060

A COMPARISON OF THE GLUCURONIDATION AND SULPHATION OF PHENOLS IN SOME MAMMALIAN SPECIES

BY

REKHADEVI MEHTA

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Department of Biochemistry,
St. Mary's Hospital Medical School,
London, W.2 1PG

To my

Parents and Brothers.

ABSTRACT

The relative extent of conjugation of phenols by the two pathways, glucuronidation and sulphation, has been studied in a number of mammalian species. Thus, the conjugation of 1-naphthol at various dose levels has been investigated in the rat, cat and guinea pig, and that of phenol and 1-naphthol compared in several species of primates. Generally, with increasing dose of the phenol, the proportion conjugated with sulphate decreased.

The activity of the enzymes, UDP-glucuronyltransferase, with 1-naphthol and phenolphthalein as substrates, have been compared in different tissues of the rat, cat and the guinea pig. The results, more or less, supported the *in vivo* findings.

To further understand the low glucuronidating capacity for phenols in the cat, detailed bisubstrate kinetic analyses of rat and cat liver UDP-glucuronyltransferase with 1-naphthol and phenolphthalein as the aglycones, and rat liver phenolsulphotransferase with 1-naphthol as substrate, have been carried out. Results show that the reduced glucuronidating capacity for 1-naphthol in the cat is due to a comparatively very low affinity of the enzyme for 1-naphthol and not the co-substrate, UDP-glucuronic acid. Conversely, the cat liver phenolsulphotransferase has a markedly higher affinity for the phenol, even higher than that in the rat.

The effect of certain membrane perturbants on rat and cat liver UDP-glucuronyltransferase has been studied. These increased the enzyme activities towards 1-naphthol both in the rat and the cat; however, in the latter, on maximal activation, the enzyme activity was still very much lower than that in the rat. Fatty acid analysis of microsomal phospholipids from rat and cat showed only slight differences between the two species. The ability of the cat to conjugate phenols with glucuronic acid and the heterogeneity of UDP-glucuronyltransferase are discussed.

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General Aspects

The living body is endowed with a natural defence mechanism to protect it from the toxic effects of the organic compounds that are present in pharmaceuticals, pesticides, food additives, and biodegradation products of industrial chemicals in the environment, all these compounds now being employed by man on a vast scale and entering into almost every phase of civilised existence. Such compounds are now termed 'xenobiotics', grouped together with the only criterion that they are alien or foreign to the living organism (from the Greek, xenos and bios for 'stranger to life'; Mason et al., 1965). That the study of xenobiotic metabolism is necessary has been shown by the two of the most outstanding examples of adverse toxic reactions that mankind has had to face so far this century, these being the thalidomide incidence of the 1960's, and more recently, the industrial accident at Seveso, near Milan, Italy, when tons of the toxic compound, dioxin, were poured out into the air.

When xenobiotics get into the body, they could undergo three possible fates, namely they could be metabolised by enzymes, they could change spontaneously into other substances without the intervention of enzymes or they could be excreted unchanged. The majority of xenobiotics are metabolised by enzymes. Most xenobiotics are lipid-soluble compounds, and cannot be excreted as such. Therefore, a general trend of processes involved in the metabolism is the conversion to more polar, more water-soluble substances which tend to be restricted to the extracellular fluid as compared with the parent compound, and thus can be put at the disposal of the excretory mechanism more readily. The metabolism of xenobiotics is usually considered to be bi-phasic, as follows:

		Oxidation,		Synthetic
	Phase I	Reduction,	Phase II	or
Xenobiotic	⟶	Hydrolysis	⟶	conjugation
	Enzymes	products	Enzymes	products

(Williams, 1959)

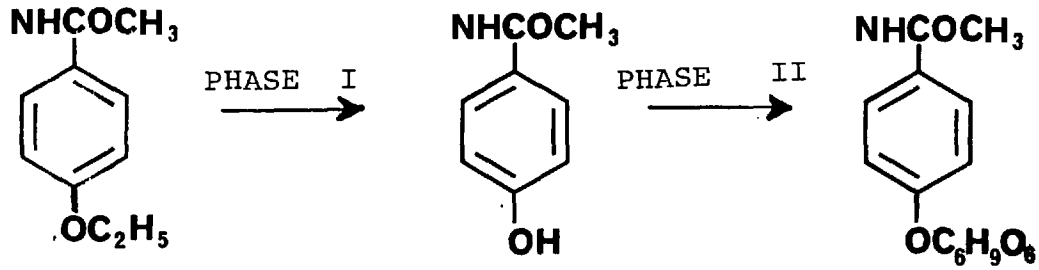
The Phase I reactions can be grouped under three different categories, these being oxidative, reductive and hydrolytic reactions (Table 1.1.). The enzymes involved in these reactions are located mainly in the endoplasmic reticulum but may also occur in mitochondria, and those for hydrolytic reactions are also found in the plasma. The reactions that occur in the endoplasmic reticulum appear to make use of the cytochrome P_{450}-linked electron transport chain, and require oxygen and either NADPH or NADH as cofactors (for review see Hutson, 1975).

In Phase I reactions, the enzymes introduce into the xenobiotic, biochemically reactive groups such as -OH, -COOH, $-NH_2$ and -SH (Table 1.1.). These groups now enable the molecule to undergo the second phase of metabolism which consists of an enzymic synthesis between the active group that has been introduced and certain compounds that are provided by the body. These endogenous compounds are referred to as conjugating agents and are usually carbohydrates or amino acids or derivatives of these. If the xenobiotic already has one of the above reactive groupings, it can undergo conjugation reactions directly without a preparatory Phase I change.

An example of a drug whose main route of metabolism occurs in two phases is the well-known analgesic and antipyretic, phenacetin (acetophenetidine). The two phases of its metabolism are as follows:

Table 1.1: The Phase I Reactions

REACTION	TYPES	GROUPS INTRODUCED OR EXPOSED
Oxidation	Aliphatic hydroxylation Aromatic hydroxylation Oxidative deamination Oxidative dealkylation S-oxidation N-oxidation Epoxide Formation	OH, COOH
Reduction	Aldehyde and Keto Reduction Azoreduction Nitroreduction	OH, NH_2, SH
Hydrolysis	De-esterification Deamidation	OH, COOH, NH_2



	Phenacetin (p-ethoxyacetanilide)	Panadol (4-Acetamidophenol)	Panadol Glucuronide (4-Acetamido-phenylglucuronide)
pKa	-	10	3.5
% ionised at pH 7.4	-	0.25%	99.99%

Thus, in Phase I, phenacetin is oxidatively de-ethylated into an active drug, panadol, which in the second phase, undergoes a synthesis with uridine 5'-diphosphate-α-D-glucuronic acid (UDPGA) to form a water-soluble, pharmacologically inactive excretory product, panadol glucuronide. Although phenacetin is an active drug itself, its main activity is due to its Phase I metabolite, panadol, which is now used as a drug in its own right (also known as paracetamol). During its metabolism, one can also notice the transition of phenacetin, a lipid-soluble, relatively neutral compound, to its glucuronide which is a water-soluble and a polar compound. Thus, panadol is a weak acid of pKa 10 and therefore, about 0.25% ionised at pH 7.4 whereas panadol glucuronide is a strong acid of pKa about 3.5 and virtually completely ionised (99.99%) at pH 7.4.

From the viewpoint of toxicity, the Phase I reactions can lead either to less active ('detoxification' - Conney et al., 1956; Miller et al., 1958; Conney, 1967) or more active (Kato, 1961; Conney, 1967; Recknagel, 1967; Mitchell and Jollows, 1975) products. In contrast, the effect of conjugation reactions is usually to produce non-toxic, highly water-soluble

and polar compounds that can be easily excreted into urine, and under certain circumstances, into the bile (Millburn, 1976; Hirom et al., 1976). The majority of conjugation reactions are therefore the true detoxication reactions.

Factors Affecting The Metabolism of Xenobiotics

Since the metabolism of xenobiotics is generally controlled by enzymes, any factor which affects the enzymes also affects the metabolism of the compound and consequently its toxicity. Several factors are now known which affect this metabolism, these being species, strain (genetic factors), age, sex, disease, diet, chronic administration, and the administration of other xenobiotics. (Table 1.2). The nature of the gut flora may also be important, since they may play a role in the metabolism of toxic compounds ingested orally or of metabolites secreted in the bile. These organisms may vary from species to species, or even within a given species, dependent upon the diet and environment.

Only those factors influencing the glucuronic acid and sulphate conjugations will be discussed, and these will be dealt with in detail later with the respective reactions.

Table 1.2: Factors that can influence xenobiotic metabolism.

Species	Diet
Sex	Stress
Strain	Temperature
Age	Time of day
Chronic Administration	Route of excretion (e.g. bile, urine)
Presence of Other Xenobiotics	Gut flora
Route of administration	Season
Size of Dose	Altitude
Disease	

(Williams, 1974).

Phase II Reactions

Phase II reactions, or conjugations, as they are commonly

known, are generally regarded as detoxication mechanisms, for compounds which undergo these reactions are converted into products which are usually less toxic and more rapidly excreted than their precursors. The concept of detoxication probably began with the observation of Baumann in 1876 that the sulphate conjugate of phenol was very much less toxic than phenol (Williams, 1959).

This concept of conjugations being 'detoxication mechanisms' is still accepted today, though there are some synthetic reactions which lead to toxic products. For example, the Millers (1966, 1970) and Weisburgers (1973) have demonstrated that the N-hydroxy derivatives of many aromatic amines including azo dyes, naphthylamine and 2-acetylaminofluorene, which were thought to be the active carcinogens may not be the potent arylating agents *per se* but may be further esterified to sulphate conjugates before they will react readily with all nucleophiles to cause cancer. Thus, the administration of acetaminophen which depletes the sulphate stores in the body, prevented the carcinogenic effects of N-hydroxy-2-acetylaminofluorene, whereas the administration of sulphate partially reversed the protective effects of acetaminophen.

Although there are occasional exceptions about conjugation reactions being detoxication mechanisms, the majority of these reactions do diminish the biological activity of xenobiotics and therefore, have important implications in pharmacology and toxicology. The common conjugation reactions that occur in mammals are:

(1) Glucuronic acid conjugation
(2) Sulphate conjugation (ethereal sulphate)
(3) Glycine conjugation
(4) Glutamine conjugation
(5) Taurine conjugation
(6) Mercapturic acid Synthesis (glutathione, cysteine)
(7) Methylation (methionine)
(8) Acetylation
(9) Thiocyanate synthesis (cyanide detoxication),

(Williams and Millburn, 1975;
James et al., 1971;
Hirom et al., 1977).

Moreover, there are some uncommon conjugation reactions which have been demonstrated only in certain species of animals and just for a certain substrate (Table 1.3).

Since conjugations are synthetic reactions, a source of energy is necessary and this is supplied through ATP. These reactions are characterised by the formation of an active intermediate which in most cases is a nucleotide and the occurrence of a transferring enzyme which catalyses the final step between the active intermediate and the conjugation product. There are two kinds of conjugation reactions depending upon whether the conjugating agent is activated (Type A; Table 1.4), or the xenobiotic is activated (Type B; Table 1.5).

Thus, glucuronic acid conjugation is of the first type, the intermediate being UDP-glucuronic acid, and the enzyme, UDP-glucuronyltransferase (E.C. 2.4.1.17). Glycine conjugation is of the second type, taking, for example, benzoic acid as the xenobiotic, the intermediate is benzoyl-CoA, the enzyme, glycine-N-acylase (E.C. 2.3.1.13), and the conjugating agent, glycine. The various enzymes involved in these conjugation reactions and their tissue and subcellular distributions are tabulated (Table 1.6).

Amino Acid Conjugations

As Table 1.6 shows, a number of amino acid conjugations have been described, and moreover, certain uncommon amino acid conjugations also exist (Table 1.7).

The nature of the amino acid utilized in a conjugation reaction varies with the animal species and chemical structure of the particular carboxylic acid undergoing conjugation. (Idle, 1976; Dixon, 1976). Glycine is commonly utilized by a wide range of species for the conjugation of a diverse range

TABLE 1.3: REFERENCES

1. Clapp, 1956
2. Sarcione and Sokal, 1958
3. Schayer, 1956
4. Boyland and Mason, 1966
5. Weinstock, Parker, Lucyszyn and Intoccia, 1969
6. Boyland, Kinder and Manson, 1961
7. Layne, Sheth and Kirdani, 1964.

