronic acid conjugates accounted for 90% of the urinary metabolites. Interestingly some 12% of the urinary metabolites from the sheep dosed with phenol was apparently excreted conjugated with phosphate. Phosphate conjugates of phenol metabolites were not detected in the urine of the pig and the rat.
FIGURE 4.6

RELATIVE % OF METABOLITES OF PHENOL FROM THE SHEEP AT DIFFERENT TIMES
In sheep urine it was also observed that there were changes in the pattern of metabolites found in the urine at various times after dosing and it was found that glucuronides were the major urinary metabolites at 3 hours after oral dosing, but subsequently sulphates were the major metabolites. However, at 8 hours the major urinary metabolites were conjugated with glucuronic acid (figure 4.6).

Studies on the ether extracts as described in Chapter 2 showed that the major radiolabelled component was phenol. Quinol was also present and was found to be about 8% in the sheep and the pig, but in the rat it was only 3%. Catechol was not found to be present by reverse isotope dilution analysis and the possible presence of resorcinol was not determined.

The 8 hour urinary metabolites of orally administered phenol which have been identified by the methods described are summarized in table 4.4. Identification of phosphate conjugates is incomplete, but for discussion see Section 4.6.

4.4 METABOLISM OF PHENOL IN URETER CANNULATED RATS

The ureters of anaesthetised rats (250 g) were cannulated and the animals dosed with $\left[U-^{14}\text{C}\right]$-phenol at 12.5 mg kg$^{-1}$ and 25 mg kg$^{-1}$ by intraduodenal injection as described.

Urine samples were collected at 10 minute intervals for a period of 2 hours and the samples were assayed for radioactivity as described. The metabolic profile of the 10 minute samples was determined by thin layer chromatography of aliquots (20-40 µl) of urine on silica gel 60 pre-coated plates (Merck) in solvent system G followed by
radiochromatogram autoradiography. The areas of the chromatograms corresponding to glucuronides ($R_f$ 0.34-0.49) and sulphates ($R_f$ 0.66-0.72) were located and compared with the corresponding dark spots on the autoradiogram. These areas were marked on the radiochromatogram cut out and placed into scintillation vials and counted as described. The radioactivity associated with each of these areas was expressed as a percentage of the radioactivity recovered from the radiochromatogram. No unconjugated metabolites were detected since ether extraction of the urine recovered little or no radioactivity in the ether fraction.

The rate of urinary elimination of radioactivity after intraduodenal administration and the percentage of the dose excreted at each time interval are summarized in Figures 4.7 and 4.8 respectively. Metabolic profiles of 10 minute urine samples are illustrated in Figure 4.9 and the rates of excretion of the sulphate and glucuronide conjugates of metabolites of phenol are shown in Figures 4.10, 4.11 and 4.12.

The results in Figure 4.7 show that at the dose levels studied 2-3% of the dose was recovered in the urine within 10 minutes after administration and that at 12.5 mg kg$^{-1}$ about 75% of the dose was recovered in 2 hours, while at 25 mg kg$^{-1}$ about 70% of the dose was recovered in 2 hours. Furthermore, it can be calculated from the linear portion of the curve that the rate of elimination of radioactivity is in the order of 11.7% of the dose per 10 minutes when the dose of phenol was 12.5 mg kg$^{-1}$ while at 25 mg kg$^{-1}$ the rate was 9.5% of the dose per 10 minutes. This would indicate that the relative rate of elimination of phenol after intraduodenal administration is dose dependent and decreases with increasing dose of phenol. In Figure 4.8, which shows the percentage of the dose excreted at each time interval, it can be seen that at 12.5 mg kg$^{-1}$ maximum excretion occurs between 20-30 minutes while at 25 mg kg$^{-1}$
FIGURE 4.7

URINARY EXCRETION OF RADIOACTIVITY AFTER ¹⁴C-PHENOL ADMINISTRATION IN RATS

RESULTS ARE PLOTTED AS THE MEAN FROM 3-4 ANIMALS AND ERROR BARS INDICATE THE RANGE
FIGURE 4.8

% 14C EXCRETED AT TIMED INTERVALS AFTER ADMINISTRATION OF 14C-PHENOL IN RATS

RESULTS ARE PLOTTED AS THE MEAN FROM 3-4 ANIMALS AND VERTICAL BARS INDICATE THE RANGE
FIGURE 4.9

% $^{14}$C EXCRETED AS SULPHATE AND GLUCURONIDE CONJUGATES OF METABOLITES OF PHENOL AT TIMED INTERVALS

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
FIGURE 4.10

URINARY ELIMINATION OF SULPHATE AND GLUCURONIDE CONJUGATES OF METABOLITES OF PHENOL

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
FIGURE 4.11

THE RATE OF URINARY ELIMINATION OF PHENYLGLUCURONIDE

○ PHENOL at 12.5 mg kg⁻¹ (33.2 μmole animal⁻¹)

○ PHENOL at 25 mg kg⁻¹ (66.4 μmole animal⁻¹)

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS.
FIGURE 4.12

THE RATE OF URINARY ELIMINATION OF PHENYL SULPHATE

○ PHENOL at 12.5 mg kg\(^{-1}\) (332 \(\mu\)moles animal\(^{-1}\))

○ PHENOL at 25 mg kg\(^{-1}\) (664 \(\mu\)moles animal\(^{-1}\))

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
maximum excretion occurs between 30-40 minutes.

At both dose levels the major metabolites were phenyl-glucuronide and phenylsulphate. Quinol glucuronide and quinolsulphate were also detected, but only as very minor metabolites. The rate of elimination of glucuronide and sulphate metabolites of phenol were calculated to be 0.23 μmole min⁻¹ and 0.18 μmole min⁻¹ respectively when the rats (250 g) were administered 33.2 μmole (12.5 mg kg⁻¹) of phenol. At a dose of 66.4 μmole (25 mg kg⁻¹) the rate of glucuronide elimination was 0.35 μmole min⁻¹ and the rate of sulphate elimination was 0.3 μmole min⁻¹ (Figures 4.11 and 4.12).

These figures show that the metabolism of phenol follows the kinetics of a first order process. The rates of elimination of both glucuronide and sulphate were dose dependent and increased with increasing dose of phenol. Saturation of the system involved in the metabolism of phenol was not observed at the dose levels used and it is likely that at saturating levels a maximum rate of elimination would be observed and at such levels toxic symptoms may be exhibited.

In Figure 4.9 where the percentage of the dose excreted as sulphate and glucuronide conjugates at different time intervals is plotted against time, it can be seen that the relative percentage of conjugate excreted at both dose levels rises rapidly to a maximum and then falls as phenol is being metabolised and excreted. At 12.5 mg kg⁻¹ maximum elimination of conjugate occurs between 20-30 minutes, while at 25 mg kg⁻¹ it occurs between 30-40 minutes after administration. Although both glucuronide and sulphate conjugates show a similar pattern of elimination, the relative amount of glucuronide eliminated at the earlier time intervals is greater than that of sulphate, but at later time
FIGURE 4.13

RATIO OF SULPHATE TO GLUCURONIDE CONJUGATES OF METABOLITES OF PHENOL IN THE URINE AFTER ADMINISTRATION OF PHENOL IN RATS

RESULTS ARE PLOTTED AS THE MEAN FROM 2 ANIMALS
intervals more sulphate was eliminated. Indeed, when the ratio of sulphate to glucuronide in the urine at different times after administration of phenol, is determined (Figure 4.13) it can be seen that this ratio changes with time. At 12.5 mg kg\(^{-1}\) sulphate elimination initially predominates, but the relative amount of glucuronide elimination increases with time until more glucuronide was eliminated than sulphate. However, as metabolism and elimination occur the relative amount of glucuronide excreted decreases again resulting in a rise in the ratio of sulphate to glucuronide with time. Similar changes in the sulphate to glucuronide ratio were observed at the higher dose level of phenol, but the changes were less pronounced.

Interestingly, the ratio of sulphate to glucuronide at 2 hours after intraduodenal administration of \(^{14}\)C-phenol was calculated to be 1.1 to 1 and 0.9 to 1 at doses of 12.5 mg kg\(^{-1}\) and 25 mg kg\(^{-1}\) respectively, indicating that at 2 hours the relative amount of sulphate conjugates eliminated was higher at the lower dose level. Figure 4.10 shows that at both dose levels the initial rate of elimination of glucuronide conjugate was greater than that of sulphate and that at the lower dose level glucuronide conjugate elimination plateaued by 2 hours, but sulphate conjugate elimination was still increasing.

4.5 METABOLISM OF PHENOL IN ISOLATED CELL PREPARATIONS

Isolated cell preparations were prepared as described in Chapter 2 and [\(_{U-{^{14}}C}\)]-phenol (100 \(\mu\)M, 5 \(\mu\)Ci) was incubated with cell suspensions (5 ml) as described.

Samples of incubate obtained at 5 minute intervals from rat hepatocytes (100 \(\mu\)l) and from intestinal mucosal cells (5 ml) at
15 minute intervals were assayed by thin layer chromatography after centrifugation to remove denatured protein. Aliquots of the supernatents (20-40 µl) were chromatographed on silica gel 60 plates (Merck) in solvent system G, and autoradiograms of the resultant radiochromatograms were prepared. The areas corresponding to phenol (R_f 0.98), phenylglucuronide (R_f 0.47), phenylsulphate (R_f 0.70), quinolglucuronide (R_f 0.36) and quinolsulphate (R_f 0.67) were located and compared with the corresponding dark spot on the autoradiogram. These areas were marked out on the radiochromatogram and were cut out and placed into scintillation vials and counted as described.

Because of the volatile nature of phenol, thin layer chromatographic estimation of small amounts of radiolabelled phenol as described was unsatisfactory, therefore the radioactivity associated with areas on the chromatogram corresponding to be non-volatile conjugates were expressed as a ratio of quinolglucuronide to phenylglucuronide to phenylsulphate. Quinol sulphate was not detected.

Aliquots of the supernatents (20 µl from liver cells and 100 µl from intestinal mucosal cells) were suitably diluted to 500 µl and extracted with ether (2 ml). Ether extraction quantitatively removes unmetabolised phenol and little or no conjugates are extracted. The radioactivity in the ether and aqueous fractions was determined by liquid scintillation counting as described and from the ratio of the non-volatile conjugates and the level of radioactivity associated with the ether and aqueous fractions, the relative proportions of phenol and phenol metabolites at different times during the incubation may be calculated.

 Autoradiograms from TLC of samples from isolated rat intestinal mucosal cells and hepatocytes are shown in Figures 4.14 and
FIGURE 4.14

AUTORADIOGRAM OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-PHENOL IN ISOLATED RAT INTESTINAL MUCOSAL CELLS

0 15 30 60 Mins.

P - Phenol

I - Glucuronide

TLC on Silica gel 60 (Mreck), in Solvent System G.
FIGURE 4.15

AUTORADIOGRAMS OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-PHENOL IN ISOLATED RAT HEPATOCYTES

I - Phenylglucuronide  
Ia - Quinol glucuronide  
II - Phenylsulphate

TLC on Silica gel 60 (Merck), in Solvent System G.

N.B. - Because of the volatile nature of phenol, unmetabolised $^{14}$C-phenol was not detected on the autoradiograms.
FIGURE 4.16

METABOLITES OF $^{14}$C-PHENOL FROM ISOLATED RAT INTESTINAL MUCOSAL CELLS

1 - $^{14}$C-Phenol (50μM) incubated with the cells for 1 hr.
2 - $^{14}$C-Phenol (100μM) incubated with the cells for 1 hr.
C - Urine from the sheep dosed with $^{14}$C-phenol as standard.
4.15 and it can be seen that both cell preparations are capable of metabolising phenol. In intestinal mucosal cells, the major metabolite was phenylglucuronide (see Figure 4.16) while in hepatocytes phenylglucuronide, quinolglucuronide and phenylsulphate were metabolites of phenol (see Figure 4.14).

Quantitative results of metabolism of phenol by hepatocytes are given in Figure 4.17 and Table 4.5 summarizes the results for intestinal mucosal cells.

The metabolism of phenol by intestinal mucosal cells was found to be very low and only 18% (0.09 μmole) of the phenol (0.5 μmole) was converted into non-volatile conjugates after incubation for 1 hour. This is equivalent to 2 μmole of phenol metabolised 10^6 cells^{-1} hr^{-1}. Phenylglucuronide was the major metabolite (15%, 0.07 μmole) and some phenylsulphate was also formed (3%, 0.02 μmole). Metabolism of phenol by intestinal mucosal cells also gave rise to an unidentified metabolite (R_f 0.58 in solvent system G, see Figure 4.16). The nature of this metabolite is not known and identification has yet to be carried out.

Phenol metabolism by hepatocytes was extremely rapid and the rate of metabolism of phenol in two separate experiments was calculated to be 25 μmole min^{-1} and 26 μmole min^{-1} and the number of viable cells in each incubation was 30 x 10^6 and 39.5 x 10^6 respectively. This indicated that under the conditions of the experiment metabolism of phenol was at a maximal rate and increases in the number of viable cells above 30 x 10^6 appears to have little effect on the rate of phenol metabolism. However, the relative amounts of glucuronide and sulphate conjugates synthesised showed considerable variation. In one experiment (Figure 4.17a) synthesis of glucuronide and sulphate was found to be approximately equal, but in
Figure 4.17

Metabolism of 14C-phenol by rat hepatocytes

(b) 

(c)
Table 4.5

Metabolism of $^{14}$C-Phenol in Isolated Intestinal Mucosal Cells

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Phenol</th>
<th>Phenylglucuronide</th>
<th>Phenylsulphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0*</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>15</td>
<td>96.0</td>
<td>2.7</td>
<td>1.3</td>
</tr>
<tr>
<td>30</td>
<td>87.0</td>
<td>9.8</td>
<td>3.2</td>
</tr>
<tr>
<td>60</td>
<td>81.9</td>
<td>14.8</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Results are expressed as % of the radioactivity found in the incubation medium corresponding to the metabolites at different time

* $100\% = 0.5 \mu$ mole of $^{14}$C-Phenol

Total No. of viable cells $40 \pm 5 \times 10^6$ incubation$^{-1}$
another experiment (Figure 4.17b) the amount of glucuronide synthesised was twice that of sulphate and the increase in glucuronide synthesis was at the expense of sulphate conjugation and was apparently due to an increase in the relative amount of quinolglucuronide (cf Figures 4.17c 4.17d).

These results showed that isolated intestinal mucosal cells and hepatocytes are capable of metabolising phenol by conjugation. The hepatocytes were also able to hydroxylate phenol to give quinol. Furthermore, the ability of the liver cells to metabolise phenol was considerably greater than that of intestinal mucosal cells.

4.6 DISCUSSION

The results of the investigations involving phenol were summarized in the previous section, and like benzoic acid (Chapter 3) a significant observation in the in vivo investigations was that some 90% of the dose of 25 mg kg\(^{-1}\) of phenol was excreted in the urine in the animals studied within 24 hours after oral administration. Indeed, the majority of the dose (84-90%) was excreted within 8 hours in these animals. Similar excretion of metabolites of phenol in the rat has been reported previously, but the pig was shown to excrete only about 51% of the dose of 21 mg kg\(^{-1}\) of phenol in 24 hours (Capel et al, 1972). Also there appears to be considerable species variation in the percentage of the dose of phenol recovered in the urine in 24 hours and at 25 mg kg\(^{-1}\) this percentage ranged from 95% in the rat to 31% in the squirrel monkey (Capel et al, 1972).

Feacal excretion of metabolites of phenol was negligible in the sheep, the pig and the rat indicating that urinary excretion is
the major pathway of orally administered phenol. Moreover, it suggests little or no biliary excretion of phenol and phenol metabolites. Indeed, Maylin (1971) was unable to demonstrate enterohepatic circulation of phenol or its metabolites in the biliary cannulated sheep. Biliary excretion of phenol and its metabolites in the rat was found to be in the order of 5%, the major biliary metabolite being phenylglucuronide (Abou-et-Makareem, et al. 1967b).