TABLE 1.3: Uncommon Conjugation Reactions

	CONJUGATING AGENT	SPECIES	SUBSTRATE	REFERENCES *
CONJUGATION AT C- AND S- ATOMS	S-β-D-Glucuronide	Dog	2-mercaptobenzo-thiazole	1
	S-Methyl	Rat	Thiouracil	2
CONJUGATION OF AMINES	β-D-Ribose	Rat and Mice	Imidazol-4-ylacetic acid	3
	Formyl	Dog, rat	2-amino-1-naphthylsulphate	4
	Succinyl	Dog, rat	1-amino-1-(4'-chlorobenzoyl) ethane	5
CONJUGATION OF PHENOLS AND ALCOHOLS	Phosphate	Dog	2-amino-1-naphthol	6
	β-N-Acetyl-D-glucosamine	Rabbit	17-α-oestradiol-3-β-glucuronide	7

* See opposite.

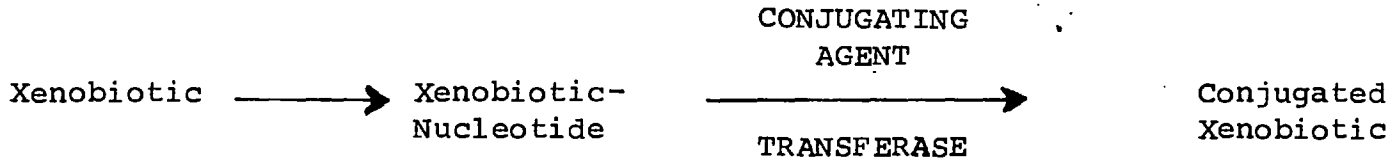
TABLE 1.4: MECHANISM OF CONJUGATION - TYPE A

Conjugating Agent → Conjugating Agent-Nucleotide (XENOBIOTIC TRANSFERASE) → Conjugated Xenobiotic

CONJUGATING AGENT	ACTIVE FORM
Glucuronic acid	Uridine diphosphate glucuronic acid (UDPGA)
Sulphate	Adenosine 3'-phosphate-5'-sulphatophosphate (PAPS)
Methyl group	S-Adenosylmethionine
Acetyl group	Acetyl-CoA
Sulphur	Thiosulphate, S = SO_3H_2

(From Williams, 1975)

TABLE 1.5: MECHANISM OF CONJUGATION - TYPE B



XENOBIOTIC	ACTIVATED XENOBIOTIC	CONJUGATING AGENT
Aromatic acid, ArCOOH	ArCO.CoA	Glycine
Arylacetic acid, $ArCH_2COOH$	$ArCH_2CO.CoA$	Glutamine
Aromatic ring, -CH = CH-	Epoxide -HC-CH- (O)	Glutathione

(From Williams, 1975)

References for Table 1.6:

(1) Dutton (1966)

(2) Roy and Trudinger (1970)

(3) Dodgson, (1977)

(4) Williams and Millburn (1975)

(5) Grover (1977)

TABLE 1.6: ENZYMES INVOLVED IN CONJUGATION REACTIONS AND THEIR TISSUE AND SUBCELLULAR DISTRIBUTIONS

REACTION	TRANSFERRING ENZYME	DISTRIBUTION (SPECIES AND TISSUE)	SUBCELLULAR LOCATION
Glucuronide Synthesis	UDP-glucuronyl-transferases	Many mammalian tissues	Endoplasmic reticulum (1)
Ester Sulphate Synthesis	Sulphotransferases	Many mammalian tissues	Cytosol (2), (3)
Methylation	Methyltransferase	Many mammalian tissues	Cytosol, Endoplasmic reticulum (4)
Acetylation	Transacetylase	Many mammalian tissues	Cytosol, mitochondria (4)
Cyanide Detoxication	Thiosulphate Sulfur-transferase	Many mammalian tissues	Mitochondria (2), (4)
Glycine Conjugation	Glycine Acyl-transferase	Many mammals - Liver and/or kidney	Mitochondria (4)
Glutamine Conjugation	Glutamine Acyl-transferase? *	Man - Kidney and Liver	Mitochondria? (4)
Ornithine Conjugation	Ornithine Acyl-transferase? *	Chicken - Kidney	? (4)
Mercapturic Acid Synthesis	Glutathione-S-transferases	Many mammalian tissues	Cytosol (5)

* Not defined. Numbers refer to references. (See Opposite)

TABLE 1.7: Occurrence of Aminoacid Conjugations

ARYL SUBSTRATE	AMINOACID (PEPTIDE)	SPECIES	REFERENCE
Aromatic acids	Glycine	Mammals, pigeon	Williams (1959) Bridges et al. (1970) Hirom et al. (1977)
Aromatic acids	Ornithine	Birds, Turtle, gecko	Bridges et al. (1970)
Arylacetic acids	Glutamine	Man, dog, primates, rabbit, sheep, cat, rat, guinea pig	Williams (1959) James et al. (1972) Bridges et al. (1974)
Benzoic acid	Glutamic acid	Rat, guinea pig Indian and African fruit bats	Strohmeyer and Klingmuller (1964) Idle et al. (1975) Collins et al. (1977)
Aromatic acids	Arginine	Arachnids	Hitchcock & Smith (1964)
Benzoic acid	Alanine	Rabbit	Schachter et al. (1955)

(continued)

Table 1.7 (continued):

ARYL SUBSTRATE	AMINOACID (PEPTIDE)	SPECIES	REFERENCE
a. Phenylacetic acid b. 1-Naphthylacetic acid c. Indol-3-ylacetic acid	Taurine	a. 23 Mammalian species b. Cat, Man, Primates c. Ferret, certain primates	a. James et al. (1972) b. Dixon et al. (1974) c. Bridges et al. (1974)
Xanthurenic acid	Serine	Rat	Rothstein & Greenberg (1957)
Quinaldic acid	a. Glycyltaurine b. Glycylglycine	Cat	a. Kaihara (1960) b. Kaihara & Price (1961)
Indol-3-ylacetic acid	Aspartic acid	Plants	Andreae and Good (1955)
o,p'-DDA*; o,p'-DDD*	Aspartic acid	Rat	Reif and Sinsheimer (1975)
Benzoic acid	L-Histidine	Peripatus nova zealandiae +	Smith (1977)

*o,p'-DDA = (2-Chlorophenyl) (4'-chlorophenyl) acetic acid
o,p'-DDD = 1-(2'-Chlorophenyl)-1-(4'-chlorophenyl)-2,2-dichloroethane
+ A small terrestrial caterpillar-like animal.

of carboxylic acids. The other mechanisms aremore restricted in occurrence either in terms of species distribution or in the type of acid conjugated. Thus, taurine conjugation occurs in a wide range of species but only for certain arylacetic acids, and ornithine conjugation occurs with a wide range of acids but only in birds and reptiles. The glutamine conjugation on the other hand, is largely confined to conjugation of arylacetic acids by anthropoid species.

The general mechanism of amino acid conjugation involves first the activation of the carboxylic acid, and secondly, the conjugation of the activated xenobiotic with an amino acid, the reaction being catalysed by the appropriate amino acid-N-acylase. Thus,

$$R.COOH + ATP + CoA \xrightleftharpoons[\text{SYNTHETASE}]{\text{ACYL CoA}} R.COS.CoA + PPi$$

$$R.COSCoA + H_2N.\underset{R'}{CH}COOH \xrightarrow[\text{N-ACYLASE}]{\text{AMINO ACID-}} R.CONH.\underset{R'}{CH}.COOH + CoA.SH.$$

The enzyme that has been studied most so far is glycine-N-acyltransferase (E.C.2.3.1.13). This enzyme has been shown to be present in mitochondria from bovine (Schachter and Taggart, 1954), rat (Brandt et al., 1968), and human livers (Tischler and Goldman, 1970). The enzyme has a narrow specificity for the amino acid, i.e. it will only transfer the acyl group from the acyl donor to glycine, but its specificity for the acyl group is less pronounced since a wide variety of aliphatic (C_{2-10}) and aromatic acids (e.g. benzoic, p-aminobenzoic, p-hydroxybenzoic, m-aminobenzoic, phenylacetic, etc.) are conjugated with glycine.

The location of the glycine conjugation system in mitochondria is unusual when it is considered that the majority

of the xenobiotic-metabolising reactions are carried out by the enzymes located in the endoplasmic reticulum. This may be due to the ability of ionic substrates to enter mitochondria whereas penetration through the microsomal membrane usually requires lipid-soluble, non-polar molecules (Smith and Williams, 1966; Idle, 1976). The glycine conjugating system is associated mainly with the liver and kidneys. Relative importance of these two organs can vary markedly with species and structure of the carboxylic acid (Wan and Riegelman, 1972a, 1972b).

Since in amino acid conjugations, the requirement for the amino acid is essential, any factor (e.g. species, diet, dose of xenobiotic) that influences the availability of the amino acid, which is usually derived from diet or endogenous metabolism, will determine to what extent a xenobiotic undergoes this type of conjugation reaction. Amino acid conjugations also compete with glucuronic acid conjugation, a much more common reaction. The extent to which this could occur depends upon several factors such as the dose of the aromatic or arylacetic carboxylic acid, lipid-solubility, ionisation, molecular size and geometry of the substrate molecule, the availability of the appropriate conjugating agents, and the affinity for intracellular conjugation sites (mitochondria or microsomal) and the associated enzymes.

Thus, the extent of glycine conjugation of substituted benzoic acids is influenced by the nature and position of nuclear substitution; in general, m- and p-substituted benzoic acids readily conjugate with glycine but this reaction occurs at a very low capacity or not at all with o-substituted derivatives (Idle, 1976). Similarly, phenylacetic acid and its simple p-substituted derivatives, 1-naphthylacetic acid and indoylacetic acids are all extensively conjugated with glycine in the rat. However, substitution of the α-methylene group, e.g. α-methylphenylacetic acid, leads to loss of affinity for glycine conjugation (Dixon, 1976; Smith, Caldwell and Idle,

1977).

Very little information is as yet available on the developmental aspects of the reaction and the regulation and structure of glycine-*N*-acyltransferase, and for the other acyltransferases much work on most enzymic aspects still needs to be carried out.

Glucuronic Acid Conjugation

The synthesis of glucuronides (β-D-glucopyranosiduronic acids) probably is quantitatively the most important conjugating mechanism in vertebrates for it occurs extensively in Man and most mammals (except the cat), and many xenobiotics with very different functional groups can be metabolised by this reaction (Table 1.8). One of the reasons for the widespread occurrence of glucuronidation is that the conjugating agent, glucuronic acid, can be made readily available from a number of carbohydrate sources in the body.

The historical aspects on the discovery of the mechanism of glucuronide synthesis have been well reviewed by Williams (1959) and Dutton (1961, 1966). Glucuronic acid may be regarded as a D-glucose molecule in which the alcoholic group at C_6 has been oxidized to that of a carboxylic acid. The reducing properties of C_1 still remain, and therefore, the molecule is still able to form glycosides, termed in this case β-D-Glucopyranosiduronic acids, or more commonly, glucuronides. Glucuronide biosynthesis involves 'active' glucuronic acid, uridine 5'-diphosphate-α-D-glucuronic acid (UDPGA) as the glucuronyl donor (Dutton and Storey, 1953; Smith and Mills, 1954; Storey and Dutton, 1955). The elucidation of its chemical structure (Figure 1.1) resolved the mechanism of glucuronide conjugation. It was shown by Strominger *et al.* (1957) that UDPGA was immediately derived from the oxidation of UDP-glucose, nicotinamide adeninedinucleotide (NAD^+) and oxygen being required with the enzyme, UDP-glucose dehydrogenase. UDP-glucose

Table I.8: Types of Groups in Xenobiotics to which Glucuronic Acid can be Transferred

GROUP	GENERAL STRUCTURE	EXAMPLE
I Hydroxyl:		
Phenolic	ArOH*	Phenol, 1-Naphthol
Alcoholic		
Primary	$-CH_2OH$*	Chloramphenicol
Secondary	CHOH*	Butan-2-Ol
Tertiary	COH*	t-Butanol
Enolic	HC=COH*	4-Hydroxycoumarin
Hydroxylaminic	NOH*	N-Hydroxy-2-acetamidofluorene
II Carboxyl:		
Aromatic	$ArCO_2H$*	Benzoic Acid
Aliphatic		
Primary	$-CH_2CO_2H$*	1-Naphthylacetic acid
Secondary	$CHCO_2H$*	Iopanoic acid
Tertiary	CCO_2H*	Trimethylacetic acid

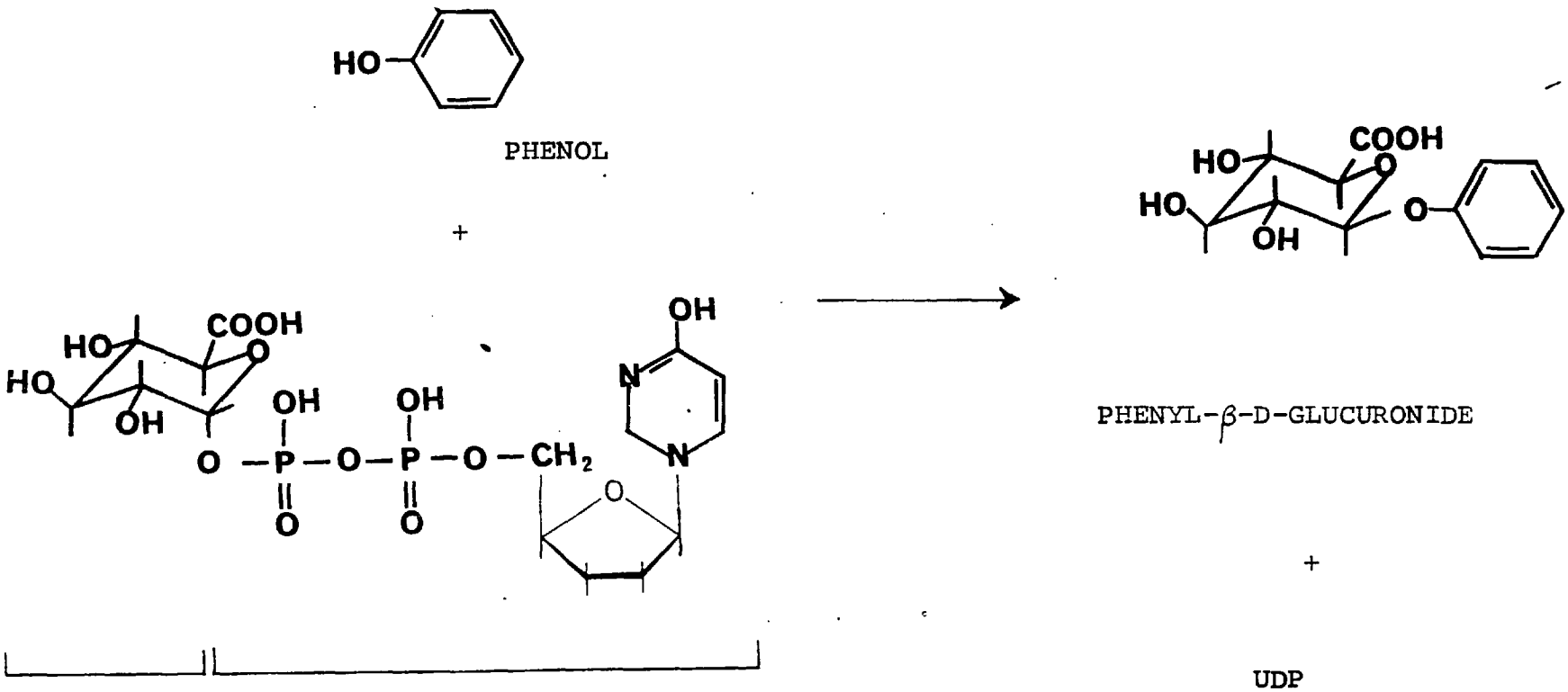
(continued)

Table 1.8: (continued)

GROUP	GENERAL STRUCTURE	EXAMPLE
III Amino:		
Aromatic	$ArNH_2$*	Aniline
Carboxyamide	$-CONH_2$*	Meprobamate
Sulphonamide	$-SO_2NH$*-	Sulphadimethoxine
Heterocyclic	NH	Sulfisonazole
IV Thiol:		
Thiol	-SH*	2-Mercaptobenzothiazole
Carbodithioic	-CSSH*	Diethyldithiocarbamic acid

* The hydrogen atoms labellled thus are replaced by the glucuronic acid residue, $C_6H_9O_6$, when glucuronidation occurs. (From Milburn (1974)).

Figure 1.1 : Glucuronyl Transfer From UDP-glucuronic acid To An Acceptor Substrate.



can be made by the action of a pyrophosphorylase on uridine triphosphate (UTP) and glucose-1-phosphate; UTP is, of course, dependent on the supply of ATP. The overall reaction mechanism can, therefore, be outlined as follows, the various enzymes involved being marked as their EC-numbers:

1(i): Glycogen $\xrightarrow[\text{E.C.2.4.1.1.}]{H_3PO_4}$ α-glucose-1-phosphate

1(ii): α-glucose-1-phosphate $\xrightarrow[\text{E.C.2.7.7.9.}]{\text{UTP}}$ UDP-glucose + PPi

1(iii): UDP-glucose $\xrightarrow[\text{E.C.1.1.1.22}]{NAD^+,\ O_2}$ UDPGA

1(iv): UDPGA + R.OH $\xrightarrow{\text{E.C.2.4.1.17}}$ R.O. glucuronic acid + UDP

E.C.2.4.1.1. - Glycogen pyrophosphorylase (1,4-α-D-glucan: orthophosphate α-glucosyltransferase).

E.C.2.7.7.9. - UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridylyltransferase).

E.C.1.1.1.22 - UDP-glucose dehydrogenase (UDP-glucose: NAD^+ 6-oxidoreductase).

E.C.2.4.1.17.- UDP-glucuronyltransferase (UDP-glucuronate β-glucuronosyl-transferase, acceptor unspecific).

Recently, it has been reported that mammalian species also conjugate endogenous and xenobiotic compounds with sugars other than glucuronic acid, for example with glucose, galactose, xylose, and N-acetylglucosamine (for references see Dutton et al.,1977; Marniemi, 1974), utilizing their corresponding UDP-derivatives as donors in similar glycosyltransferase reactions to glucuronic acid conjugation . These conjugations appear quantitatively much less significant than glucuronidation,

and the enzymes catalysing these transfer reactions are much less known than UDP-glucuronyltransferase. The ability of the animal to synthesize these glycoside conjugations depends on the levels and activities of the respective enzymes and on the amount of the respective UDP-sugars available. Thus, for example, glucosidation of a xenobiotic may become important if the available UDPGA falls. (Gessner et al., 1973).

Tissue Distribution of UDP-glucuronyltransferase

This enzyme is widely distributed in mammalian tissues. Liver appears to be the major site of conjugation but UDP-glucuronyltransferase activity has also been demonstrated in the kidney and gastro-intestinal tract (see Dutton, 1966, 1971; Aitio, 1973a), the lung (De Bernardi et al., 1972; Short et al., 1972; Aitio, 1973b; Aitio et al., 1976), spleen (Aitio, 1974a; Schachter et al., 1959), thymus (Aitio, 1974a), adrenal gland (Aitio, 1974a), and the skin (Dutton, 1966; Rugstad and Dybing, 1975). According to some workers, the placenta performs negligible glucuronidation (Aitio, 1974a;b; Burchell, 1974), whereas others have shown stimulation of UDP-glucuronyltransferase activity in the placenta by phenobarbital (Berté et al., 1969; Lucier et al., 1975b).

Recently, Powell et al. (1974) with their studies in rats fed ^{14}C-phenol have claimed that the gastrointestinal tract is the major site for the conjugation of orally-administered phenol. The gut was also noted earlier as a major site by Dutton (1966) who suggested that as the entire alimentary tract from the mouth to anus may form glucuronides, the contribution from this tissue may be considerable, and probably higher than the liver. Aitio (1973a), however, from his detailed tissue survey in the rat concluded that the rat liver exhibited the highest total glucuronidation capacity. Thus, at present, there is argument over the gut being the main site for detoxication, though it is conceivable that in vivo,

the route of administration of a xenobiotic will have an important role in determining the major site of conjugation (e.g. route of administration and conjugation of phenol by hen, Capel et al., 1974b).

As yet there is no definite evidence concerning tissue-specific UDP-glucuronyltransferases, though there are examples in the literature where the enzyme activity towards several substrates can be much less activated and more readily inactivated, when derived from the intestinal mucosa or the lung than from the liver (Del Villar et al., 1974; Aitio, 1974b; Bock and Winne, 1975, Aitio, 1973c). However, this could be due to a partial activation having already occurred through protease action during the preparation of the intestinal enzyme, or in the case of the lung, the small activation might be due to an initial activation of the enzyme by surfactants already present in the lungs and probably contaminating the microsomes (Aitio, 1973c).

Species Distribution of UDP-glucuronyltransferase

Glucuronide synthesis occurs in most mammals (Dutton, 1966; Hirom et al., 1977), birds (Wit, 1977), amphibians and some reptiles (Williams, 1968), and in fish (Sieber and Adamson, 1977). Marsupials, once thought to be defective in glucuronide conjugation, do form glucuronides, and their high urinary level of free glucuronic acid is due to a high urinary β-glucuronidase activity (Marsh, 1969; Hirom et al., 1977). No glucuronidation has been observed in either plants (Baldwin, 1977) or insects (Smith, 1977), and both these form glucose conjugates.

Glucuronic acid conjugation of phenols and some carboxylic acids, with respect to their occurrence in certain species, namely the rat, cat, pig and some primates will be discussed in conjunction with sulphate conjugation later on.

Occurrence of UDP-glucuronyltransferase during Development

Glucuronic acid conjugation is virtually absent in the early mammalian foetus and approaches adult levels only in the perinatal period, putting the foetus and the newborn at considerable risk from unwise medication or exposure to xenobiotics. Thus, for example, the toxic effects of the antibiotic, chloramphenicol, killed a number of human babies in Germany in 1960 (Williams & Millburn, 1975). The rate of appearance of glucuronide synthesis varies with species, substrate and tissue; the extrahepatic tissues possess their own developmental patterns and may be relatively more important in the perinatal than in the adult animal (see Dutton, 1966; Dutton and Burchell, 1974).

The major causes of this deficiency appear to be a lack of the enzyme UDP-glucuronyltransferase, and possibly also of UDP-glucose dehydrogenase (E.C.1.1.1.22). Various attempts have been made to induce or activate UDP-glucuronyltransferase in cases of prematurity. The enzyme can be activated in the developing liver by several agents, for example, detergents, aging, sonication and diethylnitrosamine (Burchell, 1974). In the neonate and the term foetus, administration of xenobiotic inducers such as phenobarbital has had limited success (Wilson, 1972; Yaffe & Juchau, 1974; Lathe , 1972; Burchell and Dutton, 1975). Recent studies by Wishart and Dutton (1974, 1977) in the chick embryo and mammalian foetus have suggested that the natural perinatal development of glucuronidation and UDP-glucuronyltransferase activity towards certain substrates is brought about by compounds of endogenous origin, these being glucocorticoids.

Cellular Localisation of UDP-glucuronyltransferase

On homogenisation of the liver, or any other tissue, the endoplasmic reticulum of the cell is disrupted giving small vesicles called microsomes; UDP-glucuronyltransferase

appears to be tightly bound to the microsomal membrane (Dutton, 1966). The microsomal fraction can be further subfractionated into 'rough' and 'smooth' components (Palade and Siekevitz, 1956), the former having been termed thus due to attached ribosomes.