The major urinary metabolites of phenol found in these animals were the conjugates of phenol itself, although some quinol conjugates were also detected.

The results from the rat confirms previous observations that both sulphate and glucuronic acid conjugates are excreted (Capel, et al., 1972) and at 25 mg kg⁻¹ the amount of phenylsulphate and phenyl-glucuronide found in the 8 hour urine was approximately equal (42% and 55% respectively), whereas the level of quinolglucuronide (2%) was twice that of quinolsulphate (1%).

In the pig, essentially only glucuronic acid conjugates of phenol and quinol were detected (83% and 8% respectively). This confirms previous reports that glucuronic acid conjugation is the major route of metabolism of phenol in the pig (Capel et al., 1972). However, only phenylglucuronide was reported as the metabolite of phenol, but in the present investigation appreciable amounts of hydroxylated metabolites were found in the pig urine. As pointed out previously the pig is not completely devoid of the ability to conjugate phenols with sulphate and it has been suggested that there may be several phenylsulphotransferase isoenzymes (McEvoy and Carroll, 1971) and that the pig is deficient in some of these isoenzymes since it is capable of conjugating 1-naphthol with sulphate.
to an appreciable extent (≥30%) (Capel, et al., 1974a). Sulphate conjugation of phenol in the pig is extremely low compared with glucuronic acid conjugation, at the dose level studied, sulphate conjugation has little opportunity of occurring and in the present investigation sulphate conjugation was found to be in the order of 4%.

In the sheep, phenylglucuronide was the major metabolite (49%) found in the urine. Phenylsulphate, quinolglucuronide and quinolsulphate were also detected and accounted for 33%, 2% and 4% respectively, of the urinary metabolites of phenol. Some 12% of the sheep urinary metabolites of phenol was tentatively identified to be phosphate conjugates.

Identification of phosphate conjugates was based on hydrolysis of urine samples by acid phosphatase, followed by extraction with organic solvent as described in Chapter 2. Reverse isotope dilution analysis of the resultant etheral fraction showed that phenol was essentially the only radiolabelled component, so that phenylphosphate was believed to be the phosphate conjugated metabolite, although a small amount of radiolabelled quinol was also found to be present.

Further attempts in the identification of phenylphosphate as a urinary metabolite of phenol in the sheep included the reverse isotope dilution analysis of sheep urine using authentic disodium phenylphosphate (B.D.H.). Authentic phenylphosphate was added to the urine and ether extracted at acidic pH (pH ≤ 1). Under such conditions phenylphosphate (pK₂ = 5.73) is extracted into the etheral phase and subsequent isolation and purification of phenylphosphate by conversion into the bis-cyclohexylammonium salt (m.p. 211°C) was by the addition of excess cyclohexylamine to the etheral extract (Miyano 1955). The white
precipitate formed was filtered, washed and recrystallized from hot water. Recovery was extremely low and some radioactivity was found to be associated with the bis-cyclohexylammonium derivative of phenylphosphate, but unfortunately subsequent recrystallization to constant specific radioactivity and constant melting point proved to be difficult because of the hydrolysis of the salt during recrystallization so that accurate measurements cannot be made. It should be pointed out that during the ether extraction, phenylglucuronide (pKa 3.42) present in the urine may also be extracted into the organic solvent and at acidic conditions it is expected that radiolabelled phenylsulphate found in sheep urine will be hydrolysed (Garton et al., 1949), so that free $^{14}$C-phenol will also be extracted into the ether fraction, but phenylglucuronide and phenol did not form cyclohexylammonium salts.

Attempts to identify phenylphosphate chromatographically included thin layer chromatography of the ether extract of acidified sheep urine followed by radiochromatogram scanning. This showed that free phenol and phenylglucuronide were the major radioactive components of the ether extract, but, a small radioactive peak corresponding to co-chromatographed phenylphosphate ($R_f$ 0.38-0.42 in solvent system G) was also observed. Indeed in a preliminary experiment studying the metabolism of phenol in isolated sheep hepatocytes, thin layer chromatography followed by radiochromatogram autoradiography showed the presence of a dark area on the autoradiogram corresponding to phenylphosphate. Unfortunately quinolglucuronide also has similar $R_f$ values so that separation of quinolglucuronide and phenylphosphate in solvent system G is incomplete. Chromatography in other solvent systems described in Chapter 2 was also attempted, but proved to be unsatisfactory.
The use of radiolabelled inorganic phosphate in the investigation of phenol metabolism by the sheep in vivo and in isolated sheep hepatocytes was also considered, but unfortunately such experiments have yet to be carried out. However, collectively the results obtained strongly suggest that phenylphosphate is a urinary metabolic of phenol in the sheep. Interestingly, monophenylphosphate has been reported as a metabolite of phenol in the cat (Capel, et al, 1974b). The characterization of the metabolite included paper chromatography, hydrolysis with alkaline phosphatase, incorporation of radiolabelled inorganic phosphate and mass spectrometry of the isolated metabolite.

The finding that phosphate conjugation of phenol occurs in the sheep may have important implications in the species variation in the metabolism of phenol, whereas phenylphosphate synthesis in the cat occurs in the absence of glucuronic acid conjugation of phenol. The presence of phosphate conjugates in sheep urine is not due to impaired glucuronic acid or sulphate conjugation mechanisms. The sheep appears to form glucuronide and sulphate to a similar extent with respect to phenol metabolism at 25 mg kg\(^{-1}\). Furthermore, with caution, it may be suggested that it is possible that the sheep, being a ruminant, may show unusual pathways of metabolism and metabolites of orally administered compounds.

The formation of phosphate conjugates of foreign organic compounds is a reaction which has been rarely observed in the past. Very little investigation into the possible role of phosphate synthesis in the metabolism of foreign compounds has been conducted, although the involvement of phosphate esters in biochemical reactions are numerous. For example, phosphate esters are important intermediates in the absorption and metabolism of glucose by glycolysis and gluconeogenesis which are universal biochemical pathways. Indeed, the interaction of phosphorylases
and phosphatases are important reactions in the biochemical regulation of carbohydrate metabolism.

Since inorganic phosphate is more abundant than sulphate in animal tissues, it is surprising that phosphate esters are not more common as conjugates of foreign compounds. Bis-(2-amino-1-naphthyl) hydrogen phosphate has been shown to be a metabolite of 2-naphthylamine in urine of dogs (Troll, et al., 1959, Boyland, et al. 1961) and in man (Troll, et al., 1963). Troll and Bellman (1967) also reported that bis-(2-hydroxylamino-1-naphthyl) hydrogen phosphate was another metabolite of 2-naphthylamine. 1-Naphthyldihydrogen phosphate has been found in the bodies and excreta of houseflies, blowflies and New Zealand grass grubs (Binning, et al., 1967). Houseflies and blowflies have also been found to form glucoside-6-phosphates of p-nitrophenol and 1-naphthol (Heenan and Smith 1967), but in these conjugates the phosphate group is not attached directly to the phenol. Interestingly, when rats were given large doses of ethanol intraperitoneally Tomaszewski and Buckowicz (1972) were able to isolate monomethylphosphate from the liver of these animals.

The dose level used in the present in vivo investigation was 25 mg kg⁻¹. This is well below the toxic level of phenol in these species. In the rat the oral LD₅₀ of phenol was 530 mg kg⁻¹. Information on the lethal doses of phenol in the sheep and the pig was not available, but it was observed that when the sheep was dosed with phenol at 100 mg kg⁻¹ by administration via an intestinal cannula, severe toxicity occurred (Maylin, 1971) and the symptoms included muscle tremors and weakness, rapid breathing, haemolysis of erythrocytes and anuria. Such symptoms of stress were not observed in the sheep or the pig dosed with 25 mg kg⁻¹.
of phenol and it was assumed that the dose used was below the toxic level in these species.

The metabolism of phenol was also investigated in ureter cannulated rats in an attempt to investigate the kinetics of in vivo metabolism and the possible changes in the urinary metabolic profile with time.

The kinetics of phenol metabolism have been studied previously in the rabbit (Bray et al, 1952) and in the sheep (Maylin 1971). Dosage in the rabbit experiments ranged from 0.1 g kg$^{-1}$ to 1 g kg$^{-1}$ while dosage used in the sheep experiments ranged from 10 mg kg$^{-1}$ to 100 mg kg$^{-1}$.

It was found that both the synthesis of phenylglucuronide and phenylsulphate during the metabolism of phenol approximated to a first order process. In addition, sulphate conjugation appears to be limited by the availability of sulphate so that in fasting animals, the rate of formation of sulphate conjugates is independent of the amount of phenol present in the body above a certain level, but if sulphate precursors such as cysteine are administered simultaneously with phenol, first order kinetics are once again observed.

The dose levels used in the ureter cannulated rat experiments were 12.5 mg kg$^{-1}$ and 25 mg kg$^{-1}$. Unfortunately, attempts to use higher doses of phenol resulted in severe signs of toxicity in the rats. Intruduodeneal administration phenol to anaesthetised rats as described in Chapter 2 at doses of 30 mg kg$^{-1}$, 40 mg kg$^{-1}$ and 50 mg kg$^{-1}$ resulted in immediate toxic responses which were observed as whole body convulsions. At the dose levels used first order kinetics were observed. The synthesis of both glucuronides and sulphates were dose dependent and these conjugates were eliminated in the urine at constant rates for a
period of about 1 hour after intraduodenal administration, and as the level of $^{14}$C-phenol in the body is reduced due to metabolism and elimination, the rate of elimination decreased and elimination plateaued. At 12.5 mg kg$^{-1}$, the constant rate of elimination of phenylglucuronide and phenylsulphate was calculated to be 62 $\mu$g min$^{-1}$ and 34 $\mu$g min$^{-1}$ respectively, while at 25 mg kg$^{-1}$ the corresponding values were 94.5 $\mu$g min$^{-1}$ and 56.7 $\mu$g min$^{-1}$ respectively.

Phenol with a pKa of 10 is well absorbed from the small intestine (Schanker et al., 1958). Indeed, our preliminary investigations in the rat using an in situ isolated intestinal loop technique showed that when $^{14}$C-phenol (2.5 $\mu$Ci, 1.5 mg in 0.5 ml saline) was administered to a closed intestinal loop and the blood from the corresponding branch of the mesentric vein was collected at 30 second intervals from a mesentric vein cannula, it was possible to demonstrate that radioactivity was detected in the venous blood within 30 second of administration of phenol and some 40% of the administered radioactivity was recovered in the blood in 5 minutes. Thus it is interesting to note that within 10 minutes after intraduodenal administration of $^{14}$C phenol conjugated metabolites in the order of 2% were detected in the urine and after 2 hours some 70% of the dose was excreted. This suggests that at the dose level studied, absorption from the intestine, distribution, metabolism and excretion of phenol are not rate limiting. Limiting rates are likely to occur only at high dose levels when enzyme mechanisms involved in the metabolism are saturated and such levels are likely to be toxic.

The relative amounts of glucuronide and sulphates eliminated were found to be dose-dependent so that at 2 hours after administration it was observed that sulphate conjugation predominates at
the lower dose level while at the higher dose level glucuronide conjugates predominate. This dose dependency of conjugates eliminated was also observed by other workers. Thus in the rabbit (Williams, 1938, Parke and Williams, 1953, Capel, et al. 1972), the rat and the sheep (Maylin 1971) increase in the dose of phenol resulted in a concomitant increase in the relative amounts of glucuronides and a decrease in the amount of sulphates excreted in the urine in 24 hours.

However, in the present investigation, it was also noticed that the relative amounts of glucuronides and sulphates excreted changes not only with the dose but also with time. At both dose levels, glucuronic acid conjugates were present in the urine in a greater proportion during the early phase of elimination, but as elimination proceeds the relative proportion of glucuronides decreased and sulphate increased and this is reflected in the changes in the ratio of sulphate to glucuronide found in the urine with time. Similar observations have been reported and by monitoring the hepatic venous blood plasma levels of phenol and phenol conjugates after intestinal administration of phenol in the sheep, Maylin, (1971) was able to observe changes in the relative proportion of phenol, phenylglucuronide and phenylsulphate in blood plasma with time. Thus at low dose level (10 mg kg\(^{-1}\)) glucuronide conjugates were initially present in the greatest proportion, but this decreased with time and there was an increase in sulphate conjugates accompanied by a decrease in free phenol. At a higher dose level (100 mg kg\(^{-1}\)), the proportion of sulphate conjugates remains constant, while the proportion of glucuronide conjugates increases as the proportion of free phenol decreases. Indeed, in our investigation of the in vivo metabolism of phenol by the sheep such relative changes in the proportion of glucuronides and sulphates excreted was observed (see Figure 4.6).
Thus, if it can be suggested that the relative proportion of metabolites excreted in the urine at different times reflected the relative proportion of those metabolites found in hepatic venous blood plasma, then it is possible that further investigations of this phenomenon of changes of relative proportion of conjugates eliminated with time may provide an insight into the relative roles of glucuronic acid conjugation and sulphate conjugation in the metabolism, excretion and elimination of phenol.

It has been suggested that conjugation of phenol may occur both in the liver and in the intestine (see Williams, 1959) and using isolated cell preparations from the rat it was demonstrated that both hepatocytes and isolated intestinal mucosal cells were capable of metabolising phenol. Both glucuronides and sulphates were detected as metabolites, but in the intestinal mucosal cells phenylglucuronide was the major metabolite. Other tissues are also capable of metabolising phenol and our preliminary investigation of the metabolism of phenol by pulmonary tissue preparations and isolated kidney cells suggest that phenylglucuronide and phenylsulphate are metabolites in these tissues.

Compared to the hepatocytes, intestinal mucosal cells show a limited ability to metabolise phenol so that the role of the intestine as a major site of detoxication of orally ingested phenol as proposed by Powell et al. (1974) may be questionable. Only 18% of the phenol (0.5 µmole) was conjugated by $45 \times 10^6$ intestinal mucosal cells in a 1 hour incubation, whereas under similar conditions complete conversion of phenol to its conjugates was carried out by $30 \times 10^6$ hepatocytes in about 20 minutes. It is likely that under the in vivo situation very small amounts of a dose of ingested phenol may be metabolised by the intestinal tract and as described earlier, our
preliminary investigations have shown that absorption of $^{14}$C-phenol from the intestine is extremely rapid and, furthermore, freeze drying of blood samples collected from the corresponding branch of the mesentric vein resulted in the complete loss of radioactivity. Phenol being volatile is likely to be lost during the freeze drying process so that it is suggested that free phenol was the major radioactive component found in the mesentric blood. Conjugates of phenol are nonvolatile, and unlikely to be lost during freeze drying. The rapid absorption of phenol by the intestine will limit the amount of metabolism of phenol carried out by the intestine.

Clearly further investigation is necessary before constructive discussion on the role of the intestine in the metabolism of ingested phenol can be considered. However, it is important to point out that a criteria used by Powell, et al, (1974) and Maylin, (1971) in determining intestinal metabolism of phenol was that conjugated metabolites of phenol were detected in the portal blood plasma after administration of phenol into the intestine, but portal blood samples were monitored only some time after the administration of phenol so that recirculation of hepatic metabolites into the portal blood via systemic circulation is a real possibility. Thus, phenylglucuronide and phenylsulphate detected in the portal blood 5 minutes (Maylin,1971) and 30 minutes (Powell et al 1974) after perfusion of phenol into an in situ intestinal loop may be of hepatic origin.

It was demonstrated that conjugation of phenol resulted in the detoxication of phenol, so that the toxicity of phenylglucuronide and phenylsulphate is about one-fifth and one-eighth respectively of that of phenol (Oehme 1969) and it will be recalled that when doses of phenol
ranging from 30 mg kg\(^{-1}\) to 50 mg kg\(^{-1}\) were administered intraduodenally to rats immediate toxic responses were observed. If the intestine was capable of detoxicating ingested phenol then such immediate toxic responses would not be observed. It is therefore suggested that the toxic responses resulted from the rapid absorption of free phenol into the general circulation. Fortunately the considerable ability of the liver to conjugate phenol is able to limit the potential hazard of ingested phenol with respect to the health of the whole organism. However, it is important to point out that these observations do not preclude the ability of the intestine to detoxicate phenol since, in these experiments the animals used were anaesthetized and the dose level used may be such that the intestinal conjugation mechanism was overwhelmed. Indeed, it is possible that the intestine may play a role in the detoxication of the small amounts of phenolic material ingested daily in a normal diet.