The subfractions have not been rigourously investigated for the transferase activity, though Remmer and Merker (1965) correlated the increased activity of several other drug-metabolising systems with the proliferation of the smooth membrane. Gram et al. (1968) studied the submicrosomal distribution of UDP-glucuronyltransferase in the rabbit liver with various substrates, and found that the localisation of enzyme activity in the two subfractions depends upon the xenobiotic substrate. Thus, the activity measured with p-nitrophenol and o-aminophenol as substrates was localised predominantly in the rough-surfaced microsomes, whereas that for phenolphthalein was equally present in rough- and smooth-surfaced microsomes. Mulder (1970) showed that 47% of the rat liver UDP-glucuronyltransferase with p-nitrophenol as substrate was recovered in the rough-surfaced microsomes, which also had a higher specific activity than the smooth-surfaced fraction.

Recent studies by Gorski and Kasper (unpublished, cited in Gorski and Kasper, 1977) have shown the transferase to be an integral part of the nuclear envelope as well.

UDP-glucuronyltransferase as a Membrane-bound Enzyme

Microsomal UDP-glucuronyltransferase is one of the many membrane-bound enzymes whose activity is influenced by membrane structure. Evidence for this, and the possible consequences of this close association with the membrane on the expression of enzyme activity in vivo and in vitro are presented below.

Effect of Membrane Perturbants on Enzyme Activity

The treatment of microsomes with agents that tend to perturb the membrane (Table 1.9) leads to an increase in the measurable UDP-glucuronyltransferase activity. The activation by ageing (Lueders & Kuff, 1967; Winsnes, 1969, 1972 a , 1972 b ; Hanninen and Puukka, 1970, Lucier et al., 1971; Graham and Wood, 1973), and sonication (Henderson, 1970; Vessey and Zakim, 1971; Winsnes, 1972 a , 1972 b) is also apparently due to the uncovering of the enzyme from the membrane which results in the revealing of more active sites of the enzyme accessible to the substrate.

Detergents and chaotropic agents (see Table 1.9) have a biphasic effect on the enzyme activity. Small concentrations enhance activity, but large concentrations inactivate completely. The amounts needed to yield maximum activity depend on the activator used, the source of microsomes, and the past history of the preparation (Graham and Wood, 1973; Lueders and Kuff, 1967). The action of such treatments support the idea that the activity of UDP-glucuronyltransferase is constrained in untreated microsomes.

Effect of Other Xenobiotics on Enzyme Activity

A variety of xenobiotics such as barbiturates (Bock et al., 1973; Gessner, 1976; Hanninen and Aitio, 1968; Jansen and Henderson, 1972; Mulder, 1970, 1971; Potrepka and Spratt, 1971; Vainio, 1973b), polycyclic hydrocarbons (Goldstein and Taurog, 1968; Aitio, 1973a), 1,1,1-trichloro-2, 2-bis (p-chlorophenyl) ethane (DDT; Vainio, 1974b), polychlorinated biphenyls (Vainio, 1974c; Mehendale et al., 1975), 2,3,7,8-tetrachlorodibenzo-p-dioxin (Lucier et al., 1975a; Hook et al., 1975), diethylnitrosamine (Mowat and Arias, 1970b; Nakata et al., 1975), and imipramine and desimipramine (Piper and Hargreaves, 1975) are known to increase transferase activity, probably by induction, for protein synthesis is required (Burchell

Table 1.9: Agents That Perturb The Microsomal Membrane

AGENT	REFERENCES
Surfactants, e.g. digitonin, deoxycholate, Triton X-100, cetylpyridinium chloride, Lubrol.	Lueders and Kuff (1967) Heirwegh and Meuwissen (1968) Hanninen (1968) Hanninen et al. (1968) Van Roy and Heirweigh (1968) Winsnes (1969; 1971a,b; 1972a,b) Mulder (1970, 1971) Attwood et al. (1971) Howland et al. (1971) Lucier et al. (1971) Vessey and Zakim (1971) Graham and Wood (1972, 1973) Vainio and Hanninen (1972) Marniemi (1974)
Chaotropic Agents e.g. NaSCN, KI, KNO_3, Urea	Vainio (1973a)
Organic Solvents	Vainio (1974a)
Bilirubin	Sanchez and Tephly (1973b, 1975) Del Villar et al. (1974)
Trypsin	Hanninen and Puukka (1970) Vainio (1973b) Aitio (1974b) Marniemi (1974)
Phospholipase A Phospholipase C	Aitio (1974b) Hanninen and Puukka (1971) Vessey and Zakim (1971, 1972a) Graham and Wood (1973, 1974) Winsnes (1972a, 1972b) Zakim et al. (1973c) Marniemi (1974)

et al., 1972; Bock et al., 1973).

These compounds also change other membrane-dependent properties such as kinetic behaviour (Winsnes, 1971b; Winsnes and Dutton, 1973) and relative activities between substrates (Bock et al., 1973). Induction of an enzyme would reflect changes in the concentration of either the enzyme protein of another protein which controls the enzyme activity. In the case of UDP-glucuronyltransferase where the membrane plays an important role, the effect of these xenobiotics could be to synthesize the membrane protein rather than the enzyme protein, and thus alter the expression of enzyme activity (Dutton, 1975).

Effect of Diet on Enzyme Activity

The activities of many enzymes are regulated by the dietary protein intake of an animal (Aebi, 1969; Campbell and Hayes, 1974). Therefore, studies have been carried out to determine whether the activity of UDP-glucuronyltransferase which is markedly dependent on the structure and the phospholipid composition of the membrane (Graham and Wood, 1969; Attwood et al., 1971), is also affected by a change in diet.

Thus, protein-deficient rats have enhanced UDP-glucuronyltransferase activity with p-nitrophenol and o-aminophenol as acceptors (Wood and Woodcock, 1970; Woodcock and Wood 1971). The phospholipid composition of the microsomal membrane from protein-deficient rats differs slightly from that of normally fed rats, the most striking difference being in their lysophosphatides content which in the protein-deficient animals comprised 9-14% of the total phospholipids but in normal rats, only 0-2.7%.

Lysophosphatides have a perturbing effect on microsomes (Hanninen and Puukka, 1971 ; Vessey and Zakim, 1972a), thus activating UDP-glucuronyltransferase. The large quantities of lysophosphatides present in protein-deficient rats are

sufficient to have caused the elevated levels of UDP-glucuronyltransferase. Thus, as suggested by these studies, diet-induced changes in the membrane phospholipid composition alter the enzyme activity, and it has been speculated that such changes could contribute to the control of enzyme activity *in vivo*. (Graham *et al.*, 1974b).

The Possible Role of the Microsomal Membrane in enzyme regulation

The activity of UDP-glucuronyltransferase in microsomes is relatively low, and the enzyme system can be overloaded quite easily in the intact animal. The question of the physiological significance of constraint on the activity of UDP-glucuronyltransferase in native microsomal preparation is therefore an important problem. Insight into why the activity of the transferase is less than the maximal potential activity in untreated microsomes depends on an understanding of the regulatory characteristics of this enzyme and the conditions under which it is presumed to function *in vivo*.

One approach to this problem has been the comparison of glucuronidation rates observed in perfused liver (Bock and White, 1974), liver slices (Winsnes and Dutton, 1973) or isolated hepatocytes (Moldeus *et al.*, 1975) with those in fresh microsomes exposed to a 'physiological' concentration of UDPGA and often also of UDP-*N*-acetylglucosamine, an endogenous modifier (Pogell and Leloir, 1961). From such investigations, the transferase of intact endoplasmic reticulum appears more active than in fresh microsomes, though not 'fully' activated, thus allowing positive and negative modulatory control *in vivo*.

The other approach employs a system *in vitro*, carrying out bisubstrate kinetic analysis of fresh and variously-activated microsomal enzymes. These sort of studies have been carried out mainly by Zakim's group (see reviews by Zakim and Vessey, 1974, 1977), and by Winsnes (1969, 1972b), and their

findings suggest that the following control mechanisms may influence the activity of UDP-glucuronyltransferase.

The concentration of UDPGA in the intact liver is small compared with other UDP-sugars (Keppler et al., 1970). For efficient function in vivo, the transferase, therefore, must have a high selectivity for the binding of UDPGA. The untreated form of the enzyme fulfills this requirement in that only UDPGA of all the nucleotide sugars examined, binds to the enzyme. The untreated enzyme, however, is strongly inhibited by UDP, an end-product of the reaction. Since the concentration of UDP in vivo is nearly equal to that of UDPGA (Keppler et al., 1970), end-product inhibition by UDP or UTP (which is also present in the intact liver in large amounts) could severely limit the rate of glucuronidation reactions in the intact animal.

End-product inhibition becomes negligible if UDP-glucuronyltransferase is assayed in the presence of divalent metal ions (e.g. Mg^{++} or Mn^{++}; Zakim and Vessey, 1976) and UDP-N-acetylglucosamine. The latter has all the properties necessary for an ideal modifier of the enzyme: it activates the rate of the forward reaction, inhibits the rate of the reverse reaction, limits end-product inhibition and conserves substrate specificity. (Zakim and Vessey, 1977a). The activation by UDP-N-acetylglucosamine is at least partly due to the inhibition of UDPGA breakdown by UDPGA pyrophosphatase but quite probably it also affects the accessibility to the active site of the enzyme (Pogell and Leloir, 1961; Winses, 1969, 1971a; Vessey et al., 1973b). The efficient function of UDP-glucuronyltransferase in vivo may therefore depend upon allosteric modification by UDP-N-acetylglucosamine.

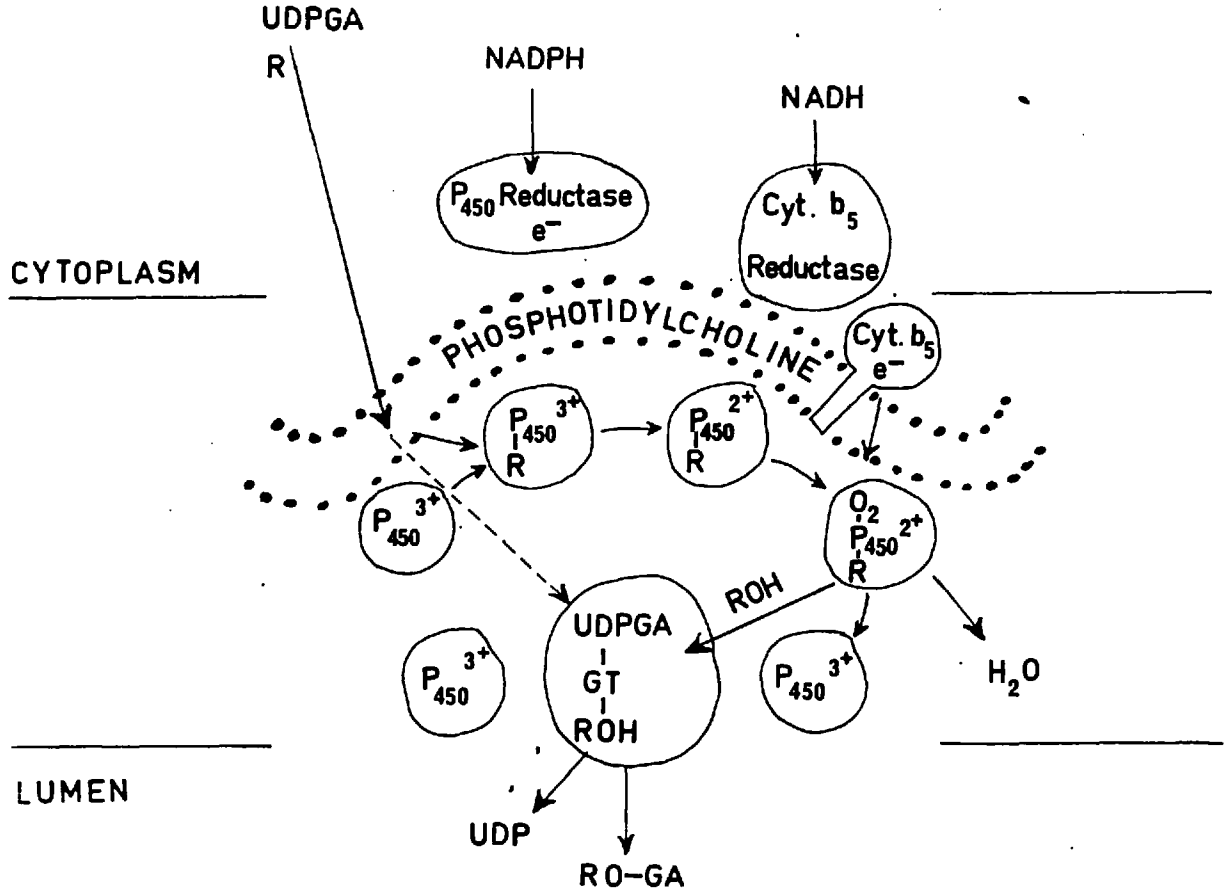
When assayed in the presence of UDP and UDP-N-acetylglucosamine, the phospholipase A-treated form of UDP-glucuronyltransferase has lower activity than the untreated enzyme. These studies therefore conclude that the form of enzyme activated

by membrane perturbants would be inhibited under physiological conditions because it has lost specificity for UDPGA and is more strongly inhibited by UDP. In vivo, transferase activity would thus be higher than in unactivated fresh microsomes but would not correspond to the state of activation achieved in vitro with perturbant procedures. Thus, both the first and the second approaches have reached similar conclusions as to the way in which UDP-glucuronyltransferase activity might be regulated in vivo, though important questions still remain unresolved.