The considerable ability of the liver to metabolise phenol was demonstrated by isolated hepatocytes. Thus it was observed that under the conditions of the experiment phenol was metabolised at a maximal rate by the liver cells and this was calculated to be in the order of 25 \(\text{n mole min}^{-1}\) at a cell density of 30 \(\times 10^6\) cells or higher. However, the metabolic profile of hepatocyte metabolism of phenol appears to show considerable variation in the relative proportion of glucuronide and sulphate conjugates synthesised. This may reflect the analogous situation in vivo where the proportion of phenylglucuronide and phenylsulphate excreted in the urine is dose dependent. In the hepatocyte experiments the "dose" of phenol was the same, i.e. 0.5 \(\text{n mole}\), but the cell density was different in the two experiments. Where glucuronide and sulphate synthesis was approximately equal (see Figure 4.17a), the cell density was 30 \(\times 10^6\) cells, whereas when twice as much glucuronide than sulphates was produced the cell density was 39.5 \(\times 10^6\) cells (see
Figure 4.17b). It is interesting to note that increase in the glucuronide conjugation observed in Figure 4.17b was apparently due to increased quinolglucuronide synthesis (Figure 4.17d) when compared with the observation of Figure 4.17c.

Clearly other factors may be involved in the variation in the metabolic profile. Indeed it may represent individual variation of the animals used since hepatocytes for the experiments were obtained from individual rats. The state of the animal prior to excision of its liver may also be involved so that in fasted animal availability of sulphates within the cell may be a limiting factor although the Leibovitz medium used in the metabolic studies is well supplied with inorganic sulphate.

The apparent increase in the level of quinolglucuronide may be due to the fact that whereas under the in vivo situation where the turnover of phenol and phenol metabolites in the liver may be extremely rapid, no such rapid turnover is possible with isolated hepatocyte incubations. Phenol and phenol metabolites are in continuous contact with hepatocytes during the incubation, so that increase in aromatic hydroxylation is a real possibility.

Thus it can be seen that although isolated rat hepatocytes can be demonstrated to metabolise phenol, further investigation of the control of conjugation and aromatic hydroxylation is necessary before understanding of the metabolism of phenol and other xenobiotics by hepatocytes is achieved.
CHAPTER 5

ANILINE
5.1 INTRODUCTION

Aniline (C₆H₇N) is an oily liquid at room temperature. It is colourless when freshly distilled, but darkens on exposure to air and light. It has a M.W. of 93.12 and m.p. of -6°C. Its flash point is 70-76°C and its pKa is 4.6 and pKb is 9.3.

It is poisonous, has a characteristic odour, is volatile with steam and is combustible. It is soluble in several organic solvents including alcohol, benzene and chloroform. It is also soluble in water to some extent and forms salts with acids. The pH of an aqueous solution of aniline is about 8.

Aniline was first obtained by the destructive distillation of indigo and later it was isolated from coal tar. It has many industrial uses, particularly in the synthesis of dyes, e.g. it is the active ingredient in shoe dyes, insoluble inks and many other of the "aniline" dyes. It is also used in the manufacture of pharmaceutical products, e.g. many of the analgesics are aniline based.

Aniline is toxic, it has an oral LD₅₀ of 460 mg kg⁻¹ in rats and the lethal doses in other animals are: - guinea pig 2.5 g kg⁻¹; rabbit 1.0 g kg⁻¹, dog 0.5 g kg⁻¹, and cat 0.2 g kg⁻¹. Toxicity in man may occur from inhalation, ingestion or cutaneous absorption of aniline. Symptoms of toxicity include cyanosis, methaemoglobinemia, anemia, vertigo, headache, nausea and mental confusion.
5.2 METABOLISM OF ANILINE

Early work on the metabolic fate of aniline appears to be very scanty. Schmiedeberg, (1878) fed dogs with aniline acetate and identified p-aminophenol in the urine after acid hydrolysis and Muller, (1887) studied a human case of poisoning by 25 g of aniline and found that the urine reduced Fehlings solution and contained conjugated p-aminophenol. Elson, et al, (1946) found that at a dose of 72.5 mg kg⁻¹ aniline is excreted exclusively as etheral sulphate, whereas the equivalent dose of p-aminophenol (90.0 mg kg⁻¹) is excreted entirely as glucuronide. They suggest that this may preclude the view that aniline is converted in the body to p-aminophenol, which is then conjugated with glucuronic acid and sulphate and that it is possible for etheral sulphate formation to take place directly in the aromatic nucleus without preliminary formation of the free phenolic compound.

In a study on the fate of aniline in the rabbit (Smith and Williams, 1949), it was shown that at dose levels of about 500 mg kg⁻¹ the metabolites of aniline included glucuronides and sulphates of o- and p-aminophenol and possibly 4-aminoresorcinol. These accounted for some 40-45% of the administered dose. Some N-acetylated derivatives were also identified, but the major metabolite was a labile glucuronide which accounted for over 50% of the administered dose.

The nature of the labile glucuronide was not known and a dihydroxyaniline glucuronide type structure was proposed as the possible labile metabolite. Later the labile conjugate was believed to be the N-glucuronide of aniline. Indeed this labile N-glucuronide was also thought to be the principle metabolite of aniline by Ishidate, et al, (1958).
N-glucuronide synthesis is interesting because in physiological conditions it can proceed enzymically or non-enzymically. Bridges and Williams (1962) using free glucuronic acid and several arylamines including aniline as substrates were able to demonstrate that N-glucuronide formation was non-enzymic both in vitro and in vivo. Enzymic synthesis of N-glucuronides of arylamines including aniline has been demonstrated by Axelrod et al (1957, 1958). They showed that in the presence of UDPGA and UDPGA transferase from guinea pig liver microsomes, N-glucuronides are formed from several arylamines. This observation has also been confirmed by Arias (1961) and Isselbacher et al (1962). Arias (1961) was able to show that Gunn rats, which have defective glucuronyl transferase activities were able to synthesise the N-glucuronide of aniline both in vitro and in vivo. This therefore suggests that the enzymes responsible for the synthesis of ethereal and ester glucuronides may be different from those involved in the synthesis of N-glucuronides. Indeed Isselbacher et al (1962) reported the solubilization of glucuronyl transferases from rabbit liver microsomes, by treatment with snake venom, which were unable to catalyse the formation of aniline N-glucuronide but readily catalysed the formation of ester and ether glucuronides. On the other hand, Leventer et al (1965) have solubilized N-glucuronyl transferase for aniline from guinea pig liver microsomes by sonic oscillation.

In an investigation of the metabolism and disposition of aniline in the isolated, blood perfused liver of the rat (Boobis and Powis 1975) it was found that the major metabolite produced by the perfused liver was an acid labile conjugate which represented about 1/3 of the perfused aniline. This acid labile conjugate was also believed to be the N-glucuronide of aniline. However, Parke (1960) using $^{14}$C
aniline showed that at an oral dose of 160-500 mg kg$^{-1}$ the amount of N-glucuronide excreted in the urine was about 3-10% and that the major pathway of metabolism of aniline appears to be aromatic hydroxylation followed by conjugation with glucuronic acid and sulphate.

Other aniline conjugates have also been observed. For example, phenylsulphamic acid (N-sulphate of aniline) was detected in the urine of rabbits dosed with aniline, but it was not found in the rat (Boyland et al 1957). It is also acetylated to a small extent to give acetanilide, but it is not methylated (Williams 1959) and interestingly a novel metabolite, $\delta$-glutamyl-anilide has been identified recently as the major metabolite of aniline in the cattle tick - Boophilus microplus (Willox et al 1976).

It has been stated that the major pathway of aniline metabolism is hydroxylation of the aromatic nucleus, but hydroxylation of the amino group can also occur to a small extent to give phenylhydroxylamine. Indeed Kiese (1959) has identified phenylhydroxylamine as the metabolite of aniline responsible for the production of methaemoglobin following injection of aniline to the dog and it is suggested that the toxic effects of aniline are due to the metabolite, phenylhydroxylamine (Lipschitz 1920, Von Isselentz 1939, Schlimine 1943 and Jenkin et al 1972). N-Hydroxylation of aniline has been demonstrated in the isolated perfused liver and lung of the cat (Kiese and Uehleke 1961) and in washed rat liver microsomes in the presence of NADPH and oxygen (Uehleke 1961), but evidence for phenylhydroxylamine as a urinary metabolite of aniline has not been shown (Von Jagow et al 1966, Parke 1960), although it may be detected in the blood (Kiese 1959). However, hydroxylamines of p-substituted derivatives of aniline are apparently excreted in the urine (Kiese 1966).
Phenylhydroxylamine readily rearranges to p-aminophenol in the presence of acid. Indeed a chemical synthesis of p-aminophenol utilises this property of phenylhydroxylamine giving yields of about 90% of p-aminophenol (Vogel 1956, Fieser and Fieser 1961).

Chemically, phenylhydroxylamine may be obtained by the reduction of nitrobenzene with zinc or iron powder and dilute acid or electrolytically and interestingly, methaemoglobin formation due to nitrobenzene results from the enzymic reduction of nitrobenzene to nitrosobenzene and phenylhydroxylamine (Parke 1968). Thus it is possible that the urinary p-aminophenol may be derived from the acid catalysed chemical rearrangement of the N-hydroxylated metabolite of aniline. Indeed conversion to p-aminophenol has been shown to be the major metabolic change of phenylhydroxylamine and at an oral or subcutaneous dose of 40 mg kg\(^{-1}\) about 45% of the dose of phenylhydroxylamine was recovered in 2 days as doubly conjugated p-aminophenol, probably the glucuronide and sulphate of N-acetyl-p-aminophenol (Williams 1959). The mechanism of reaction was unknown, but was believed to involve the reduction of phenylhydroxylamine to aniline and subsequent oxidation to p-aminophenol and conjugation.

Phenylhydroxylamine might also rearrange to o-aminophenol since it has been pointed out that arylamines are converted to o-aminophenols via enzymic N-hydroxylation and enzymic or acid catalysed rearrangement of the N-hydroxy derivatives (Miller, et al., 1960, Boyland and Booth, 1962, Booth and Boyland, 1964). However, enzymic rearrangement of phenylhydroxylamine to o-aminophenol has not been demonstrated, although N-hydroxyacetanilide has been shown to rearrange to N-acetyl-o-aminophenol (Booth and Boyland, 1964). Arylhydroxylamine substrates, which have been shown to rearrange to o-aminophenols through the action of an enzyme of the soluble fraction of the liver, tended to be p-substituted aniline and acetanilide derivatives.
and the rearrangement is believed to proceed via the corresponding quinolimines and quinolimides. (Miller et al 1960, Boyland and Booth 1962).

Aniline, besides forming sulphate and glucuronide conjugates may also form cysteine conjugates. Traces of o- and p-aminophenyl and N-acetyl-p-aminophenyl-mercapturic acids have been detected in the urine of rats and rabbits dosed with aniline (Boyland et al 1963), and it is suggested that the synthesis of the mercapturic acid may arise from reaction of phenylhydroxylamine, with some sulphhydryl compounds such as glutathione, cysteine and acetylcysteine, in vivo through the intermediate formation of quinolimine (Boyland and Booth, 1962). Quinolimines are also oxidative products of aminophenols. Indeed neutral or alkali solutions of aminophenols readily darken with the formation of quinolimines. Quinolimines are extremely sensitive and are transient in aqueous solution undergoing hydrolysis to the quinones.

Probably one of the most studied foreign compound metabolism reactions is aromatic hydroxylation to give phenols. It has been shown to occur in all species examined, such as insects, fish, birds, reptiles and mammals including man (Smith, 1968, Parke, 1968, Williams, 1967). Although this reaction occurs in most species, the orientation of the phenolic metabolite formed by the hydroxylation reaction in mammals shows considerable species variation. The relative amounts of the o- and p-aminophenol excreted and the ratio of the total p-aminophenol to total o-aminophenol found in the urine after complete hydrolysis with acid have been determined in several animal species receiving aniline orally. It was shown to vary from 15 in the gerbil to 0.4 in the cat (Parke, 1960) and it appears that carnivores have a p-/o- ratio of 1 or
less while the $p$-$o$- ratio in rodents is much higher (Williams 1967) (See Table 5.6).

The species variation in $o$- and $p$-hydroxylation of aniline also shows an apparent correlation with the toxicity of aniline. Thus cats, dogs and other carnivores, to which aniline is very toxic produce mostly $o$-aminophenol, whereas rabbit and other herbivores to which aniline is less toxic produce mostly $p$-aminophenol (Parke 1960). Therefore it would appear that the toxicity of aniline may be due to $o$-aminophenol, this metabolite being more toxic than the $p$-isomer. It has been suggested that two aniline hydroxylases are present and species variation is due to the variation of the activities of these enzymes from species to species (Williams 1967).

Aniline is a so-called type II substrate (Schenkman et al. 1967), that is, it produces a type II difference spectrum by interacting with the haem iron of cytochrome $P_{450}$. However, a type I difference spectrum for aniline has also been demonstrated (Vainio and Henninen, 1972). It is also one of a few type II substrates which can be demonstrated to be hydroxylated in vitro by microsomes, but this may be due to its type I component, since it can be shown that in the absence of acetone, aniline gives a type I difference spectrum under conditions in which the type II sites on the microsomes are saturated with imidazole. Thus spectral observations and microsomal metabolism may be difficult to interpret. Nevertheless, aniline-4 hydroxylase is an enzyme system widely measured in vitro and indeed it is often used to evaluate the properties of the microsomal mixed function mono-oxygenase system of enzymes.

In general aniline hydroxylase activity is monitored by incubating microsomes with a NADPH regenerating system and aniline.
Aniline and its metabolite p-aminophenol are extracted from the incubation mixture into ether and subsequently separated from each other by additional extraction with alkali containing phenol which reacts with p-aminophenol in alkali to form a blue phenol-indophenol complex. This blue complex is quantitatively determined spectrophotometrically ($\lambda_{\text{max}}$ 630-670 nm).

The activity of microsomal aniline hydroxylase determined as described is influenced by a number of factors. It is induced by pretreatment with the classical inducers of microsomal enzymes such as phenobarbital and 3-methylcholanthrene, (Kato, et al 1970, Lu, et al 1972) and it has also been demonstrated to be stimulated in vitro by several compounds including ethylisocyanide (Imai and Sato, 1966), acetone (Anders, 1968, Uainio, and Hanninen, 1972), 22'-bipyridine (Anders, 1969), paraoxon (Stevens, et al, 1972), metyrapone (Kahl, et al, 1973) and haemoglobin (Jonen, et al, 1976). Interestingly, acetone (Anders, 1968) 22'-bipyridine (Anders, 1969), metyrapone (Leibman, 1969) and some pyridyl ketones (Leibman and Ortiz, 1973) also enhance microsomal hydroxylation of acetanilide.

It appears that these compounds only stimulate the in vitro hydroxylation of the so-called type II (and reverse type I) substrates and either have no effect or inhibit the other in vitro activities of the microsomal mixed function mono-oxygenase system. Thus acetone and haemoglobin apparently have no effect on the N-demethylation of ethylmorphine, aminopyrine and NN'dimethylaniline (Anders, 1968, Jonen, et al, 1976) while in vitro metabolism of type I compounds such as hexobarbital and aminopyrine are invariably inhibited by metyrapone (Leibman, 1969, Netter, et al, 1969). On the other hand 22'-bipyridine, besides stimulating aniline hydroxylase in vitro was also shown to stimulate N-dealkylation of N-methyl- and NN'dimethyl-aniline, but
inhibited N-de-ethylisation of ethylmorphine and aminopyrine (Anders 1969). Interestingly, ethylisocyanide was shown to exhibit both stimulatory and inhibitory effects on microsomal hydroxylase (Imai and Sato 1966) and the inhibitory effect was found to be due to a competition between ethylisocyanide and oxygen for the microsomal enzyme.

The mechanism of *in vitro* stimulation of aniline hydroxylase by these compounds remains to be elucidated, but it has been proposed that since type II substrates such as aniline produce an inhibition of cytochrome P₄₅₀ reduction by NADPH cytochrome P₄₅₀ reductase (NADPH cytochrome c reductase), whereas type I compounds stimulate this reduction (Gigon, *et al* 1969), it is possible that the mechanism of *in vitro* stimulation of aniline hydroxylase may be related to the rate of reduction of cytochrome P₄₅₀. That is, the presence of compounds such as acetone *in vitro* removes the inhibition of cytochrome P₄₅₀ reductase by type II substrates (Anders 1969, Vainio and Hanninen 1972). A number of possible explanations for the enhancement of aniline hydroxylase by haemoglobin were discussed by Jonen, *et al* (1976).

By using reconstituted microsomal enzyme systems, it has been demonstrated that besides NADPH and molecular oxygen, three microsomal components are essential for aniline hydroxylase activity; namely: cytochrome 'P₄₅₀', NADPH cytochrome P₄₅₀ reductase and a lipid fraction (Lu *et al* 1972). However, it has been shown that numerous haemoproteins and a flavin could replace the native enzyme systems and achieve considerable rates of aniline hydroxylation and also of nitro-reductase activity (Symms and Juchau, 1974). These findings were initiated by the realization that aniline hydroxylation in the rat and human placenta was due to the catalytic activity of contaminating haemoglobin with NADPH as the electron donor (Juchau and Symms, 1972).
This illustrated that it is unsuitable to study mono-oxygenase activities in homogenates of many extrahapatic tissues from which blood has not been efficiently perfused. The haemoproteins which are capable of catalysing aniline hydroxylation included cytochrome b$_5$, methaemoglobin, metmyoglobin, cytochrome c, peroxidase and catalase and in the presence of flavins such as FMN, FAD or riboflavin, the reaction rates are markedly increased.

From the works of Symms and Juchau, it can be shown that the constituents of an aniline hydroxylating system are: an electron donor such as NADH or NADPH, a suitable haemoprotein and molecular oxygen together with the substrate, indicating that hydroxylation of aniline may be carried out by an "artificial" system, and the fact that the elements of these "artificial" systems comprised only of biological compounds, e.g. haemoproteins which are analogous to the components proposed to be functional in the hepatic microsomal mixed function oxidase system suggested that common catalytic mechanisms may be involved. Indeed numerous "artificial" model systems capable of effecting the hydroxylation of organic compounds have been proposed and investigated in attempting to gain some insight into the mechanism of oxygenation of substrate in microsomal mixed function oxidation reactions. Such systems have been reviewed recently by Ullrich and Standinger, (1971) and Hamilton, (1974). Moreover, it is particularly interesting to note that microsomal haemoproteins such as cytochrome b$_5$ are also capable of catalysing p-hydroxylation of aniline (Symms and Juchau, 1974). Clearly it can be seen that the mechanism of microsomal aniline hydroxylation to give p-aminophenol is complex, possibly involving more than one pathway of reaction and may even give rise to in vitro artifacts.
The ability of various tissues to metabolise aniline has been considered and by determining aniline hydroxylase activity in microsomal preparations it has been demonstrated that besides the liver, other tissues such as the intestine, the lungs, the kidneys and the brain are able to metabolise aniline by converting it to p-aminophenol. (Feuer et al, 1971, Hook et al, 1972, Bend et al 1972, Litterst et al, 1975, Hook and Bend, 1976). However, in view of the preceding discussion regarding microsomal aniline hydroxylase activity, the validity of these observations may be questionable and further investigations are necessary in order to establish the ability of extrahepatic tissues to metabolise aniline.

The rate of in vitro metabolism of aniline in the rat has been investigated recently (Wisniewska-Knypl and Joblonska, 1975) by determining the unmetabolised aniline remaining in the animal tissue at several timed intervals after administration of a dose of aniline at 50 mg kg\(^{-1}\). Unmetabolised aniline was determined colourimetrically following steam distillation of aliquots of homogenates of the whole animal (i.e. blood and carcass, minus the skin and tail), and it was found that at 50 mg kg\(^{-1}\) the biological half life of aniline was about 67 minutes. Also it was found that metabolism of a single dose of aniline was considerably accelerated if the animals were pretreated with either phenobarbital or 3.4 benzpyrene, but was inhibited after administration of SKF525A. The biological half lives of aniline in these situations were calculated to be 42, 37 and 86 minutes respectively. Indeed using indirect methods such as hexabarbital sleeping time (Conney, and Burns, 1963) and antipyretic activity of phenacetin (Conney et al, 1966) for the assessment of in vivo rates of metabolism and assay of microsomal aniline hydroxylase activity, it was observed that repeated
FIGURE 5

COMPOUNDS IDENTIFIED AS METABOLITES OF ANILINE

\[ R = \text{GLUCURONIC ACID OR SULPHATE} \]

\* THE GLUCURONIDE WAS THE MAJOR METABOLITE ACCORDING TO SMITH AND WILLIAMS (1949), ISHIDATE ET AL (1958)

\+ MAJOR METABOLITE ACCORDING TO PARKE (1960)
exposure of rats to aniline at a daily dose of 50 mg kg$^{-1}$ for 1 month results in a stimulation of drug metabolism as manifested by an increase in the aniline hydroxylase activity, shortening of hexabarbitral sleeping time and a reduction of the antipyretic effects of phenacetin. Thus suggesting that prolonged exposure to aniline stimulates its own metabolism (Wisniewska-Knypl et al., 1975). Rabbits exposed to low doses of aniline for 18-26 days showed an increase rate of excretion of p-aminophenol in the urine. (Minkina et al., 1970).

5.3 IN VIVO INVESTIGATION OF ANILINE METABOLISM

[U-14C]-aniline, 50 mg kg$^{-1}$ was administered to the animals as described. Urine and faeces were collected and assayed as described in Chapter 2.

The $R_f$ values and methods of detection of aniline and some of its metabolites are given in Table 5.1 and the results of orally administered $^{14}$C-aniline metabolism in the sheep, the pig and the rat are summarized in Tables 5.2, 5.3 and 5.4.

The tables showed that after oral administration of $^{14}$C-aniline a significant proportion of the dose was excreted by these animals in 24 hours. In the rat some 96% of the administered dose was recovered in the urine within 24 hours while, in the sheep the recovery was in the order of 80%. However, at 24 hours the radioactivity found in pig urine corresponded to about 56% of the administered dose of aniline and furthermore, where urinary excretion from the pig was monitored to 31 hours, the recovery of radioactivity between 24 and 31 hours after administration was only in the order of 2%. Faecal excretion of radioactivity during the first 24 hours was found to be in the order
Table 5.1  
R<sub>f</sub> Values and Colour Reactions of Aniline and Some of its Possible Metabolites

<table>
<thead>
<tr>
<th>Compounds</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; Values from Thin Layer Chromatography in Solvent Systems</th>
<th>Colour Reactions with Spray Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B*</td>
<td>G&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Aniline</td>
<td>0.91-1.00</td>
<td>0.62-0.86</td>
</tr>
<tr>
<td>Acetaniline</td>
<td>0.94-0.98</td>
<td>0.78-0.84</td>
</tr>
<tr>
<td>N-Acetyl-p-aminophenol</td>
<td>0.94-0.98</td>
<td>0.75-0.81</td>
</tr>
<tr>
<td>o-Aminophenol</td>
<td>0.80-0.86</td>
<td>0.59-0.62</td>
</tr>
<tr>
<td>m-Aminophenol</td>
<td>nd.</td>
<td>nd.</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>0.61-0.65</td>
<td>0.50-0.57</td>
</tr>
<tr>
<td>Phenylsulphamic acid</td>
<td>0.59-0.62</td>
<td>0.50-0.53</td>
</tr>
</tbody>
</table>

* Silica gel 60 (Merck)
+ Silica gel G (Schleicher & Schuell)

nd. Not determined
st. Streak

184
### Table 5.2

**Urinary Excretion of $^{14}$C-Aniline by Sheep and Pig**

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose $\text{mg Kg}^{-1}$</th>
<th>$\mu$Ci.</th>
<th>Time (hr)</th>
<th>Volume of Urine (ml)</th>
<th>% of Administered Dose Excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep 1</td>
<td>50</td>
<td>94.1</td>
<td>0-3</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3-5</td>
<td>176</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5-7</td>
<td>564</td>
<td>20.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7-24</td>
<td>2800</td>
<td>29.5</td>
</tr>
<tr>
<td>Sheep 2</td>
<td>50</td>
<td>135.3</td>
<td>0-2</td>
<td>100</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2-4</td>
<td>165</td>
<td>26.8</td>
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<td></td>
<td></td>
<td></td>
<td>4-6</td>
<td>110</td>
<td>22.3</td>
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<td></td>
<td>8-24</td>
<td>250</td>
<td>10.6</td>
</tr>
<tr>
<td>Pig 1</td>
<td>50</td>
<td>98.4</td>
<td>0-3</td>
<td>68</td>
<td>7.8</td>
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<td>3-5</td>
<td>65</td>
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<td>5-7</td>
<td>54</td>
<td>10.7</td>
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<td>7-24</td>
<td>150</td>
<td>16.5</td>
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<td></td>
<td></td>
<td></td>
<td>24-31</td>
<td>2200</td>
<td>1.9</td>
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<td>Pig 2</td>
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<td>80.9</td>
<td>0-3</td>
<td>152</td>
<td>20.1</td>
</tr>
<tr>
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<td>3-6</td>
<td>150</td>
<td>20.8</td>
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<td>6-9</td>
<td>94</td>
<td>4.2</td>
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<td></td>
<td></td>
<td>9-21</td>
<td>560</td>
<td>6.6</td>
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<td></td>
<td>21-24</td>
<td>56</td>
<td>0.9</td>
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</table>
Table 5.3

R_f Values and Colour Reactions of Radiolabelled Metabolites from Thin Layer Chromatography of Urine Samples

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>R_f Values in Solvent Systems</th>
<th>Colour Reactions with Spray Reagents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>G</td>
</tr>
<tr>
<td>I</td>
<td>0.62-0.66</td>
<td>0.59-0.61</td>
</tr>
<tr>
<td>II</td>
<td>0.29-0.31</td>
<td>0.28-0.31</td>
</tr>
<tr>
<td>III</td>
<td>0.50-0.53</td>
<td>0.47-0.48</td>
</tr>
<tr>
<td>IV</td>
<td>0.12-0.15</td>
<td>0.11-0.13</td>
</tr>
<tr>
<td>V</td>
<td>0.40-0.42</td>
<td>0.43-0.45</td>
</tr>
<tr>
<td>VI</td>
<td>0.47-0.50</td>
<td>0.51-0.53</td>
</tr>
<tr>
<td>VII</td>
<td>0.91-0.94</td>
<td>0.90-0.92</td>
</tr>
</tbody>
</table>

TLC on Silica gel 60 (Merck) - No colour
<table>
<thead>
<tr>
<th>Species</th>
<th>% Dose in 24 hr. Urine</th>
<th>G</th>
<th>S</th>
<th>NPAG</th>
<th>NPAP</th>
<th>A</th>
<th>PAPG</th>
<th>PAPS</th>
<th>0APG</th>
<th>0APS</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>sheep</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>81.7</td>
<td>68.8</td>
<td>21.4</td>
<td>63.7</td>
<td>15.7</td>
<td>2.1</td>
<td>2.1</td>
<td>3.6</td>
<td>1.0</td>
<td>1.5</td>
<td>4.7</td>
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<tr>
<td>2</td>
<td>79.1</td>
<td>70.4</td>
<td>22.1</td>
<td>59.2</td>
<td>14.6</td>
<td>1.2</td>
<td>3.4</td>
<td>8.0</td>
<td>0.8</td>
<td>3.2</td>
<td>6.7</td>
</tr>
<tr>
<td>pig</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>60.0</td>
<td>77.3</td>
<td>10.6</td>
<td>72.8</td>
<td>6.1</td>
<td>4.7</td>
<td>0.6</td>
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<td>0.7</td>
<td>3.0</td>
<td>3.8</td>
</tr>
<tr>
<td>2</td>
<td>52.6</td>
<td>63.8</td>
<td>14.8</td>
<td>60.4</td>
<td>12.7</td>
<td>13.1</td>
<td>1.6</td>
<td>0.9</td>
<td>0.6</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Rat*</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>96.0</td>
<td>15.7</td>
<td>76.9</td>
<td>12.6</td>
<td>55.6</td>
<td>6.5</td>
<td>0.5</td>
<td>2.1</td>
<td>13.7</td>
<td>1.0</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>(87.9-101.6)</td>
<td>(11.8-15.7)</td>
<td>64.0-</td>
<td>12.5-</td>
<td>51.7-</td>
<td>4.2-</td>
<td>0.4-</td>
<td>0.9-</td>
<td>12.3-</td>
<td>0.4-</td>
<td>6.6-</td>
</tr>
<tr>
<td></td>
<td>(17.9)</td>
<td>(12.9)</td>
<td>78.6-</td>
<td>7.8)</td>
<td>(60.7)</td>
<td>7.8)</td>
<td>1.0)</td>
<td>(2.6)</td>
<td>16.2)</td>
<td>(1.3)</td>
<td>8.4)</td>
</tr>
</tbody>
</table>

G - Glucuronides  
S - Sulphates  
NPAG - N-Acetyl-p-aminophenylglucuronide  
NPAP - N-Acetyl-p-aminophenol  
NPAPG - N-Acetyl-p-aminophenylglucuronide  
NPAPS - N-Acetyl-p-aminophenylsulphate  
A - Acetanilide  
PAPG - p-Aminophenylglucuronide  
PAPS - p-Aminophenylsulphate  
OAPG - o-Aminophenylglucuronide  
OAPS - o-Aminophenylsulphate

* Results are expressed as the mean from 6 animals and the range is given in parentheses.
of 1% in the sheep and 2% in the pig. Faecal excretion in the rat was not determined, but was believed to be negligible.

Identification of metabolites of aniline was based on chromatography, differential enzymic hydrolysis, solvent extraction and reverse isotope dilution analysis as described in Chapter 2.

Thin layer chromatography followed by radiochromatogram scanning of urine samples from the species studied showed the presence of several peaks (Figure 5.1). Radiochromatograms of urine from the sheep showed the presence of peaks I, II and IV, while peaks I, II, V, VI and VII were obtained in the radiochromatograms from pig urine and from rat urine peaks I, II and III were produced. The $R_f$ values of these peaks and their colour reactions with several spray reagents are given in Table 5.3.

Colour reaction with spray reagent (a) showed that positive naphthoresorcinol reactions were given by II, IV and V indicating the presence of glucuronides. The use of spray reagents (h) and (i) which are typical reagents for detecting free aromatic amino groups showed that III, IV and V contained components with free aromatic amino groups and when chromatograms were first sprayed with reagent (j) i.e. 0.5M HCl and heated at 100°C for a few minutes, subsequent colour reactions with (h) and (i) showed that positive reactions were also given by I, II VI and VII, indicating that aromatic amino groups are exposed as the result of acid hydrolysis in the area of the radiochromatogram corresponding to these peaks. Using spray reagent (g) positive colour reactions were observed in the area corresponding to VI indicating the presence of divalent sulphur and suggesting the possible presence of a radiolabelled mercapturic acid.
FIGURE 5.1

THIN LAYER RADIOCHROMATOGRAM SCAN OF URINE FROM ANIMALS DOSED WITH $^{14}$C-ANILINE
Following enzymic hydrolysis, thin layer chromatography of urine samples and their hydrolysates, together with radiochromatogram autoradiography revealed that the dark areas on the autoradiogram corresponding to peaks II, IV and V disappeared after hydrolysis with \(\beta\)-glucuronidase and there was a concomitant appearance of a dark area corresponding to peak VII. On the other hand, hydrolysis with arylsulphatase resulted in the disappearance of dark areas corresponding to peaks I and III, and with a concomitant appearance of a dark area corresponding to VII (Figures 5.2, 5.3 and 5.4). Thus II, IV and V are glucuronic acid conjugates, while I and III are sulphate conjugates and the aglycones being components of VII which can be extracted into organic solvent. Interestingly peak VI, which was observed only from radiochromatogram of pig urine was not hydrolysed by either \(\beta\)-glucuronidase or arylsulphotase (Figure 5.3).