Having established that some constraint operates on the transferase in microsomes, and probably also in vivo, studies have been carried out to determine the nature of this constraint. The first suggestion as to the nature of this constraint is that the enzyme is totally enclosed in the membrane and access to it is dependent on diffusion or transport mechanisms, being unrestricted only after the unpeeling of the membrane by perturbation processes. The studies of Hanninen and Alanen (1966) showing increased inhibition of the transferase by alcohols as they became more lipid-soluble were the first to suggest the location of the enzyme behind a lipophilic membrane. Hanninen and Puukka (1970) have reported that trypsin treatment of rat liver microsomes activates the transferase, the data having been taken as evidence that the active site of the enzyme is within the membrane, covered by a layer of protein. Treatment with trypsin is thought to remove this layer thereby enhancing the access of the substrate to the enzyme. However, activation by trypsin has been observed only in the rat for in the guinea pig, trypsin inactivated the enzyme (Wood and Graham, 1972). Vainio (1973c, 1975) has speculated that UDP-glucuronyltransferase is buried deep within the microsomal membrane, and may be both functionally and topochemically closely related to the microsomal mixed-function oxidase system (Figure 1.2), perhaps forming a vectorially

Figure 1.2: The Proposed Clusterlike Arrangement of the Drug-metabolising Enzymes within the Microsomal Membrane

(After Vainio, 1975)



orientated multienzyme complex in the membrane. Phospholipid is believed to play an important role in the regulation of enzyme activity as various studies have shown (Graham et al., 1974(a); Berry et al., 1975, 1976; Jansen and Arias, 1975), and as suggested by Vainio (1975), the phospholipid bilayer could affect the enzyme in two ways:

allotopically, i.e. the membrane as a matrix may modify the chemistry of the protein, or

topologically, i.e. the membrane as a physical structure may orientate the molecule in a specific direction.

As far as the xenobiotic-metabolising enzymes are concerned, both allotopy and topology seem to be involved in activity control. The lipid surrounding the enzymes might also facilitate the metabolism of lipid-soluble xenobiotics by concentrating them at the active sites of the enzymes (Bock and White, 1974; Vessey and Zakim, 1975).

UDPGA is synthesized in the cytosol and because free penetration by UDPGA (a charged molecule) of a lipid membrane is not likely (Nilsson et al., 1973; Jato-Roderiguez and Mookerjee, 1974; Depierre and Dallner, 1975), Winsnes (1972a) suggested a permease to be involved, with UDP-N-acetylglucosamine acting, not as previously suggested, as an allosteric effector, but assisting this permease. This concept of compartmentation has been further elaborated by Berry and Hallinan (1974), and Berry (1975), who in their 'coupled transglucuronidation' studies found that o-aminophenol was glucuronidated faster with added UDP and p-nitrophenyl glucuronide than with added UDPGA; they proposed that since UDPGA is excluded from the inside of the vesicle,the UDP-dependent hydrolysis of p-nitrophenyl glucuronide generates a greater amount of UDPGA on the inside of the vesicle than can be achieved by the diffusion of this substrate from the cytosol. A serious difficulty with this interpretation is that p-nitrophenyl glucuronide has a direct activating effect

on the enzyme catalysing glucuronidation of o-aminophenol and other aglycones, and it does so by enhancing the affinity of the enzyme for UDPGA (Vessey and Zakim, 1974).

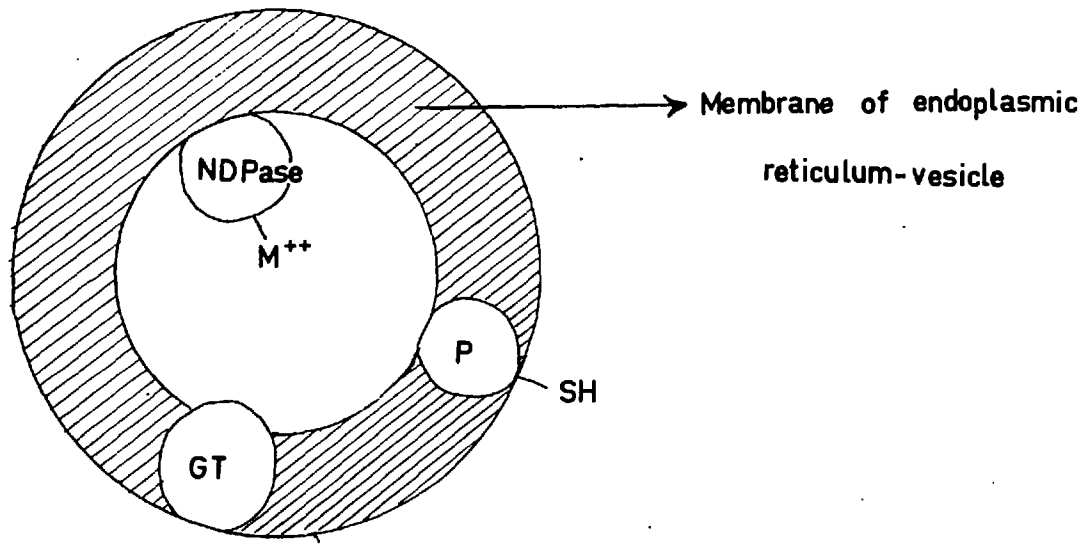
Berry and Hallinan (1977) have further proposed a selective 'UDPGA-permease' which is suggested as the SH-dependent site for the 'glucuronyl-transferase effector', UDP-N-acetylglucosamine (figure 1.3). Nucleoside diphosphatase (NDPase), which is probably also a cisternal enzyme of the endoplasmic reticulum, may have as one of its functions, the divalent metal ion (M^{++}) dependent hydrolysis of UDP, thus helping to make UDP-glucuronyltransferase irreversible *in vivo*.

An attractive feature of the compartmentation model of activity is its apparent simplicity. However, treatments with membrane perturbants have more complex effects on the kinetic parameters of UDP-glucuronyltransferase than a simple increase of enzyme activity (Zakim and Vessey, 1975a), each treatment producing a form of enzyme with unique kinetic properties which differ from the enzyme in untreated microsomes.

Thus, Zakim's group argues instead that the transferase exists in several different conformational isomers, each with unique kinetic properties (Vessey and Zakim, 1971; Zakim and Vessey, 1977). According to this view, the conformation of UDP-glucuronyltransferase would depend in part on lipid-protein interactions, and altering the nature of these interactions by modification of the lipid phase would produce a redistribution between the conformational isomers of the enzyme. Although the conformational model has not been proven by direct evidence, it accounts for the differential effects of membrane perturbants as well as the binding of substrates and inhibitors, the sensitivity to allosteric effectors, the reactivity of -SH groups and the temperature-dependence of enzyme activity (Zakim and Vessey, 1977), which the compartmentation model cannot do.

The conformational mechanism is not unusual, as the results obtained with several membrane-bound enzymes in pro-

Figure 1.3: A Three-Component Regulatory Model For UDP-Glucuronyltransferase (From Berry and Hallinen, 1977).



caryotic and eucaryotic cell membranes suggest that the regulation of allosteric behaviour by membrane lipid could be a general biological phenomena (Farias et al., 1975). The membrane lipids are known to be in a dynamic state of equilibrium (Singer, 1971), and in vivo local changes in the lipid composition mark a possible allosteric control. Such changes would be more rapid and also less expensive for the cells than the total synthesis de novo of an enzyme or a membrane adapted to new metabolic events.

Thus, at present, there are arguments for and against both the compartmentation and conformational models for the regulation of UDP-glucuronyltransferase activity by the membrane. The classical approach to the study on enzyme is to first obtain a pure enzyme, but the work on UDP-glucuronyltransferase illustrates that in order to understand the molecular basis of enzyme function in vivo, the presence of the membrane environment which is an integral part of the regulatory mechanism of UDP-glucuronyltransferase, is a necessity, since the removal of the enzyme from this environment produces modified forms of the enzyme.

The Heterogeneity of UDP-glucuronyltransferase

So far it is generally believed that several forms of transferases exist. As already stated previously, there are a variety of functional groups participating in glucuronidation reactions and within each group the number of different types of substrates is large. There is uncertainty, for example, about the number of specific enzymes catalysing the glucuronidation of phenolic aglycones. The realisation that the uniqueness of the properties of UDP-glucuronyltransferase is related in large part to its location within the membrane has made the heterogeneity of the enzyme questionable.

The idea of heterogeneity in terms of there being several substrate-specific types of transferases has mainly arisen from indirect evidence, this having been listed in Table 1.10.

TABLE 1.10: Evidence for UDP-glucuronyltransferase Heterogeneity

SOURCE OF EVIDENCE	REFERENCES
Species Differences, e.g. Cat and the Gunn Rat- Have the ability to conjugate some xenobiotics and not others.	Dutton and Greig, 1957 Jansen and Henderson, 1972 Jacobson et al., 1975 Hirom et al., 1977
Developmental Studies, Differences in rates of appearance of UDP-glucuronyltransferase activity in many species.	Dutton et al., 1964 Dutton, 1966a
Differences in submicrosomal distribution.	Gram et al., 1968 Howland and Burkhalter, 1970
Differences in response of rate of conjugation to metals and other compounds including membrane perturbants.	Howland et al., 1971 Zakim et al., 1973a; 1973b Tomlinson and Yaffe, 1966 Vessey et al., 1973a Halac and Reff, 1967
Selective stimulatory effects on microsomal UDP-glucuronyltransferase of enzyme inducers such as 3-methylcholanthrene and phenobarbital.	Bock et al., 1973 Sanchez and Tephly, 1973a Jacobson et al., 1975
Kinetic Studies, (i) Absence of mutual inhibition of glucuronidation between various substrates. (ii) Absence of mutual product inhibition.	(i) Dutton ,1956 Lathe and Walker, 1958 Storey, 1965 Temple et al., 1968 Sanchez and Tophly, 1973a (ii) Zakim et al., 1973a Vessey and Zakim, 1974
Separation of some enzyme activities from each other during purification.	Vollrath et al., 1974 Del Villar et al., 1975 Jansen and Arias, 1975

As suggested by Mulder (1974) and further developed by Dutton and Burchell (1976), the microsomal enzyme heterogeneity may be due to:

(i) Sequential heterogeneity, i.e. due to differences in the primary protein structure and detectable only by isolation and amino acid sequencing of the different enzyme proteins.

(ii) Conformational heterogeneity, i.e. due to changes in the tertiary or quaternary protein structure, with or without an associated change in the physical state or composition of the attached lipid, thus suggesting a possibility that a single protein may exhibit changes in substrate specificity on changes in the micro-environment of the enzyme.

(iii) Accessional heterogeneity, i.e. due to differential access of substrates or modulators to a single enzyme protein through a micro-environment that could be constantly changing with time. Thus, different xenobiotics, e.g. o-aminophenol and p-nitrophenol have very different physico-chemical properties, and therefore, each of these may have different influences on the micro-environment of the enzyme, thereby showing differences in the glucuronidation rates of the two substrates (Mulder, 1974; Mulder and Van Doorn, 1975). Therefore, the heterogeneity of the enzyme may be best studied by using substrates endogenous to the species or occuring in their natural diet (Dutton, 1971).

(iv) Artefactual heterogeneity, i.e. the techniques that are employed to study the enzyme heterogeneity are very limited mainly due to the very close association of the enzyme with the membrane, and these may therefore produce erraneous data towards the heterogeneity of the enzyme.

Thus, the above classification suggests that there are two possibilities concerning the transferase heterogeneity: either that there may be different enzyme proteins for different substrates, or that different enzyme activities may

result from there being a single enzyme protein in different micro-environments.

Having reviewed the possible reasons for UDP-glucuronyltransferase hetergeneity, we may now consider the validity of the evidence (see also Dutton et al., 1977) that has been put forward to show that there may be several forms of the enzyme (Table 1.10). Most of this evidence is indirect and does not establish that there is a family of transferases for the glucuronidation of phenols, though it is difficult to reconcile with the existence of a single enzyme.

Differences in enzyme activity between different species, strain or tissue could indicate that the enzyme protein or its constraining membrane differs genetically, as for example between the rat and the chick, which would perhaps be expected. Such studies, therefore, do not contribute much towards assesing the heterogeneity of the enzyme, though genetic defects, for example the Gunn rat, (see later) may help in the understanding of the enzyme mechanism and the way in which the membrane environment may influence the enzyme activity (Vessey and Zakim, 1972a; Zakim et al., 1973d; Nakata et al., 1975, 1976).

Evidence from other sources like developmental studies, and studies with inducers, membrane perturbants and other chemicals is of little value since all of these factors could involve membrane changes of various degrees; and therefore, suggest different substrate-specific enzymes when one could only be observing membrane-dependent changes in the specificity of the enzyme. Kinetic studies, on the other hand, lead to multiple interpretations with membrane-bound enzymes (Engasser and Horvath, 1974), specially when such studies are carried out with crude enzyme systems since in such systems many factors such as physico-chemical properties of the substrates (Mulder, 1974; Mulder and Van Doorn, 1975) and their effects on the membrane, and possible compartmentation and microsomal sequestration of substrates (Vessey and Zakim,

1975) could play a role in giving misleading evidence towards heterogeneity.

Thus, as the fore-going suggests, the microsomal membrane to which UDP-glucuronyltransferase is bound has so far acted as 'barrier' for providing definite evidence for heterogeneity. The only valid evidence for different forms of enzyme would be provided by physical separation of the various enzyme activities, and at present the few examples that we have are separable activities that have been demonstrated towards p-nitrophenol and o-aminophenol (Howland et al., 1971), and p-nitrophenol and bilirubin (Jansen and Arias, 1975), though some of these activities could not be obtained in an active form. Vollrath et al., (1974) from their studies on pig intestinal oestrogen glucuronyltransferase have suggested that this enzyme may exist in the microsomal membrane in different forms or states of combinations with lipids and protein or as isoenzymes. Thus, it is hoped that soon direct evidence for the heterogeneity of UDP-glucuronyltransferase will be provided with a highly purified enzyme, though at present this appears to be a little difficult.

Purification of UDP-glucuronyltransferase

It is because the transferase is very closely associated with the endoplasmic reticulum that attempts to separate active forms of the enzyme have been unsuccessful. Various studies claiming UDP-glucuronyltransferase purification have been published (Mowat and Arias, 1970a; Gregory and Strickland, 1973; Berry 1975; Howland et al., 1971; Rao et al., 1972; Bock et al., 1973; Lucier et al., 1975a; Puukka et al., 1975; Del Villar et al., 1975; Graham et al., 1974b; Jansen and Arias, 1975; Hanninen et al., 1974; Vollrath et al., 1974; Halac and Reff, 1967). However, none of these methods have satisfactorily purified the enzyme though these attempts have yielded little better purification than the original study of Isselbacher et al. (1962).

Recently, Burchell and Burchell (1976), using successive ion-exchange chromatography have succeeded in associating enzyme activity with only six bands of protein. Gorski and Kasper (1977) have also purified p-nitrophenol conjugating activity from rat liver 150- to 200-fold. The enzyme was purified as a phospholipid-protein complex and was shown to consist of a single polypeptide chain of M.W. 59000. Amino-acid analysis indicated approximately 531 mol of amino acid per 59,000g of enzyme and a molar ratio of nonpolar to polar residues of 1.08. The active lipo-protein complex showed both size and charge heterogeneity, and three forms of the enzyme could be resolved by isoelectric focusing. However, the poly-peptide composition of these electrophoretically distinct phospholipid-protein complexes were indistinguishable suggesting that the charge heterogeneity may be the result of different phospholipid contents of the complexes.

Sulphate Conjugation

It seems certain that sulphur has played an important biological role since the beginnings of life on Earth. It is an extremely reactive element and participates in an extraordinary range of chemical linkages in a wide variety of biological compounds. Sulphur, as a constituent of amino acids such as cysteine and methionine and of cofactors such as biotin, thiamin, Coenzyme A, and lipoic acid is essential for the structure and function of all living cells. There are, however, many other sulphur-containing compounds, the functions of which are still ill-defined. For example, isothiocyanates, sulphones, sulphoxides and sulphonic acids all of which occur widely in plants have not as yet been ascribed precise roles. A similar situation occurs with naturally occurring sulpho-conjugates, only a few of which have well-established biological functions.

Sulphoconjugates constitute a very diverse and widespread group of compounds, these being found in micro-organisms,

lower and higher plants, and throughout the animal world. The sulphoconjugates exist in a variety of linkages, namely, $P-O-SO_3^-$, $N-SO_3^-$, $N-O-SO_3^-$, $S-SO_3^-$, and $C-O-SO_3^-$. Table 1.11 lists these various types of linkages together with examples and the roles that they may play in living organisms.

Source of sulphate for conjugation

Microorganisms and plants can obtain inorganic compounds of sulphur directly from the environment, and subsequently transform them into organic forms. Animals, on the other hand, require preformed sulphur-containing amino acids and vitamins. For the most part, their requirements for sulphate are met by oxidation of the sulphur-containing amino acids. The only exception to this generalisation is that ruminants, and probably also birds, can utilize inorganic compounds of sulphur through their symbiotic association with micro-organisms in their digestive tract; whether this occurs in other mammals as well is not known.

Evidence has been produced which suggests that L-cysteine and L-methionine are more effective starting points as sources of sulphate than inorganic sulphate *in vivo* (Wellers, 1964; cited by Dodgson, 1977). Thus, Roe (1971) has shown that either L-methionine or inorganic sulphate can supply the sulphur for the synthesis of indoxyl sulphate following the administration of indole to rats, but that L-methionine is required to prevent the toxicity of large doses of indole. Since such conclusions are based on feeding experiments, the results probably reflect the relatively inefficient gastro-intestinal absorption of L-cysteine or L-methionine. In fact, Astudillo *et al*. (1964, cited by Dodgson, 1977) have shown that PAPS may be involved in the transport of sulphate through the intestinal wall in the rabbit, presuming, of course, that L-cysteine and L-methionine serve as sulphur donors for PAPS synthesis. Thus, higher animals, for their source of inorganic sulphate, would

TABLE 1.11: Various Types of Linkages Occurring In Sulphoconjugates

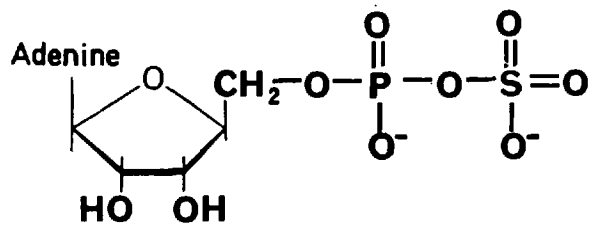
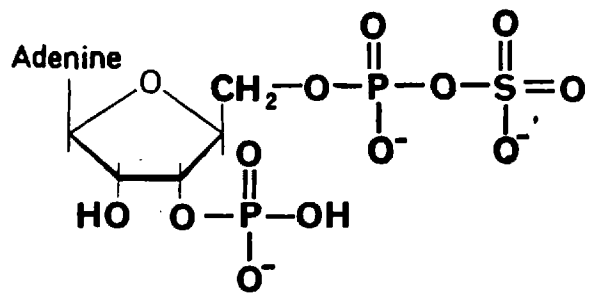
LINKAGE TYPE	EXAMPLES OF CONJUGATES	POSSIBLE ROLE	OCCURRENCE
$P-O-SO_3^-$	PHOSPHOSULPHOCONJUGATES: Adenosine-5'-Phosphosulphate (APS):  3'-Phosphoadenosine-5'-phosphosulphate (PAPS):  	'Activated' forms of sulphate. (1) Both have a role in biosynthesis of all other sulphoconjugates. (2) A vital role in general recycling of sulphur in nature.	Wide variety of micro-organisms, Animals, Plants.

TABLE 1.11 (contd.): Various Types of Linkages Occurring in Sulphoconjugates

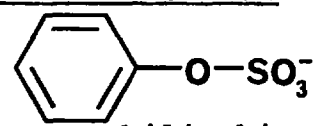
LINKAGE TYPE	EXAMPLES OF CONJUGATES	POSSIBLE ROLE	OCCURRENCE
$C-O-SO_3^-$	(I) ARYLSULPHATE ESTERS - Compounds having a phenolic hydroxyl group: e.g. Ester sulphate esters of: Phenol  Also of: Oestrogens, bilirubin, tyrosine, indoxyl, Serotonin, 3:5-3'-Triodo-L-thyroxine	(1) Detoxication of xenobiotics. (2) Little information on role of naturally occurring conjugates.	Invertebrates, Birds, Reptiles, Amphibia, Mammals.
	(II) ALKYLSULPHATE ESTERS (1) Steroid Alkyl Sulphates e.g. Cholesteryl sulphate Bile Alcohol sulphates. (2) Simple Alkyl Sulphates e.g. (a) Choline-O-Sulphate (b) Sulphates of alcohols	(1) Detoxication or end-products of steroid metabolism. (2a) As non-ionised stores of sulphur. (2b) Detoxication	ANIMALS (2a) Plants and Micro-organisms (2b) Amphibia and Mammals.

TABLE 1.11 (contd.): Various Types of Linkages Occurring in Sulphoconjugates

LINKAGE TYPE	EXAMPLES OF CONJUGATES	POSSIBLE ROLE	OCCURRENCE
$C-O-SO_3^-$	(III) CARBOHYDRATE SULPHATE ESTERS: e.g. Glycolipid sulphate esters; Sulphated glycosamino-glycans; Sulphated polysaccharide chains	Structural components of living organisms.	Most living organisms.
$N-SO_3^-$	ARYLAMINE SULPHATE ESTERS OF: e.g. Aniline, 2-Naphthylamine, tyramine, serotonin, noradrenanline, heparin and heparan.	(1) Detoxication (2) For heparin, the $N-SO_3$ grouping is essential for anticoagulant action in vivo.	Spiders Mammals
$N-O-SO_3^-$	Mustard-oil glycosides - Sulphate esters of substituted hydroxylamines.	?	Plants
$S-SO_3^-$	S-sulphate conjugates of cysteine and glutathione	Role obscure. S-sulphoconjugate of glutathione is a component of the lens of the eye	Animals

be expected to be very much dependent on the intake of these organic sulphur compounds, and their subsequent catabolism to provide inorganic sulphate.

The metabolic sequence for the activation of inorganic sulphate leading to the synthesis of PAPS, was clarified almost entirely by the work of Wilson and Bandurski (1956) and Robbins and Lipmann(1956 , 1958a), who showed that two enzymes, ATP-sulphurylase and APS-kinase, were involved, these two enzymes together making up the sulphate-activating system. (Details of historical aspects-Bostrom and Vestermark, 1961; De Meio, 1975; Roy and Trudinger, 1970).