Quantitative estimations of ethereal extracts for radioactivity showed that after \(\beta\)-glucuronidase hydrolysis of 24 hour urine samples some 70% of the radioactivity of the hydrolysate from the sheep and the pig was extracted into the ethereal phase, whereas from the rat only 16% was extracted. On the other hand, the amount of radioactivity extracted after arylsulphotase hydrolysis was in the order of 21%, 15% and 77% from the urine hydrolysates of the sheep, the pig and the rat respectively. When the 24 hour urine samples were incubated with acid phosphatase at pH 4.5 and the hydrolysates were solvent extracted as described, the amount of radioactivity extracted into the ether phase was found to be essentially the same as control level. Control levels which are taken into account when results from enzymic hydrolysis experiment are calculated, ranged from about 4% in the sheep, 7% in the rat and 10% in the pig. These values corresponded to the
FIGURE 5.2

ENZYMIC HYDROLYSIS OF URINE FROM THE SHEEP DOSED WITH $^{14}$C-ANILINE

A - Untreated Urine
B - "Control"
C - $\beta$-Glucuronidase Hydrolysis
D - Arylsulphatase Hydrolysis

II & IV - Glucuronides
I - Sulphates
VII - "Deconjugated" Metabolites
FIGURE 5.3

ENZYMIC HYDROLYSIS OF URINE FROM THE PIG DOSED WITH $^{14}$C-ANILINE

A - Untreated Urine
B - "Control"
C - β-Glucuronidase Hydrolysis
D - Arylsulphatase Hydrolysis

II & V - Glucuronides
I - Sulphates
VII - "Deconjugated" Metabolites
VI - "Mercapturic Acid"
FIGURE 5.4

ENZYMIC HYDROLYSIS OF URINE FROM THE RAT DOSED WITH $^{14}$C-ANILINE

A - Untreated Urine
B - "Control"
C - β-Glucuronidase Hydrolysis
D - Arylsulphatase Hydrolysis

II - Glucuronides
I & III - Sulphates
VII - "Deconjugated" Metabolites
level of radioactivity found in the ether fraction when untreated urine from these animals was solvent extracted. This indicated the presence of metabolite of aniline which are not conjugated with glucuronic acid or sulphate, and are organic solvent extractable.

Thus from the enzymic hydrolysis experiments it can be deduced that orally administered aniline at 50 mg kg$^{-1}$ is excreted in the 24 hour urine essentially as glucuronides and sulphates and no phosphate conjugates were detected. In the rat 16% of the 24 hour urinary metabolites was found to be glucuronide and 77% to be sulphates. In the sheep the corresponding figures are 70% and 21% while in the pig they are 70% and 15% respectively.

Qualitative and quantitative investigation of etheral extracts of hydrolysates from the enzymic hydrolysis of urine samples by thin layer chromatography, radiochromatogram scanning and reverse isotope dilution analysis revealed that the major radiolabelled component of all ether extracts was N-acetyl-p-aminophenol, although o- and p-aminophenol were also present, but they accounted for only a small proportion of the radioactivity of the ether extract (Figure 5.5). On the other hand, investigation of etheral extract from untreated urine samples showed that both N-acetyl-p-aminophenol and acetanilide were radiolabelled components from the sheep and the pig while in the rat N-acetyl-p-aminophenol was found to be the major radiolabelled component.

From these observations it was deduced that VII is essentially N-acetyl-p-aminophenol while I being the major radiolabelled component in rat 24 hour urine is N-acetyl-p-aminophenylsulphate and II being the major radiolabelled component of pig urine is N-acetyl-p-aminophenylglucuronide. Other peaks are identified as p-aminophenylsulphate,
THIN LAYER RADIOCHROMATOGRAM SCAN OF ETHER EXTRACTS OF ARYLSULPHATASE HYDROLYSED URINE FROM THE RAT DOSED WITH $^{14}$C-ANILINE

A = ANILINE
B = ACETANILIDE
C = o-AMINOPHENOL
D = N-ACETYL-p-AMINOPHENOL
E = p-AMINOPHENOL
III; p-aminophenylglucuronide, IV; o-aminophenylglucuronide, V; and mercapturic acids, VI. A peak corresponding to o-aminophenylsulphate was not identified, but compared with the chromatographic behaviour of other metabolites in the solvent system used, o-aminophenylsulphate is likely to have a $R_f$ value greater than I but less than VII. (See Addendum to Chapter 5). m-Aminophenol was not found to be a phase I metabolite of aniline by reverse isotope dilution analysis and N-glucuronides, and N-sulphates of aniline were not detected. The possible presence of free aniline in the urine of the animals studied was not determined, although free aniline may be derived from the hydrolysis of N-conjugates of aniline which are relatively labile. However, from the results obtained it is likely that little or no free aniline would be found in the urine of these animals.

The 24 hour urinary metabolites of orally administered aniline which have been identified by the methods described are summarized in Table 5.4, and mercapturic acids as possible metabolites of aniline are discussed in Section 5.6.

Interestingly it was also observed that in both sheep and pig urine there were changes in the pattern of metabolites found in the urine at various times after dosing (Figures 5.6). Thus in the sheep the relative proportion of glucuronides in the urine increased with time together with a concomitant decrease in the proportion of metabolites not conjugated with glucuronic acid or sulphate. Sulphate conjugated metabolites apparently remain constant with time. In the pig on the other hand, there is an apparent decrease in the relative proportion of glucuronic acid conjugates with time together with a concomitant increase in the proportion of metabolites not conjugated with glucuronic acid or sulphate. Again, sulphate conjugates appear to remain constant with time.
RELATIVE % OF URINARY METABOLITES OF ANILINE AT DIFFERENT TIMES

SHEEP 2

0-24 hrs

RELATIVE % OF URINARY METABOLITES

2-4 hrs

4-6 hrs

6-8 hrs

0-24 hrs

GLUCURONIDES

SULPHATES

NOT CONJUGATED AS S OR G

0-3 hrs

3-6 hrs

6-9 hrs

0-24 hrs

PIG 2

RELATIVE % OF URINARY METABOLITES
5.4 METABOLISM OF ANILINE IN URETER CANNULATED RATS

The ureters of anaesthetised rats (250 g) were cannulated and dosed with \([U-^{14}C]\)-aniline at 25 mg kg\(^{-1}\) and 50 mg kg\(^{-1}\) by intraduodenal injection as described.

Urine samples were collected at 15 minute intervals for a period of 3-4 hours and the samples were assayed for radioactivity as described. The metabolic profiles of 15 minute samples were determined by thin layer chromatography of aliquots (20-40 \(\mu l\)) of urine on silica gel 60 precoated plates (Merck) in solvent system B followed by radiochromatogram autoradiography. The areas corresponding to N-acetyl-p-aminophenylsulphate (I, \(R_f 0.64\)), N-acetyl-p-aminophenylglucuronide (II, \(R_f 0.3\)), p-aminophenylsulphate (III, \(R_f 0.52\)), p-aminophenylglucuronide (IV, \(R_f 0.13\)) and N-acetyl-p-aminophenol (VII, \(R_f 0.93\)) were located and compared with the corresponding dark spots on the autoradiogram. These areas were marked on the radiochromatogram, cut out and placed into scintillation vials and counted as described. The radioactivity associated with each of these areas was expressed as a percentage of the radioactivity recovered from the radiochromatogram. Other possible metabolites of aniline were not detected by the methods used.

The rate of urinary elimination of radioactivity after intraduodenal administration is summarized in Figure 5.7. Metabolic profiles of 15 minute urine samples are illustrated in Figures 5.8 and 5.9, and the rates of excretion of various metabolites of aniline are shown in Figure 5.10 and 5.11.
RESULTS ARE PLOTTED AS THE MEAN FROM 3 ANIMALS AND ERROR BARS INDICATE THE RANGE.
FIGURE 5.8

% $^{14}C$ EXCRETED AS METABOLITES OF ANILINE
AT TIMED INTERVALS AT 25 mg kg$^{-1}$

NPAP = N-Acetyl-p-aminophenol
PAP = p-Aminophenol
S = Sulphate
G = Glucuronide

RESULTS ARE PLOTTED AS THE MEAN FROM 3 ANIMALS
FIGURE 5.9

9% 14C EXCRETED AS METABOLITES OF ANILINE
AT TIMED INTERVALS AT 50 mg kg⁻¹

NPAP = N-Acetyl-p-aminophenol
PAP = p-Aminophenol

S = Sulphate
G = Glucuronide

RESULTS ARE PLOTTED AS THE MEAN FROM 3 ANIMALS
**FIGURE 5:10**

RATE OF URINARY ELIMINATION OF METABOLITES OF $\text{^{14}C-ANILINE}$ AT 25 mg kg$^{-1}$

RESULTS ARE CALCULATED FROM THE MEAN FROM 3 ANIMALS.
FIGURE 5.11

RATE OF URINARY ELIMINATION OF METABOLITES OF $^{14}$C-ANILINE AT 50 mg kg$^{-1}$

RESULTS ARE CALCULATED FROM THE MEAN FROM 3 ANIMALS.
The results in Figure 5.7 showed that at both dose levels studied only about 50% of the dose was excreted in the urine in 3 hours, although radioactivity in the order of about 0.5% of the dose was detected in the urine within 15 minutes of administration and from the linear portion of the graphs it can also be calculated that the rate of elimination of radioactivity was in the order of about 3% of the dose per 10 minutes at both dose levels. The major metabolite found at both dose levels of aniline was N-acetyl-p-aminophenylsulphate (I). N-Acetyl-p-aminophenylglucuronide (II) and N-acetyl-p-aminophenol (VII) were found as minor metabolites while p-aminophenylsulphate (III) was found to contribute quantitatively to the urinary metabolic profile of aniline (Figures 5.8 and 5.9). p-aminophenylglucuronide (IV) as a metabolite was found to be negligible and at 3 hours after intraduodenal administration the level of p-aminophenylglucuronide was less than 1% of the administered dose (0.4% at 25 mg kg$^{-1}$ and 0.9% at 50 mg kg$^{-1}$).

The rates of elimination of these metabolites, (calculated from the linear portion of the plots in Figure 5.10 and 5.11) were found to be 0.18 μmole min$^{-1}$ for I, 0.02 μmole min$^{-1}$ for II and 0.07 μmole min$^{-1}$ for III when the rats (250 g) were given 67.2 μmole (25 mg kg$^{-1}$) of aniline. At 134.4 μmole (50 mg kg$^{-1}$) the rates of elimination of I, II and III are calculated to be 0.23, 0.05 and 0.15 μmole min$^{-1}$ respectively. The rate of elimination of N-acetyl-p-aminophenol (VII) was not determined and the level of VII found in the urine 3 hours after intraduodenal administration of $^{14}$C-aniline was about 1 μmole at the lower dose level and 2.5 μmole at the higher dose level.

The results obtained showed that the elimination of metabolites of aniline was dose dependent and that they increased with increasing dose of aniline, and the dose levels used in the present
RATIO OF N-ACETYLP-AMINOPHENOL SULPHATE (NPAPS) TO P-AMINOPHENOL SULPHATE (PAPS) IN THE URINE AFTER ADMINISTRATION OF ANILINE IN RATS.

25 mg kg$^{-1}$

50 mg kg$^{-1}$
investigation did not saturate the systems involved in the metabolism of aniline. In Figures 5.8 and 5.9 where the percentage of the dose excreted as different metabolites at different time intervals are plotted, it is interesting to note that maximum elimination of p-aminophenylsulphate (III) occurred at about 1 hour after administration whereas maximum elimination of N-acetyl-p-aminophenylsulphate occurred at about 1½ to 2 hours after administration. When the ratio of N-acetyl-p-aminophenylsulphate to p-aminophenylsulphate in the urine at different times after administration was determined (Figure 5.12), it can be seen that the ratio changed with time. At either dose level, elimination of both sulphate conjugates was in the same order initially, but as metabolism and elimination occurred, the relative amount of N-acetyl-p-aminophenylsulphate found in the urine increased so that this ratio increased with time. Indeed, this increase is best illustrated at the lower dose level. At the higher dose level this change in the ratio in less pronounced but in Figures 5.10 and 5.11 it can be seen that elimination of p-aminophenylsulphate has plateaued by 3 hours after administration at the lower dose level, whereas at the higher dose level elimination of p-aminophenylsulphate is just beginning to plateau. At both dose levels, the amount of N-acetyl-p-aminophenyl conjugates found in the urine was still increasing 3 hours after intraduodenal injection of 14C-aniline.

5.5 METABOLISM OF ANILINE IN ISOLATED CELL PREPARATIONS

Isolated cell preparations were prepared as described in Chapter 2 and [U-14C]-aniline (100 μM, 5 μCi) was incubated with cell suspension (5 ml) as described.
Samples of incubates obtained at 5 minute intervals from rat hepatocytes (100 μl) and from gut cells (5 ml) at 15 minute intervals were assayed by thin layer chromatography after centrifugation to remove denatured protein. Aliquots of the supernatant (20-40 μl) were chromatographed on silica gel 60 plates (Merck) in solvent system B and autoradiograms of the resultant radiochromatograms were prepared. The areas corresponding to N-acetyl-p-aminophenylsulphate (I, Rf 0.64); N-acetyl-p-aminophenylglucuronide (II, Rf 0.3); p-aminophenylsulphate (III, Rf 0.52); p-aminophenylglucuronide (IV, Rf 0.13) and N-acetyl-p-aminophenol (VII, Rf 0.93) were located and compared with the corresponding dark areas found on the autoradiograms. The autoradiograms of radiochromatograms from samples from isolated rat intestinal mucosal cells and hepatocytes thus obtained are shown in Figures 5.13 and 5.14 respectively.

It can be seen that rat hepatocytes are capable of metabolising aniline progressively, and after 1 hour, 5 dark areas on the autoradiogram (Figure 5.14) corresponding to the 5 metabolites of aniline which have been previously identified were observed. Intestinal mucosal cells on the other hand, showed only one dark area on the autoradiogram (Figure 5.13) which corresponded to VII. A dark area on the autoradiograms corresponding to the origin was also seen, but the nature of this radioactive band was not known and it is believed to be due to the association of radiolabelled materials with components of cell debris which are not chromatographed in solvent system B.