Irrespective of the chemical nature of the final sulphated product, sulphoconjugation follows the same general pathway:

2(i) activation of inorganic sulphate to yield adenosine-5'-phosphosulphate (APS):

$$ATP + SO_4^{=} \underset{}{\overset{\text{E.C.2.7.7.4}}{\rightleftharpoons}} APS + PPi;$$

2(ii) activation of APS to 3'-phosphoadenosine-5'-phosphosulphate (PAPS):

$$APS + ATP \xrightarrow{\text{E.C.2.7.1.25}} PAPS + ADP;$$

2(iii) the transfer of sulphate group from PAPS to a suitable acceptor (e.g. a phenolic group, R-OH), forming the sulphate ester and adenosine 3', 5'-diphosphate (PAP),

$$PAPS + R\text{-}OH \xrightarrow{\text{E.C.2.8.2.1}} PAP + R\text{-}O\text{-}SO_3^{-}.$$

E.C.2.7.7.4 - ATP - Sulphurylase (ATP: Sulphate Adenylyltransferase.

E.C.2.7.1.25 - APS - Kinase (ATP: Adenylylsulphate 3' -Phosphotransferase).

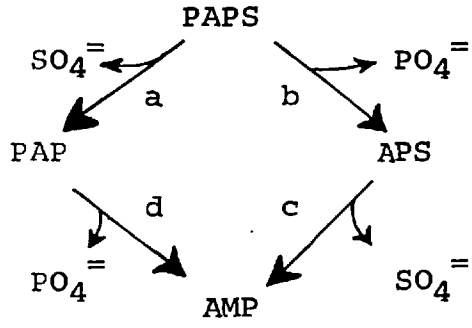
E.C.2.8.2.1 - Aryl (or Phenol) Sulphotransferase (3'-Phosphoadenylylsulphate: Phenol Sulphotransferase).

The first step, the splitting of ATP by inorganic sulphate, has an equilibrium unfavourable to the formation of APS. This indicates that the sulphate in this bound form has a high group potential, far above that of the high-energy phosphate in ATP. As a result, the formation of PAPS will occur to a small extent, and APS will be converted to PAPS in appreciable amounts only because the products of the reaction, APS and pyrophosphate are removed by subsequent reactions. The enzyme catalysing the second step (APS-kinase) has such a high affinity for APS that it can act on the small concentrations of this substrate normally present, and this characteristic of the enzyme helps to drive the first step. (Robbins and Lipmann,1958a, 1958b).

The enzymes catalysing sulphate activation are thought to be present in most mammalian tissues based on the assumption that they are present in tissues which form PAPS and sulphate ester since synthesis of sulphate esters via PAPS is the only known pathway for this conjugating mechanism (Roy and Trudinger, 1970). In the cell, the enzymes for sulphate activation are present in the cytosol.

The knowledge of the mechanism involved in the breakdown of APS and PAPS is of physiological importance for the understanding of the control of sulphate activation and in the study of their biosynthesis and utilisation in tissues. This also has practical implications especially when crude tissue preparations are used for synthesis of PAPS or as a source of enzyme for sulphoconjugation since these enzymes are present in similar parts of the cell as the PAPS-synthesising and sulphoconjugating enzymes. Dodgson and Rose (1975) have recently summarised the nature of these hydrolytic enzymes, and have established the existence in the cytosols of bovine and rat liver of at least two alternative enzymic routes for PAPS degradation to AMP (Figure 1.4).

Figure 1.4



(From Dodgson & Rose, 1975)

Enzymes:

a - PAPS-sulphohydrolase
b - PAPS-3'-nucleotidase and/or PAPS-phosphohydrolase
c - APS-sulphohydrolase
d - PAP-phosphohydrolase.

Different APS- and PAPS-degrading enzymes have also been shown to be present in lysosomes (Fry and Koritz, 1972) as well as associated with the particulate components of the cell (Austin et al., 1967). At present, the role of the hydrolytic enzymes is regarded as participating in the degradation of active sulphate to produce AMP though their precise physiological role and the significance of their presence is unknown. (Dodgson and Rose, 1975).

The Phenolsulphotransferases

Enzymes transferring sulphate from PAPS to a suitable acceptor are known as sulphotransferases. These are very widely distributed in nature and although there is uncertainty as to the exact number of enzymes that exist, due to such diversity of substrates that can be sulphated (Table 1.11) leaves little doubt that there must be a multiplicity of sulphotransferases with different degrees of specificity. Few of these enzymes have been purified because they are difficult enzymes to study, being unstable and far from easy to separate from one another. Here, only the sulphotransferases that are involved in the conjugation of phenolic xenobiotics will

be considered, though it is uncertain at present whether these transferases differ from those involved in the sulphation of endogenous phenolic compounds.

Cellular Localisation

The sulphotransferases involved in the conjugation of phenols, along with those for oestrogens, androstenolone, aetiocholanolone, testosterone, simple alcohols, arylamines, lactose, neuramine lactose and ascorbic acid are located in the cytosol. Some sulphotransferases (e.g. for the formation of chondroitin-4-sulphate, heparin and heparan sulphate) are not expected to play a role in the conjugation of xenobiotics. Thus, the transferases responsible for the sulphation of small molecules are freely soluble in the cytosol, whereas those involved in the assembly oflarger molecules are arranged in assembly-line fashion on the membrane of the endoplasmic reticulum and Golgi apparatus (Dodgson, 1977).

Species Distribution

From the indirect evidence that is available on the presence of ester sulphates in the excreta, it is considered that phenolsulphotransferases are present in all mammals, including several species of whales (Schmidt-Nielsen and Holmsen,1921). The activity of these enzymes vary in different species. According to Stekol (1936), the conjugation of iso-barbituric acid with sulphate, and similarly, the conjugation of various phenols (Capel et al., 1972, 1974a)has been found to be low in pigs. Birds, amphibia, molluscs, invertebrates and fish form sulphoconjugates as well (De Meio, 1975; Roy and Trudinger, 1970; Sieber and Adamson, 1977).

Tissue Distribution

In mammals, the sulphating activity is present in many tissues including the liver, adrenals, kidney, spleen, intestine, brain and lung (Holcenberg and Rosen, 1965; Bostrom and Wengle,

1967; Balasubramanian, 1975; Wong, 1976a;Hidaka and Austin, 1972; Cohen et al., 1976).

Occurrence during Development

Various studies have indicated that sulphoconjugation of vital structural components occurs in foetal life but that the appearance of phenol- and steroidtransferases is delayed until the late foetal stage (Percy and Yaffe, 1964; Krasner and Yaffe, 1967; Wengle, 1966; Carroll and Spencer, 1965; Spencer and Raftery, 1966; Moore, 1970; Carroll and Armstrong, 1976). During two or three weeks following birth, the activity increases four- to five-fold and then declines to adult levels.

Recent studies during development of rat intestinal and rat liver phenolsulphotransferases with p-nitrophenol as acceptor have shown that activity in both tissues are absent in the foetus five days before birth but rise rapidly thereafter. However, in the case of the gut, activity falls to zero between day before birth and birth. No activity is detected over the period of next three days. The reason for such a dramatic loss in activity has been suggested to be a presence of an inhibitor which makes its appearance at or near the time of birth. The nature of such an inhibitor has not as yet been ascertained, and its physiological significance is not known.(Dodgson, 1977).

Enzyme purification, heterogeneity and regulation

Phenolsulphotransferases catalyse the type of reaction represented by Equation 2(iii). In the case of simple arylsulphates, e.g. phenyl or naphthyl sulphates, the reaction is essentially irreversible. However, if the 'sulphate group potential' is high, as in p-nitrophenylsulphate or the dinitrophenyl sulphates, the reaction is reversible, thus:

$$\text{p-NPS} + \text{PAP} \xrightarrow{\text{Phenolsulphotransferase}} \text{p-NP} + \text{PAPS}$$

(p-NPS = p-nitrophenylsulphate; p-NP = p-nitrophenol).

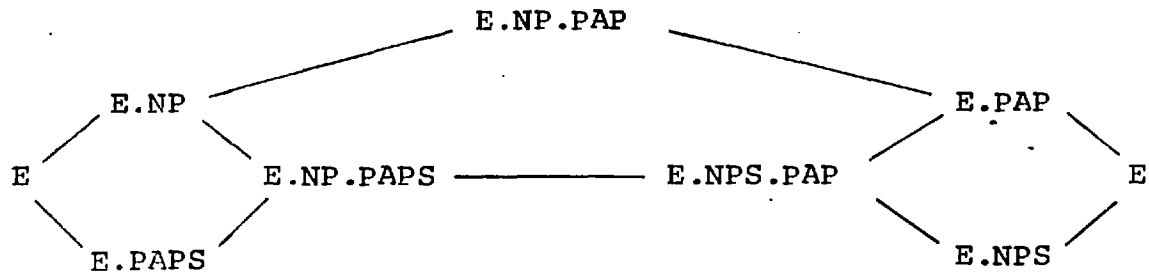
Gregory and Lipmann (1957) and Brunngraber (1958) have shown that in such a system if an additional sulphate acceptor, the 'sulphate potential' of which is far below that of p-NPS is also provided (e.g. phenol or m-aminophenol), with PAP as a cofactor, then the phenolsulphotransferase can catalyse the transfer of sulphate from p- NPS to the phenol, thus:

$$\text{p-NPS} + \text{phenol} \xrightarrow[\text{PAP}]{\text{Phenolsulphotransferase}} \text{p-NP} + \text{phenylsulphate.}$$

They have also suggested that such a reaction can be used to assay either PAP or phenolsulphotransferase, though its general validity is doubtful following the failure of Bannerjee and Roy (1968) to achieve a similar transfer with a purified phenolsulphotransferase from guinea pig liver.

How many individual phenolsulphotransferases occur in mammalian tissues is not known. From the considerable variations that have been reported in their properties, it would appear that there are more than one enzyme. With these enzymes, even accurate kinetic data are not available because very frequently the assays have not been carried out with known concentrations of the cofactor, PAPS, but rather the latter has been generated under arbitrary conditions, by a sulphate-activating system present during the actual assay of the enzyme. In such systems, and also when crude enzyme systems are used, PAP, which may inhibit the enzyme, and the PAPS-degrading enzymes, which may compete with phenolsulphotransferase for PAPS, are likely to be present making such procedures quite valueless for kinetic work. However, with the commercial availability now of ^{35}S-labelled PAPS (Davis, 1975), and of chemical synthetic procedures (Cherniak and Davidson, 1964) and recently, of facile synthesis (Horwitz et al., 1977) for the preparation of PAPS, it should now be possible to obtain reliable kinetic data on the sulphotransferase.

The sulphotransferase isolated and purified from guinea pig liver is the only example of this class of enzymes which has been investigated in any detail from the standpoint of reaction kinetics (Bannerjee and Roy, 1968). The enzyme has a pH optimum of 5.6, the enzyme being very unstable at this pH; it does not require Mg^{++}, it is a sulphydryl enzyme sensitive to metals or oxidation, and it is not inhibited by EDTA. The estimated molecular weight is 70,000. The studies of the forward and reverse reactions (with p-nitrophenol as substrate) show that there are independent binding sites for the phenol and PAPS, and that the reaction catalysed by the enzyme is of a rapid equilibrium random bi-bi reaction (Cleland's nomenclature, 1963) with one dead-end ternary complex of enzyme-3'-phosphoadenylic acid-p-nitrophenol. The reaction can be represented thus:



The specificity studies of Bannerjee and Roy (1968) show that of the various phenols tested (i.e. p-nitrophenol, 1-naphthol, 2-naphthol, 5,6,7,8-tetrahydro-2-naphthol, 4-nitro-1-naphthol, 2-phenanthrol, equilin and equilenin) only oestrone is not a substrate of the enzyme, suggesting that only phenols having an essentially planar structure can combine with the enzyme. It is possible that oestrone sulphate was formed by this enzyme but at a rate too low to be detected by the methods used (Bannerjee & Roy, 1968).

Attempts that have been made to purify the phenolsulphotransferases and study their properties are as follows. Nose

and Lipmann (1958) partially separated the sulphotransferases of rat liver that were responsible for the formation of dehydroepiandrosterone sulphate, oestrone sulphate and p-nitrophenylsulphate but these enzymes were not further characterised. Carroll and Spencer (1964) also separated two groups of enzymes, one of which catalysed the formation of dehydroepiandrosterone and p-nitrophenyl sulphates, and the other of alkyl sulphates and aryl sulphamates. However, they were unable to obtain any separation of the various enzyme activities in rabbit liver.