Interestingly the dark areas on Figure 5.14 corresponding to VII showed changes with time. Initially, it can be seen as a diffused dark area which gradually appears more discreet and at 1 hour a distinct dark area was observed corresponding to VII. This indicated that in hepatocytes, the areas on the chromatograms corresponding to VII
FIGURE 5.14

AUTORADIOGRAMS OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-ANILINE IN ISOLATED RAT HEPATOCYTES

I - N-Acetyl-p-aminophenylsulphate
II - N-Acetyl-p-aminophenylglucuronide
III - p-Aminophenylsulphate
IV - p-Aminophenylglucuronide
VII - N-Acetyl-p-aminophenol (Aniline & Acetanilide)

TLC on Silica gel 60 (Merck),
in Solvent System B.
FIGURE 5.13

AUTORADIOGRAM OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-ANILINE IN ISOLATED RAT INTESTINAL MUCOSAL CELLS

VII - Aniline, Acetanilide & N-Acetyl-p-aminophenol
(see text)

TLC on Silica gel 60 (Merck),
in Solvent System B.
may contain more than one component and that the relative proportions of these components are subject to changes with time. Similar observations were also obtained from the autoradiogram from the intestinal mucosal cell samples (Figure 5.13). However, it may be recalled that aniline and acetanilide have the same $R_f$ value as N-acetyl-p-aminophenol in solvent system B (see Table 5.1) and furthermore, aniline appears as a diffused area when developed in this solvent system. Thus it is likely that the dark areas in Figure 5.13 and 5.14 which corresponded to VII may consist of components which are mixtures of aniline, acetanilide and N-acetyl-p-aminophenol. Indeed, when aliquots (20-40 µl) of supernatent from the hepatocytes were chromatographed on silica gel G plates (Schleicher and Schull) in solvent system L and autoradiograms were prepared as described (Figure 5.15), three dark areas corresponding to aniline ($X, R_f 0.69$), acetanilide ($Y, R_f 0.64$) and N-acetyl-p-aminophenol ($Z, R_f 0.43$) were observed and the relative proportions of these three metabolites apparently changed with time so that the dark areas corresponding to aniline decreased with time and at 1 hour essentially only acetanilide and N-acetyl-p-aminophenol were observed. This indicated that the dark area corresponding to VII in solvent system B may be resolved into aniline, acetanilide and N-acetyl-p-aminophenol in solvent system L. The dark areas on the autoradiogram (Figure 5.15) which corresponded to $R_f$ values ranging from 0 to 0.1 are O-conjugated metabolites of aniline and they are not chromatographed in solvent system L. On the other hand, when aliquots (20-40 µl) of supernatent from intestinal mucosal cells were chromatographed in solvent system L, aniline and acetanilide were observed to be the components in the supernatents which are radiolabelled indicating that acetanilide was the intestinal metabolite of aniline. Indeed when aliquots (1 ml) of the supernatents from intestinal mucosal cell samples were solvent
FIGURE 5.15

AUTORADIOGRAMS OF TLC SHOWING THE TIME COURSE OF METABOLISM OF $^{14}$C-ANILINE IN ISOLATED RAT HEPATOCYTES

X - Aniline
Y - Acetanilide
Z - N-Acetyl-p-aminophenol
C - "Conjugated" Metabolites

TLC on Silica gel G (Schleicher & Schull), in Solvent System L.
extracted with ether (2 x 5 ml), quantitative extraction of radioactivity (95% - 102%) into the ethereal fraction was observed and subsequent reverse isotope dilution analysis of the ethereal fraction with authentic acetanilide and N-acetyl-p-aminophenol revealed that acetanilide was the intestinal mucosal cellular metabolite of aniline (Table 5.5a). Negligible amounts of N-acetyl-p-aminophenol were detected (<0.1%) and unmetabolised aniline was assumed to be the major radiolabelled component in the ethereal fraction. When on the other hand, an aliquot (1 ml) of the supernatent from the isolated rat hepatocytes which had been incubated with aniline for 1 hour was similarly extracted with ether, it was found that only 25% or so of the radioactivity was extracted into the ether fraction and subsequent reverse isotope dilution analysis with acetanilide and N-acetyl-p-aminophenol showed that both compounds are ether extractable metabolites of aniline of rat hepatocytes. These metabolites accounted for >52% and >48% of the radioactivity in the ether extract respectively (Table 5.5b) and the remaining radioactivity was believed to be due to free aniline.

Free aminophenols were not detected as metabolites of aniline in either isolated rat hepatocytes or intestinal mucosal cells and the possible synthesis of aniline N-glucuronide either as a metabolite of cellular metabolism or chemical reaction was not observed. However, it should be pointed out that aniline N-glucuronide is very labile under acid conditions and chromatography in the acidic solvent system B will effectively hydrolyse any N-glucuronide which may be present giving rise to free aniline, whereas chromatography in the basic solvent system L will conserve any N-glucuronide which may be present, but aniline-N-glucuronide is likely to remain at the origin when chromatographed on silica gel, thin layer plate in solvent system L.
Table 5.5

**Ether Extractable Metabolites of $^{14}$C-Aniline from Isolated Cells**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>N-Acetyl-p-aminophenol</th>
<th>Acetanilide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>µmole</td>
</tr>
<tr>
<td>0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>15</td>
<td>&lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>&lt;0.1</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>0.1</td>
<td>-</td>
</tr>
</tbody>
</table>

**a) Isolated Intestinal Mucosal Cells from Rat**

**b) Isolated Hepatocytes from Rat**

$100\% = 0.5$ µmole of $^{14}$C-Aniline

Ether extraction of radioactivity from

- a) 100% (95-105%)
- b) 25%

Total No. of viable cells incubation

- a) $40 \pm 5 \times 10^6$
- b) $35 \div 5 \times 10^6$
Quantitative analysis of the metabolism of aniline by isolated hepatocytes was performed by thin layer chromatography of aliquots of samples of supernatents, followed by the determination of the $^{14}$C content of areas on the resultant radiochromatogram corresponding to the various metabolites of aniline which have been identified. The methods used were as described previously and the radioactivity associated with each of these areas was expressed as a percentage of the radioactivity recovered from the radiochromatogram.

The preliminary results are summarized in Figure 5.16 and it should be pointed out that these results must be viewed with some caution since possible loss of $^{14}$C-aniline from the chromatograms during chromatography and autoradiography was not taken into account so that the relative proportion of the metabolites of aniline reported for the early stages of the incubation may be artificially high when compared with unmetabolised aniline. Unfortunately detailed investigations by organic solvent extraction and reverse isotope dilution analysis of the ether extractable metabolites from samples of incubates at various times have yet to be carried out. However, after incubation for 1 hour, the relative proportion of metabolites of aniline from isolated rat hepatocytes was found to be: $\text{N-acetyl-p-aminophenylsulphate (25\%); }$ $\text{N-acetyl-p-aminophenylglucuronide (14\%); }$ $\text{p-aminophenylsulphate (14\%); }$ $\text{p-aminophenylglucuronide (12\%); }$ $\text{N-acetyl-p-aminophenol (12\%) and acetanilide (13\%).}$ The radioactivity at the origin of the radiochromatograms developed in solvent system B was found to be in the order of 5-10% of the $^{14}$C recovered from the radiochromatogram.

Figure 5.16 illustrates the time course of metabolism of aniline by rat hepatocytes. With the exception of acetanilide, all metabolites of aniline are found to increase with time of incubation until a plateau
METABOLISM OF $^{14}$C-ANILINE BY RAT HEPATOCYTES

- N-ACETYL-$p$-AMINOPHENOL
- ACETANILIDE
- ANILINE
- VII - SEE TEXT

Radiochromatogram

% Radioactivity Recovered

SULPHATES
GLUCURONIDES

- N-ACETYL-$p$-AMINOPHENOL CONJUGATES
- $p$-AMINOPHENOL CONJUGATES
is reached and after incubation for about 1 hour, maximum synthesis of these metabolites was observed. Maximum synthesis of acetanilide, on the other hand, appears to occur in about 25 minutes, but interestingly subsequent incubation resulted in a fall in the level of acetanilide. This indicated that acetanilide is further metabolised by isolated hepatocytes probably to N-acetyl-p-aminophenol conjugates. Throughout the time course of the metabolism, synthesis of N-acetyl-p-aminophenylglucuronide and p-aminophenylglucuronide was approximately equal whereas synthesis of the sulphate conjugates of N-acetyl-p-aminophenol and p-aminophenol was in the ratio of about 1.7 to 1.

These results showed that isolated rat intestinal mucosal cells and hepatocytes are capable of metabolising aniline. In the intestinal cells, this was found to be low and the metabolic pathway was N-acetylation. In the hepatocytes, metabolism of aniline involved N-acetylation, aromatic hydroxylation, and O- conjugation with glucuronic acid and sulphate. N-Conjugation with glucuronic acid and sulphate was apparently not observed.

5.6 DISCUSSION

The results of the investigation involving aniline are summarized in the previous sections and in the following discussion, these results will be considered in terms of the metabolic profile of orally administered aniline at 50 mg kg⁻¹, the pathways involved in the metabolism of aniline and its relationship to toxicity with reference to methaemoglobin formation. A brief discussion on the kinetics of in vivo metabolism of aniline and the ability of various tissues to metabolise aniline will also be considered.
In the sheep some 80% of the dose of 50 mg kg\(^{-1}\) of aniline was excreted in the urine within 24 hours after administration, but in the pig only 56% of the administered dose was excreted in the urine during the first 24 hours. When urinary excretion in the pig was monitored during the second day, only about 2% of the dose was excreted between 24-31 hours (see Table 5.2), suggesting that excretion of the remaining dose of aniline in the pig may be very slow. Indeed, compared to benzoic acid and phenol urinary elimination of aniline in both species appears to be slow. In the rat, 96% of the dose was excreted within 24 hours of administration. Faecal excretion was in the order of 1-2% in the sheep and the pig during the first day. Unfortunately, subsequent faecal excretion was not monitored, but it is believed that since biliary excretion of metabolites of aniline is generally small, then faecal excretion of aniline metabolites may be negligible compared to urinary excretion. Biliary excretion of aniline and its metabolites in the rat is reported to be in the order of about 6%, the major biliary metabolite being p-aminophenylglucuronide (Abou-el-Makarem et al., 1967).

The major urinary metabolites of aniline found in these animals were glucuronic acid and sulphate conjugates of N-acetyl-p-aminophenol leading to the unusual appearance of double conjugates in the urine. In the sheep and the pig, N-acetyl-p-aminophenylglucuronide was the major urinary metabolite (60% and 69% respectively), while in the rat the sulphate conjugate was the major metabolite accounting for some 56% of the total urinary metabolites. Urinary N-acetyl-p-aminophenyl-sulphate in the sheep and the pig was about 15% and 9% respectively, while urinary N-acetyl-p-aminophenylglucuronide in the rat was roughly 13%. Thus, it can be seen that N-acetylation appears to be the major conjugation mechanism for the metabolism of aniline at 50 mg kg\(^{-1}\) in
these three species. Indeed acetanilide and free N-acetyl-p-aminophenol were also detected as minor urinary metabolites of aniline together with the glucuronides and sulphates of o-, and p-aminophenol in each species. Urinary metabolites of aniline which are N-acetylated accounted for 82%, 85% and 76% in the sheep, pig and rat respectively, and conjugates of aniline, i.e. the labile N-glucuronide and phenylsulphamic acid were not detected as urinary metabolites in the present investigation. Interestingly, N-acetyl-p-aminophenol is the pharmacologically active compound, paracetamol (Acetaminophen) and the synthesis of this compound during the metabolism of aniline give rise to an example of in vivo drug activation. Furthermore, the finding that N-acetylation is an important pathway in the metabolism of aniline may rationalise the observation of Elson et al. (1946) who showed that equivalent amounts of aniline and p-aminophenol are excreted exclusively as O-sulphates and O-glucuronides respectively.

The results obtained from the present in vivo investigations fail to confirm previous observations, where it was shown that the major urinary metabolite of aniline was either the glucuronic acid and sulphate conjugates of aminophenols (Parke 1960) or the acid labile N-glucuronide (Smith and Williams 1949, Ishidate et al. 1958). It should be noted that acetylation of aniline has been shown to occur in vitro and an acetyltransferase from pigeon liver which is capable of carrying out this reaction has been isolated and purified (Jacobson 1961). Interestingly, acetyl-CoA or acetanilide derivatives were shown to be capable of acting as the acetyl-donor.

A common feature in the previous investigations was the use of relatively strong acids in the hydrolysis of glucuronic acid and sulphate conjugated metabolites to obtain the phase I metabolites
for identification. However, such strong hydrolytic procedures will also deacetylate any N-acetylated metabolites and may account for the previous observations of low levels of N-acetylated derivatives of aniline as urinary metabolites. In the present investigation conjugate hydrolysis was by specific enzymes under much milder hydrolytic pH conditions than those previously used and the chemical environment of the enzymic experiments were such that in the absence of enzymes no significant chemical hydrolysis of even relatively labile conjugates appeared to occur. Furthermore, the differences between the reported metabolic profiles of aniline and the present results may also be partially due to the dose levels used. In earlier experiments using the rabbit and the rat, the dose levels used ranged from 200-500 mg kg\(^{-1}\) (Smith and Williams, 1949, Parke, 1960, Boyland et al 1963) that is, considerably higher than the 50 mg kg\(^{-1}\) dose used in the present investigation. At the high dose levels used, the animals showed symptoms of toxicity and indeed some animals died in about 3 days after dosing with 500 mg kg\(^{-1}\) of aniline (Parke, 1960). Toxic symptoms were not observed in the present in vivo investigations and it was assumed that 50 mg kg\(^{-1}\) was well below the toxic level of aniline in these species.

The identification of acetylated metabolites, i.e. N-acetyl-p-aminophenol and acetanilide was based on co-chromatography and reverse isotope dilution analysis with authentic metabolites of the ether extracts obtained from enzyme hydrolysed urine. Enzymic hydrolysis with \(\beta\)-glucuronidase and arylsulphatase which was carried out in an acetate buffer resulted in the deconjugation of glucuronides and sulphates respectively leaving the aglycone which was organic solvent extractable. The use of ketodase as the commercial preparation of \(\beta\)-glucuronidase, which was supplied as a solution of the enzyme in an
acetate buffer necessitated the use of 0.1 M acetate buffer pH 5.0 and this raises the possibility that the N-acetyl derivatives may be artifacts formed during the enzymic incubations as the result of interaction with the acetate ions. However, studies using a different source of β-glucuronidase (i.e. from Helix pomatia, Sigma type I) and in the presence of a citrate buffer showed that N-acetyl derivatives are found to be the major urinary metabolites of aniline. Indeed since chemical synthesis of N-acetyl derivatives of aromatic amines generally required drastic conditions such as refluxing with acetic anhydride, spontaneous acetylation of aniline and its metabolites is unlikely to occur under the mild conditions of enzymic hydrolysis so that N-acetylation is fundamental in the pathway of metabolism and urinary elimination of administered aniline, and may play an important role in the metabolism and detoxication of aniline.

The excretion of mercapturic acid conjugates as minor metabolites of aniline has been previously suggested (Boyland, et al 1963). In the present investigation, an unidentified sulphur containing metabolite from the pig was observed. This metabolite was not hydrolysed by β-glucuronidase or arylsulphatase, but gave a positive reaction with potassium dichromate - silver nitrate spray reagent (Knight and Young 1958) which indicated the presence of divalent sulphur atoms. It is therefore suggested that this unknown metabolite may be a mercapturic acid. Indeed, since N-acetyl-p-aminophenol, the pharmacologically active compound paracetamol was identified as the major metabolite in the present investigation and since Mitchell et al (1975) have shown that paracetamol induced liver necrosis was a result of the depletion of glutathione by paracetamol in forming a readily excreted mercapturic acid, this unknown metabolite may be a mercapturic acid derived from
N-acetyl-p-aminophenol conjugation with glutathione. Unfortunately complete identification of this metabolite has yet to be completed. Another interesting observation in the pig compared with the other two species studied was that whereas urinary excretion of the administered aniline in the sheep and the rat was in the order of 80-90% of the dose in 24 hours, only about 56% of the dose was eliminated in the urine of the pig in 24 hours, suggesting that elimination of aniline in the pig is much slower than the other two species. Furthermore, if mercapturic acid of N-acetyl-p-aminophenol was indeed a metabolite of aniline in the pig, this may reflect important differences in the pathways of metabolism of aniline in these species and possible consequences in terms of detoxication. Indeed the fact that the pig shows deficiencies in sulphate conjugation may be of significance in alternative pathways of metabolism in the pig which will merit further investigation.

Since N-acetyl-p-aminophenol was previously identified only as a minor metabolite of aniline (Smith and Williams 1949, Parke 1960), the importance of acetylation in the metabolism of aniline has not been considered, but it was proposed (Smith and Williams 1949) that acetylation occurred after aniline has been oxidised to p-aminophenol which was followed by O-conjugation and there may be no direct acetylation of aniline. However, the present investigation has shown that N-acetylation was important in the metabolism of aniline and that O-conjugates of N-acetyl-p-aminophenol were the major metabolites of aniline indicating some similarities between the metabolism of aniline and acetanilide. Indeed, at one time a generally accepted belief was that aniline and acetanilide are interconvertible in vivo by N-acetylation and N-deacetylation, but based on their observation that aniline is metabolised mainly to an
acid labile glucuronide, Smith and Williams (1949) suggested that acetanilide and aniline are not metabolically equivalent. Acetanilide is metabolised and excreted in the urine as glucuronic acid and sulphate conjugates of N-acetyl-p-aminophenol. (Smith and Williams 1948).

From the present observation it may be possible to propose that in the species studied, orally administered aniline at 50 mg kg$^{-1}$ may be primarily N-acetylated and subsequently metabolised as acetanilide by aromatic hydroxylation and O-conjugation, and that O-conjugates of aminophenols may arise from either direct aromatic hydroxylation of aniline followed by O-conjugation or via N-deacetylation of the acetyl derivatives. Indeed it has been suggested that acetylation of arylamine and deacetylation of N-acetylarlylamine derivatives may be catalysed by closely related enzyme systems (Bray et al 1950) and the rate of acetylation and deacetylation may be a function of the electronic configuration of the aniline and acetanilide derivatives (Perault and Pullman 1963). However, aromatic hydroxylation of aniline to aminophenols followed by N-acetylation as suggested by Smith and Williams (1949) still remains an alternative route of metabolism of aniline, and indeed in a study of the metabolism of aminophenols (Bray et al 1952a) it was shown that 17% and 45% of a dose of about 400 mg kg$^{-1}$ of o- and p-aminophenol respectively was excreted as N-acetyl-p-aminophenol in free and O-conjugated forms.
Attempts to rationalize these two pathways of formation of \(N\)-acetyl-\(p\)-aminophenol from the observations of the present investigation were unsuccessful but interestingly acetanilide was identified as a metabolite of aniline in isolated rat hepatocytes and intestinal mucosal cells. Indeed it was the only metabolite detected in isolated intestinal mucosal cells. Observations from these hepatocytes (see Figure 5.16) suggest that acetanilide formed is further metabolised, probably to O-conjugates of \(N\)-acetyl-\(p\)-aminophenol. Clearly further investigation of the interrelationship between acetylation and deacetylation pathways must be considered before understanding of the involvement of acetylation in the \(in\ \text{vivo}\) metabolism of aniline can be achieved.

It has already been suggested that the dose level of aniline administered may influence the metabolite pattern. Thus at the high dose levels used in previous investigations, it is possible that either the acetylation pathway is saturated (although acetylases are not known to be readily saturatable enzymes) or enzymic deacetylation of the products of the acetylase enzyme is extensive due to "activation" of deacetylase by high levels of acetyl derivatives. High levels of
acetyl derivatives may arise as the result of the failure of the kidneys to eliminate the acetyl derivatives rapidly. Thus on the basis of the percentage of the dose, metabolites excreted as acetyl derivatives will be low. Unfortunately few systematic studies have been carried out on the interrelationship of the roles of N-acetylation and N-deacetylation pathways in the metabolism of foreign compounds. However, some preliminary investigations on the deacetylation of acetyl derivatives of sulphanilamide have been conducted with reference to the metabolism of sulphanilamide (Bridges and Williams 1963) and recently the in vivo metabolism of a number of [acetyl -14C]-acetanilides in the rat has been studied (Smith and Griffiths 1976). Using the criteria that excretion of 14CO2 in expired air is a measure of N-deacetylation of N-14C-acetyl-derivatives, Smith and Griffiths (1976) were able to demonstrate that acetyl -14C-acetanilides are metabolised to a considerable extent by N-deacetylation although the products may subsequently undergo re-acetylation. In the present investigation very little O-conjugates of aminophenols were detected in the urine whereas O-conjugates of N-acetyl-p-aminophenol were the major urinary metabolites. This suggests that the in vivo acetylation of aniline in the species studied is high whilst the deacetylation of acetylated metabolites of aniline is low.

Urinary N-acetyl-p-aminophenol conjugates may also arise from another metabolic pathway, i.e. N-hydroxylation of aniline to give phenylhydroxylamine. Phenylhydroxylamine or its N-hydroxy conjugates have not been identified as a urinary metabolite of aniline, but it is metabolised to glucuronic acid and sulphate conjugates of N-acetyl-p-aminophenol (Williams, 1959). The mechanism of reaction is believed to involve reduction of the hydroxylamine to the parent amine which is subsequently metabolised by N-acetylation and aromatic hydroxylation.
The reduction of phenylhydroxylamine to aniline was believed to be a non-enzymic reaction, but recently it has been shown that this reduction is catalysed by a microsomal enzyme (Kadlubar, et al., 1973) and indeed an NADH dependent reductase has been isolated from pig liver microsomes which is capable of converting arylhydroxylamine to aromatic amines (Kadlubar and Zieglar, 1974). Phenylhydroxylamine has been demonstrated to be an intermediate in aniline metabolism and is the principle metabolite responsible for the toxicity of aniline in causing methaemoglobin formation (Kiese, 1959). However, whether N-hydroxylation of aniline is an important pathway in the overall metabolism of aniline remains to be investigated. It may be possible to gain indirect information here by studying the metabolism of aniline in rat hepatocytes in the presence of erythrocytes and monitoring the metabolic profile and methaemoglobin formation in the erythrocytes.

Aromatic hydroxylation has been demonstrated to be the major pathway in the metabolism of aniline in that both o- and p-aminophenol are phase I metabolites. As the relative proportion of these phenols is subject to considerable species variation (Parke, 1960), it was suggested that two aniline hydroxylases are responsible for the synthesis of the two isomeric phenols and that the relative activities of these enzymes vary from species to species, thus giving a possible explanation for the species variation in o- and p-hydroxylated metabolites of aniline (Parke, 1960, Williams, 1967). In the present investigation, urinary o-hydroxylated metabolites of aniline in the three species studied accounted for only about 4-10% of the urinary metabolites, whereas the remaining urinary metabolites were essentially p-hydroxylated derivatives. Now, since it has been demonstrated that N-acetyl derivatives constituted the major metabolites of aniline in
these animals, it is therefore proposed that species variation in o- and p-hydroxylation may be influenced by the acetylation of aniline. If metabolism of aniline involved primary N-acetylation, then the acetanilide so formed would be preferentially hydroxylated in the p-position. This is because of the steric hindrance of the o-position by the acetamino group and the greater inactivation of the o-position due to the inductive effect of the acetamino substituent. Thus species which show extensive acetylation of aromatic amines, e.g. aniline, will give rise to higher levels of p-hydroxylated metabolites relative to o-hydroxylated metabolites, whereas species which eliminate a relatively higher level of o-hydroxylated metabolites will tend to show lower activities towards acetylation of aromatic amines. On the other hand, if aromatic hydroxylation occurs prior to N-acetylation of aniline, then the relative activities of the proposed aniline-2-hydroxylase and aniline-4-hydroxylase and not the activities of N-acetylase will have the major influence on the orientation of the hydroxylation. However, if one considers the N4-acetylation of sulphanilamide, which is an example of acetylation of an aromatic amino group (see Table 5.6), it can be seen that with the exception of the guinea pig and the cat, there is an apparent correlation between the acetylation of aromatic amines and the ratio of p- to o-hydroxylation of aniline in vivo in the species shown. Thus a high p-/o- ratio correlates with high acetylation of aromatic amino groups while a low p-/o- ratio correlates with low in vivo aromatic N-acetylating activities. This suggests that N-acetylation of aniline may be the important factor in determining the formation of o- and p-hydroxylated metabolites and indicates that the primary reaction in metabolism of aniline is N-acetylation. It should be noted, however, that this apparent correlation is necessarily only tentative since species variation in deacetylation reactions and other metabolic interconversions of aniline has not been considered.
Table 5.6

Species Variation in o- & p- Hydroxylation of Aniline and N4-Acetylation of Sulphanilamide

<table>
<thead>
<tr>
<th>Species</th>
<th>p-/o- Ratio of Aniline Hydroxylation</th>
<th>Lethal Dose of Aniline g Kg⁻¹</th>
<th>N4-Acetylation of Sulphanilamide (%) (Total N4⁺ N⁻ NN⁻)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat</td>
<td>0.4</td>
<td>0.2</td>
<td>14 (65)</td>
</tr>
<tr>
<td>Dog</td>
<td>0.5</td>
<td>0.5</td>
<td>0 (82)</td>
</tr>
<tr>
<td>Ferret</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mouse</td>
<td>3.0</td>
<td>-</td>
<td>20 (49)</td>
</tr>
<tr>
<td>Hen</td>
<td>4.0</td>
<td>-</td>
<td>25 (47)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>6.0</td>
<td>1.0</td>
<td>43 (22)</td>
</tr>
<tr>
<td>Rat</td>
<td>6.0</td>
<td>-</td>
<td>38 (36)</td>
</tr>
<tr>
<td>Hamster</td>
<td>10.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>11.0</td>
<td>2.5</td>
<td>14 (65)</td>
</tr>
<tr>
<td>Gerbil</td>
<td>16.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Expressed as % of dose excreted in urine.

High proportions of sulphanilamide were excreted as the free drug in these species. The values are given in parentheses.

N.B. Species differences in deacetylation reactions are not taken into consideration.

Table adapted from Parke 1960 and Bridges & Williams 1963
Indeed, deacetylase activities may show considerable species variation (Bridges and Williams, 1963, Williams, 1967) and also the extent of deacetylation may be substrate dependent. Thus Smith and Griffiths, (1976) have shown that in the rat in vivo deacetylation of acetanilide (25-31%) was considerably higher than the deacetylation of N-acetyl-p-aminophenol (6%) when equivalent amounts (1.4 mmole kg$^{-1}$) of the acetyl derivatives were administered.

Besides the direct hydroxylation of aniline catalysed by "aniline hydroxylase", the introduction of a hydroxyl group into the aromatic nucleus may be achieved via N-hydroxylation to give phenyl-hydroxylamine followed by rearrangement to either o- or p-aminophenol. Although this rearrangement to p-aminophenol in the presence of acid has been demonstrated (Fieser and Fieser, 1961), such rearrangement of phenylhydroxylamine to p-aminophenol has not been demonstrated either chemically or during the metabolism of aniline. However, since it has been pointed out that acetanilide derivatives are converted to o-aminophenols via enzymic N-hydroxylation and enzymic or acid catalysed rearrangement of the N-hydroxy derivatives (Miller et al, 1960, Boyland and Booth, 1962, Booth and Boyland, 1964), then N-hydroxylation of N-acetylated metabolites of aniline such as acetanilide and N-acetyl-p-aminophenol may also be a possibility in the biotransformation and elimination of administered aniline. Indeed, the first unequivocal demonstration of N-hydroxylation in vivo was that N-acetyl-N-2-fluorenylhydroxylamine was the major metabolite of the acetyl derivative: 2-acetylaminofluorene (Cramer, et al, 1960). However, Booth and Boyland, (1964) were unable to demonstrate the N-hydroxylation of acetanilide using rabbit liver microsomes, although they were able to show that N-acetyl-N-phenylhydroxylamine (N-hydroxyacetanilide) was isomerized
to N-acetyl-o-aminophenol by the soluble fraction of rabbit liver. Now since o-substituted acetanilides are readily deacetylated (Bray et al 1950) then, deacetylation of N-acetyl-o-aminophenol will give o-aminophenol which has been identified as a metabolite of aniline. N-Hydroxylation of N-acetyl-p-aminophenol to give a toxic quinolimide intermediate (see Figure 5.17a) which reacts preferentially with glutathione has been postulated as the pathway of formation of the mercapturic acid of N-acetyl-p-aminophenol (Mitchell et al 1975). Quinolamine intermediates from N-hydroxylation of aniline and aminophenol are also considered to be precursors in the formation of mercapturic acid metabolites from aniline by reacting with glutathione by Boyland and his colleagues (1962, 1963). Clearly, additional investigations are necessary in order to elucidate the relationship between N-acetylation, N-hydroxylation and aromatic hydroxylation in the metabolism of aniline and the pathways of biotransformation of aniline are summarized in Figure 5.18.

It has been proposed that since species which produce mostly o-aminophenol tend to be very susceptible to the toxic effects of aniline so that the toxicity of aniline may be due to o-aminophenol which is more toxic than the p-isomer (Parke 1960). Indeed the formation of a reactive quinolimine intermediate may be a contributing factor in the toxicity of aniline. However, the apparent correlation between acetylation of the aromatic amino group and o- and p-hydroxylation suggests that acetylation of aniline may have a role in the detoxication of aniline.

The mechanism of methaemoglobin formation from administered aniline results from N-hydroxylation of aniline to give phenylhydroxylamine.
POSTULATED PATHWAY OF FORMATION OF MERCAPTURIC ACIDS FROM QUINOLIMINIDE INTERMEDIATES

\[
\begin{align*}
\text{R-} & \quad \text{R-} \\
\text{N-H} & \quad \text{N-H} \\
\text{O} & \quad \text{HO-N-} \\
\text{X} & \quad \text{OH} \\
\end{align*}
\]

\[X = \text{GLUCURONIDE OR SULPHATE}\]

ADAPTED FROM MITCHELL et al (1975)

FIGURE 5.17 b

ENZYMIC CYCLE FOR FERRIHAEMOGLOBIN FORMATION BY PHENYLHYDROXYLAMINE IN RED CELLS

\[
\begin{align*}
\text{FERRO-HAEMOGLOBIN} & \quad \text{H-N-OH} \\
\text{O}_2 & \quad \text{DIAPHORASE} \\
\text{NO}^- & \quad \text{NADPH} \\
\text{FERRI-HAEMOGLOBIN} & \quad \text{G-6-P DEHYDROGENASE} \\
\text{G-6-P} & \quad \text{6-P-GA}
\end{align*}
\]
METABOLIC PATHWAYS OF ANILINE BIOTRANSFORMATION

Ac = -COCH₃
R = GLUCURONIDE OR SULPHATE
GSH = GLUTATHIONE
Phenylhydroxylamine is involved in an enzymic cycle (see Figure 5.17b) in which oxidation of phenylhydroxylamine to nitrosobenzene is coupled to the conversion of ferrohaemoglobin to ferrihaemoglobin. In the red cells nitrosobenzene is enzymically reduced back to phenylhydroxylamine and so starting a cycle of events by which one molecule of phenylhydroxylamine transforms many equivalents of ferrohaemoglobin to ferrihaemoglobin. This will continue until either the phenylhydroxylamine and nitrosobenzene are removed or further metabolised by other metabolic pathways or until all ferrohaemoglobin has been oxidised (Kiese, 1971 and cited references).

Now if acetylation prevents the interconversion of phenylhydroxylamine and nitrosobenzene either by preventing the formation of phenylhydroxylamine from N-hydroxylation of aniline or by giving rise to the formation of N-acetyl-N-phenylhydroxylamine (N-hydroxyacetanilide), then this would reduce the toxicity of aniline in methaemoglobin formation via the enzymic hydroxylamine/nitrosobenzene interconversion. Thus it is possible that species which actively acetylate aromatic amino groups may be less sensitive to aniline intoxication, whereas, species with slow aniline acetylase activities may be more sensitive to aniline induced methaemoglobin formation. Interestingly it has been suggested that deacetylation is a requisite step for the formation of methaemoglobin by acetanilide analogues and the rate of deacetylation may be the limiting factor in the expression of their haemotoxicity (Heymann, et al., 1969, Beattie, et al. 1973, Mitchell, et al. 1973) and since it has been shown that N-acetyl-p-aminophenol is not deacetylated to any great extent (Smith and Griffiths, 1976), then the formation of N-acetyl-p-aminophenol from aniline may indeed be a detoxication reaction with respect to methaemoglobin formation.
Following from the observation that the urinary metabolic profile of aniline in the sheep and the pig showed changes with time, the metabolism of aniline was also investigated in ureter cannulated rats in an attempt to study not only the possible changes in the urinary metabolic profile of aniline with time, but also the kinetics of \textit{in vivo} metabolism.

The kinetics of \textit{in vivo} metabolism of aniline at 50 mg kg\(^{-1}\) in the rat have been investigated previously by Wisniewska-Knypl and Jablonska, (1975). By using a relatively crude methodology it was found that the biological half life of aniline was in the order of about 1 hour. However, in the present investigation using dose levels of 25 and 50 mg kg\(^{-1}\), it was found that 50\% of the dose of aniline was eliminated in the urine in about 3 hours and that urinary metabolites of aniline may be detected within 15 minutes of intraduodenal administration, although this represented only about 0.5\% of the dose. Aniline with a pKa of 4.6 is well absorbed from the small intestine (Schanker, \textit{et al.}, 1958), but its slow rate of elimination and high lipophilicity suggest that distribution and metabolism may be limiting factors in the rate of elimination of orally administered aniline. An interesting observation from the ureter cannulation experiments was that at both dose levels studied O-conjugates of p-aminophenol were eliminated maximally before maximal elimination of the N-acetyl derivative (see Figure 5.8 and 5.9) and that by about 3 hours elimination of p-aminophenylsulphate has plateaued whereas elimination of N-acetyl derivatives was still increasing (see Figures 5.10 and 5.11). Thus, the fact that the urinary metabolic profile of aniline in the rat showed changes with time suggests that the \textit{in vivo} metabolism of aniline is very complex and a more detailed investigation of distribution and metabolism by various tissues is
necessary to the understanding of the roles of various pathways and the kinetics of the metabolism of aniline.

In an attempt to investigate the ability of various tissues to metabolise aniline, some preliminary experiments were carried out in isolated rat hepatocytes and intestinal mucosal cells. It was found that whereas rat hepatocytes metabolise aniline to glucuronic acid and sulphate conjugates of both N-acetyl-p-aminophenol and p-aminophenol, such conjugates were not detected as metabolites in rat intestinal mucosal cells. Indeed the only metabolite of aniline detected in rat intestinal mucosal cell was acetanilide. Acetanilide and free N-acetyl-p-aminophenol were also detected as metabolites in rat hepatocytes.

Up till now, the ability of various tissues to metabolise aniline has been demonstrated by showing the formation of p-aminophenol from aniline in tissue homogenates or microsomal preparations. However, from the works of Symms and Juchau discussed in Section 5.2, it was shown that p-hydroxylation of aniline may be achieved by an "artificial" system and the requirements of such systems may be simply, an electron donor, a suitable haemoprotein and molecular oxygen together with the substrate, so that the presence of "aniline hydroxylase" activity in a particular tissue homogenate or microsomal preparation may not necessarily reflect the ability of that tissue to metabolise aniline in vivo. Indeed the finding that N-acetyl-p-aminophenol is the major metabolite of aniline suggests that N-acetylation may be a more relevant enzymic activity for consideration. Moreover, in vitro studies of aniline metabolism using microsomes and NADPH regenerating system have always shown that p-aminophenol and not N-acetyl-p-aminophenol was the metabolite of
aniline and furthermore, there are apparently no reports of o-aminophenol as a metabolite of microsomal metabolism of aniline. This failure to observe N-acetyl derivatives in microsomal metabolism stems from the fact that N-acetylation involves the transfer of the acetyl moiety from acetyl-CoA to the aromatic amino group catalysed by N-acetyltransferase enzyme systems which are present in the soluble fraction of cells (Weber and Cohen 1967). Such systems will not be present in the in vitro incubation medium involving microsomes, so that microsomes may not be the best tissue preparation for the study of aniline metabolism. Indeed the mechanism of microsomal aniline hydroxylation is poorly understood and as discussed in Section 5.2 it is influenced by a number of factors such that constructive interpretation of in vitro aniline hydroxylase activity may prove to be difficult and give rise to erroneous hypotheses which have no bearing on the in vivo situation. The use of isolated cells in the present investigation provides an advantage over other in vitro investigations in that a full complement of biochemical reactions are present in the intact cells so that metabolism involving more than one metabolic pathway in different cellular compartments may be monitored at the same time and this may provide a closer relationship to the in vivo situation.

In the present studies using isolated rat hepatocytes, it was observed that the relative proportion of O-conjugates of p-aminophenol in the incubation was apparently higher than that observed in the in vivo situation. At the end of 3 hours' incubation some 25% of the aniline (0.5 μmole) in the rat hepatocyte incubates was detected as O-conjugates of p-aminophenol whereas under in vivo conditions roughly 16% of the urinary metabolites of aniline in the rat were O-conjugates of p-aminophenol. The remaining metabolites
in both the in vivo and in vitro experiments were N-acetylated derivatives of aniline. This apparent difference may be similar to the observation of phenol metabolism in isolated rat hepatocyte discussed in Chapter 4 (Section 4.6) and result from the continuous contact of N-acetylated metabolites with the hepatocytes in the incubation mixture so that higher deacetylation may occur. Under in vivo conditions the clearance of metabolites from the liver may be such that deacetylation is very low. Also it was noted that during the thin layer chromatographic investigation of aniline metabolites from rat hepatocytes some 5-10% of the radioactivity remained at the origin (see Figure 5.14). The nature of this is not known, but the presence of N-conjugated metabolites and the binding of radioactive metabolites to cellular macromolecular components may be real possibilities and merits further investigations.

Thus from the foregoing discussion it can be seen that metabolism of aniline is very complex and involves several metabolic pathways. Clearly, understanding of the nature and the role of the various metabolic pathways involved in the detoxication and elimination of administered aniline requires a more detailed investigation and such investigations must take into account the dose level of aniline in relation to metabolic reactions such as N-acetylation, N-deacetylation and N-hydroxylation together with aromatic hydroxylation and O-conjugations. Furthermore, a re-examination of the metabolic profile of o- and p-aminophenol, acetanilide, phenylhydroxylamine and N-acetyl-p-aminophenol may be helpful in providing a clearer understanding of the pathways of aniline metabolism.
ADDENDUM

Subsequent to the completion of Chapter 5, some preliminary investigations on the metabolism of aniline by isolated dog hepatocytes were performed. The methodology was essentially as described in Section 5.5 and although quantitative determinations have yet to be carried out, thin layer chromatography followed by radio-chromatogram autoradiography revealed that aniline was metabolised by dog liver cells to a major metabolite which had a $R_f$ value of 0.71-0.73 on silica gel 60 F$_{254}^*$ precoated plates (Merck) when developed in solvent system B. Enzymic hydrolysis with arylsulphatase followed by thin layer chromatography in solvent systems K and L showed that this metabolite was o-aminophenylsulphate. Small amounts of acetanilide, N-acetyl-p-aminophenol and p-aminophenolsulphate together with traces of free o- and p-aminophenol were also tentatively identified as metabolites of aniline in isolated dog hepatocytes.

It has been shown that the dog metabolises aniline predominately by $o$-hydroxylation (Parke 1960). It is also highly susceptible to the toxic actions of aniline (see Table 5.6) and it shows a deficiency in acetylating aromatic amino groups (Williams 1967). Thus the observation that $o$-aminophenylsulphate was the major metabolite of aniline in isolated dog hepatocytes and that only small amounts of acetyl derivatives were produced may be presented as additional support for the involvement of N-acetylation in detoxication of aniline and the orientation of aromatic hydroxylation as discussed in Section 5.6.
CHAPTER 6

FINAL DISCUSSION
In the last chapter a comparison of the metabolic capabilities between farm animals (sheep and pig) and laboratory animals (rat) will be made. The discussion will also attempt to assess the importance of studying kinetics of in vivo metabolism, the ability of different tissues to metabolise the compounds investigated and their possible influence on the observed urinary metabolic profile of these compounds. Furthermore, some suggestions for future investigation will be proposed.

In this thesis, the in vivo metabolism of three "simple" monosubstituted benzenes has been investigated. In the sheep, the pig and the rat it has been demonstrated that in these species both qualitative and quantitative similarities in the metabolism benzoic acid, phenol and aniline are generally observed. However, some differences are also shown and these may have important significance in species variation.

Rates of Metabolism

Following a single oral dose of these "model" compounds urinary elimination in all three species is normally rapid, some 80-100% of the dose being recovered in the urine during the first 24 hours after administration of the compound. Indeed it is noticeable that even with the sheep and pig, a major portion of the dose is eliminated in the first voiding of urine. However, the pig when dosed with aniline at 50 mg kg\(^{-1}\) is an exception in that only about 56% of the administered dose is recovered in the urine during the first 24 hours. Faecal excretion during this time in these species was very small, being about 2% of the dose for the compounds investigated. This therefore suggests that intestinal absorption is largely complete and that urinary excretion
is the major pathway for the elimination of these compounds.

It is generally believed that large animals tend to be slower in metabolising foreign compounds than small animals because of their slower metabolic rate. Although little or no kinetic information in the in vivo metabolism and elimination of these compounds is available our data on 24 hour excretion figures would not support this contention. It was also observed that in all three species the elimination of orally administered aniline was slower than that of administered benzoic acid and phenol. This is due partly to the more lipophilic nature and probably as a consequence, the more complex pathway of metabolism of aniline compared to phenol and benzoic acid.

Route of Metabolism

A comparison of the urinary metabolic profile from all three species reveals the fate of a particular "model" compound is rather similar. Thus hippuric acid is the major metabolite of benzoic acid, phenylglucuronide and phenylsulphate are the major metabolites of phenol and the unusual double conjugates of N-acetyl-p-aminophenylglucuronide and N-acetyl-p-aminophenylsulphate are the major metabolites of aniline in all three species. However, some significant differences were also observed. The limited sulphate conjugating ability of the pig with respect to phenol does not appear to extend to the phenolic metabolite of aniline, thus implying that the widely reported deficiency of sulphate conjugation in the pig is highly substrate dependent. This is supported by the finding of Capel et al. (1974a) who showed that substantial amounts of 1-naphthylsulphate, but not 2-naphthylsulphate are excreted by the pig following injection of 1-naphthol and 2-naphthol respectively. The unusual formation of significant amounts of phosphate
conjugate of phenol in the sheep has also been demonstrated. This apparently is not due to any deficiency in other conjugating mechanism. Phosphate conjugation may be a peculiarity of the sheep, or, perhaps ruminants in general. This clearly merits further investigation. In vivo investigation in the sheep using 2-naphthylamine which in the dog has been shown to give rise to phosphate conjugates (Troll et al 1959, Boyland et al 1961) may prove to be interesting. Considering the plentiful supply of "high energy phosphate" (e.g. ATP, creatine phosphate) in all mammalian species and the importance of phosphorylation of OH groups of endogenous compounds, it is perhaps surprising that phosphate conjugation of foreign compounds is such an apparent rarity. It may be that it is the necessity to conserve these high energy components which has lead to their very restricted use in detoxication. If this is the case the nature and physiological function of the enzyme involved in phosphorylation of phenol in the sheep may be of considerable biochemical relevance. The use of isolated cell preparations may be a valuable model for studying formation of unusual conjugates. Contrary to previous reports acetylation appears to be an important pathway of metabolism of aniline in all three species. Besides conjugation by acetylation, metabolites of aniline are also conjugated with glucuronic acid and sulphate and interestingly an apparent species difference between the rat and the farm animal is observed at the dose level studied in that glucuronide conjugates are the major metabolites of aniline in the sheep and the pig while sulphates predominate in the rat. Benzoic acid, in contrast to aniline and phenol forms only a very small amount of benzoylglucuronide and no sulphate conjugate but is metabolised extensively by glycine conjugation. Although all the compounds investigated in the present thesis contained a benzene nucleus, only aniline was metabolised extensively by aromatic hydroxylation. Aromatic hydroxylation of benzoic
acid has also been shown to occur to a small extent by previous
workers and trace amounts of hydroxy metabolites were found (Acheson
and Gibbard, 1962). In the present investigation using isolated cells from
the rat, dog and ferret, small amounts of metabolites of benzoic acid
which have been designated tentatively as hydroxy metabolites were found
in kidney cell incubates, but not in liver cell incubates. This
observation requires further investigation particularly in view of the fact
that the kidney is generally regarded as being deficient in its ability
to carry out oxidative metabolism of foreign compounds. Differences in
the metabolism of these compounds may be partly related to their
lipophilic properties which may influence their intracellular distribution.
Thus aniline and phenol will be metabolised by the microsomal enzymes
(e.g. "aromatic hydroxylase" and glucuronyl transferase) and the
enzymes of the soluble fraction (e.g. acetylase and sulphotransferase),
while the more water soluble benzoic acid may partition to a greater
extent into mitochondria which are the site of glycine conjugation.
Although these compounds are relatively "simple" from a chemical point
of view, their metabolism is extremely complex and the extent to which
some of these metabolic reactions are interdependent is unclear.

In the pig, aniline unlike the other compounds studied
was only eliminated to an extent of about 56% of the dose in the first
24 hours whereas in the sheep and the rat the 24 hour urinary elimination
was in excess of 80% of the dose. The slow rate of elimination in the
pig compared with other species could result from several factors such
as poor absorption, limited renal excretion, alternative pathways of
metabolism leading to the same urinary metabolite, extensive entrohepatic
recirculation of metabolites or possible tissue binding in the pig.
The actual cause(s) is difficult to pin point in the absence of kinetic
data on the metabolite profile.

In vivo observations in these species demonstrate that a different urinary metabolic profile is obtained at different times after administration of each compound. These changes in the urinary metabolic profile of a compound with time after dosing may stem from different rates of formation of different metabolites, as the result of differences in the site of formation of different metabolites, further metabolism of metabolites and different rates of renal elimination of different metabolites. Preliminary investigations using anaesthetised ureter cannulated rats have confirmed that aniline (50% of the dose in 3 hours) is eliminated much more slowly than phenol or benzoic acid (∼80% of the dose in 2 hours). Also it is possible to demonstrate that different metabolites are eliminated at different rates so that the ratio of these metabolites changes with time. Furthermore, the in vivo metabolism of these compounds at the dose levels investigated indicates apparent first order kinetics. By linking these ureter cannulation experiments with studies of blood metabolite profile with time useful information may be derived regarding the role of the kidney in governing the rate of elimination of various metabolites of a given compound. The use of anaesthetised animals for such studies has a potential disadvantage since such animals may differ from normal animals. This aspect requires clarification by accompanying our results with those obtained in conscious post-operative ureter cannulated animals. Experiments in which blood and urine samples are determined at various times in ureter cannulated animals from various species may help to pinpoint some of the sources of species differences in drug metabolism.

Preliminary investigations using isolated cells from various species to study metabolism of these "model" compounds have
shown that besides the liver, other organs are also capable of metabolising these compounds. Thus glycine conjugation of benzoic acid is brought about by both the liver and the kidneys of the rat whereas in the dog and the ferret it is essentially carried out by the kidney. Also liver cells from the dog and the ferret apparently have a limited ability to metabolise benzoic acid by other means. Using isolated intestinal mucosal cells from the rat it was possible to demonstrate that phenol was conjugated to a small extent and aniline was converted to acetanilide by the small intestine. Clearly such observations may have functional significance in species variation. Indeed, the use of isolated cells from different organs may provide a useful means to ascertain both the site(s) and the rate of formation of different metabolites and provide a more valid model for comparing species differences in biotransformations than microsomes or tissue homogenates. However, it must be remembered that although the viable cells used contained a full complement of biochemical activities, the extrapolation directly to in vivo situation must be made with caution since physiological factors such as the bloodflow through the organs concerned are absent and various cell types may be absent or under represented.

In conclusion, it can be seen that using these "simple" monosubstituted benzenes, it has been possible to demonstrate that the sheep and the pig metabolise these compounds in a largely similar manner to the rat. It is likely that the sheep being a ruminant may show new metabolites for orally administered compounds which are poorly absorbed and are therefore likely to be exposed to the gut microorganisms.
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