Bannerjee and Roy (1966) have obtained, by chromatography on DEAE-Sephadex, from guinea pig liver, three fractions showing phenolsulphotransferase activity. One of these is a true phenolsulphotransferase in that it only catalyses the sulphation of p-nitrophenol, while the other peaks of activity are associated with androstenolone and oestrone sulphotransferases. They also suggest that both the latter enzymes also sulphurylate p-nitrophenol and 2-naphthylamine. Similar findings have been made with rat liver (Mattock and Jones, 1970; Barford and Jones, 1971) and bovine kidney (Carroll and Armstrong, cited by Dodgson, 1977). In each case, there is a major peak of enzyme activity which can be separated from activity towards oestrone and dehydroepiandrosterone. However, the major peak shows activities towards simple phenols (e.g. p-nitrophenol) as well as towards L-tyrosine methyl ester and tyramine.

McEvoy and Carroll(1971), on the other hand, have been successful in obtaining a highly purified enzyme from rat livers which showed activity only towards simple phenols with no activity towards either steroids, aliphatic alcohols, aromatic amines or L-tyrosine methyl ester, though this study has also contributed towards the recent view that a single enzyme may be involved in the sulphation of p-nitrophenol and L-tyrosine methyl ester or tyramine as shown below. Thus, the above

(McEvoy and Carroll's) studies showed that glutathione was a potent inhibitor of the purified phenolsulphotransferase but had no effect on the enzyme activity of the freshly prepared liver supernatant, though the activity of the latter preparation can be stimulated by thiol-binding agents, e.g. p-chloromercuribenzoate, iodoacetamide (Carroll, 1969). This suggested that in the cell, phenolsulphotransferase existed in the reduced state and became oxidized during purification. It has been further demonstrated that in a partially purified enzyme, the activity towards L-tyrosine methyl ester can be reduced to virtually nothing by various treatments whereas the activity towards p-nitrophenol is not markedly affected (Mattock and Jones, 1970). Barford and Jones (1971) have also shown the selective loss of activity towards L-tyrosine methyl ester as a result of oxidation of the enzyme -SH groups, the loss being restored by treatment of the oxidized enzyme with dithiothreitol.

Recently, attempts have again been made to separate sulphotransferase activities toward p-nitrophenol and tyramine by methods that distinguish between charge and molecular weight (Dodgson, 1977). Preparations showing only three protein bands on gel electrophoresis have all the activity towards both substrates localised in the main band only, and the enzyme activity exhibited by this preparation depends on its oxidation/reduction state (Table 1.12).

Thus, maximum activity of the oxidized enzyme is obtained when electrophilic substituent groups are present in the benzene ring and declines when nucleophilic groups are present. In fact a reasonable correlation can be shown between the substitution constant (σ) and the observed maximum velocity of the enzyme towards the appropriate substrate. In contrast, maximum activity of the reduced enzyme is obtained when nucleophilic substituents are present though in this case there is

TABLE 1.12: Relative Maximum Velocities for the Sulphoconjugation of some Substituted Phenols by Oxidized and reduced forms of a purified preparation of the main Phenolsulphotransferase fraction from rat liver.

(From Dodgson, 1977)

SUBSTITUENT GROUP	SUBSTITUTION CONSTANT (σ^-)	Relative Vmax	
		OXIDIZED ENZYME	REDUCED ENZYME
p-NO_2	+1.27	8.6	3.5
m-Cl	+0.37	6.4	3.7
p-Cl	+0.24	5.6	2.3
H	0	4.0	7.5
m-NH_2	-0.04	2.1	6.2
p-OCH_3	-0.11	3.0	7.2
p-$CH_2CH_2NH_2$	-0.45	1.0	6.3

no correlation between σ^- and enzyme activity. It is further assumed that in the liver cell, the enzyme is present in the reduced state, and it might therefore be concerned with sulphating compounds of endogenous origin such as tyramine, serotonin and dopamine, although some activity would be exhibited towards other phenols. The other possibility is that the enzyme can modify its oxidation-reduction state in order to deal with different types of acceptors.

Some support for the view that the phenolsulphotransferase may exist in the cell in a reduced state is perhaps provided by the *in vivo* studies of Williams (1938) in rabbits which were fed phenols with different substituent groups. It was found that the electrophilic ortho substituent groups tended to reduce sulphation, whereas the nucleophilic ortho substituent groups had a similar but lower effect as the various ortho groups while para groups had relatively little effect. The 'ortho effect' was attributed to the lack of sulphation of *o*-hydroxybenzoic acid by the rabbit. Thus, if it is assumed that the enzyme exists in the cell in a reduced state and as shown by the data in Table 1.12, the reduced enzyme has a high affinity for phenols with nucleophilic substituent groups, then *in vivo* findings of Williams (1938) could perhaps be explained by suggesting that the poor sulphation of phenols with electrophilic substituent groups was due to the presence in the *in vivo* situation of an enzyme predominantly in a reduced state.

Whether there exist tissue-specific phenolsulphotransferases with entirely different properties and specificities is not known, and also no further work has been done on minor peaks of enzyme activity that have been observed on chromatographic columns in the purification studies to see if they represent separate enzyme entities with distinct specificities. Thus, as it would appear at present, much work still remains to be done on various aspects of sulphoconjugation.

Species Differences in the Conjugation of Phenols With Glucuronic Acid and Sulphate

The extensive studies of Williams and his collegues have shown that whilst the safety of pharmaceuticals and other xenobiotics used by Man is largely assessed by tests on laboratory animals such as mice, rats, rabbits, guinea pigs, dogs, cats and occasionally primates, the activity and toxicity of these chemicals are not always the same in animals as they are in man (Williams, 1974). Also, situations do arise when,

(a) a relatively common route of metabolism is deficient or absent in a certain species (Table 1.13); and

(b) a route of metabolism is found which appears to be specific to one species or closely related species and does not occur generally in other species (Table 1.3 - see above).

Such situations are found with conjugation reactions, and as far as we know at present they may be less common with reactions involving oxidation, reduction and hydrolysis, although future research may modify this view.

Conjugation of Phenols in the Rat, Cat and Pig

Phenols are conjugated substantially with both glucuronic acid and sulphate in most mammalian species except the pig and the cat. Thus, the pig tends to excrete phenols mainly as glucuronides with small amounts of sulphate conjugate, the exception being 1-naphthol where 32% of the urinary excretion was observed to be 1-naphthyl sulphate (Capel et al., 1972, 1974a; Table 1.14). Thus, the pig forms significant amounts of 1-naphthyl sulphate but not of its isomer 2-naphthyl sulphate.

In the cat, on the other hand, some phenols, but not all, are eliminated mainly as sulphate with traces of glucuronide conjugates. The in vivo studies of Hartiala (1955) and Dutton and Greig (1957) with cat liver organ slices and o-aminophenol as substrate were the first to show that the

Table 1.13: Species 'defects' in some common Conjugation Pathways (Williams, 1974)

CONJUGATION REACTION	SPECIES IN WHICH REACTION AT LOW LEVEL OR ABSENT	REFERENCES
O-Glucuronide	Cat	See Text Later
N-Glucuronide (e.g. Madribon)	Rabbit	Bridges et al., 1964
Glycine	Hens, ducks, geese, turkeys, arachnids	Baldwin et al., 1960 Smith, 1962
Mercapturic acid	Man, guinea pig, hen	Wainer and Lorincz, 1963 Boyland and Sims, 1958 Grover and Sims, 1964 Bray et al., 1959
Sulphate	Pig	Capel et al., 1972, 1974a Stekol, 1936
Acetylation	Dog, guinea pig	Williams, 1964

TABLE 1.14: Conjugation of Some Phenols in the Pig

COMPOUND	% OF DOSE IN URINE IN 24h.	% OF 24h. URINARY EXCRETION AS:			
		GLUCURONIDE	SULPHATE	UNKNOWN METABOLITES	UNCHANGED
Phenol	97	94	6	0	0
1-Naphthol	81	66	32	0	0
2-Naphthol	84	94	6	0	0
4-Hydroxy-amphetamine	89	51	6	0	43
Phenacetin	32	55	11	20	16

Data taken from Capel et. al. (1972, 1974a)
Dose 25mg/Kg intraperitoneally except for 4-hydroxyamphetamine (0.1mg/Kg)

cat had a low capacity for glucuronidation. Lathe and Walker (1958) also demonstrated that no o-aminophenyl glucuronide was formed in the cat liver *in vitro*, though it could synthesize bilirubin glucuronide. These observations were confirmed *in vivo* by Robinson and Williams (1958) who administered a number of xenobiotics, namely phenol, borneol, 4-methylumbelliferone, 2-amino-4-nitrophenol, 8-hydroxyquinoline and 2-methoxybenzoic acid to cats and showed that only negligible quantities of glucuronide conjugates were excreted in urine.

Recent studies with various phenols have suggested that the molecular structure of the phenol may play a role in determining the extent of glucuronidation in the cat (Capel *et al.*, 1972, 1974a). Thus, phenol, 1- and 2-naphthols, morphine and 4-acetamidophenol are conjugated almost entirely with sulphate, whereas with phenolphthalein substantial amounts of glucuronide conjugates are formed (Table 1.15). Even with the other phenols, glucuronic acid conjugation is not entirely absent since it occurs in small but definite amounts (1-2% of dose). No glucuronide conjugate of morphine was detected, though Yeh *et al.*, (1971) and Smith *et al.* (1973) have shown small amounts of morphine-3-glucuronide in the urine and bile of cats after the administration of the drug. Similarly, quinol and 2,6-dimethoxyphenol are excreted in the urine of cats conjugated mainly with sulphate together with small amounts of their glucuronide conjugates (Miller *et al.*, 1973), and the carcinogen 2-acetamidofluorene is also excreted by cats to a small extent (about 3% of dose) as the glucuronide of the hydroxylated metabolite, the major conjugate in urine being sulphate (12-16% of the dose; Weisburger, *et al.*, 1964).

By contrast, the female rat appears to utilize glucuronic acid and sulphate in about equal proportions for the conjugation of these phenols (Table 1.16). With 2,6-dimethoxyphenol administered intravenously at three dose levels , however, in

TABLE 1.15: Conjugation of Some Phenols in the Cat

COMPOUND	DOSE (mg/Kg) (i.p.)	% OF DOSE IN URINE IN 24h.	% OF 24h. URINARY EXCRETION AS:		
			GLUCURONIDE	SULPHATE	OTHER METABOLITES
Phenol	25	63	1	88	12
1-Naphthol	25	91	1	98	0
2-Naphthol	25	73	3	98	0
Morphine	5	88	n.d.	71	28 (FREE MORPHINE)
Phenacetin*	25	56	3	86	11
Phenolphthalein	25	27 (URINE)	60	40	0
	10+	13 (BILE)	95	n.d.	0

i.p. means intraperitoneally.
n.d. means not detected.
* Urine contains mainly conjugates of Phase I metabolite of phenacetin, i.e. 4-acetamidophenol.
+ Dose was injected intravenously into bile-duct-cannulated cats and bile collected for 3h.
(Adapted from Hirom et.al. 1977; data from Capel et al., 1972, 1974a)

TABLE 1.16: Conjugation of Some Phenols in the Rat

COMPOUND	DOSE (mg/Kg) (i.p.)	% OF DOSE IN URINE IN 24h	% OF 24h URINARY EXCRETION AS:		
			GLUCURONIDE	SULPHATE	OTHER METABOLITES
Phenol	25	95	44	55	0
1-Naphthol	25	59	47	53	0
2-Naphthol	25	86	52	48	0
Morphine*	5	72 (BILE 49; URINE 23)	57 (BILE : 40 ; URINE:17)	n.d.	43
Phenacetin +	100	58	24	72	4
Phenolphthalein	50	100(BILE)	98	n.d.	0

i.p. means intraperitoneally; n.d. means not detected.

* Morphine was injected intravenously into bile-duct-cannulated rats and bile and urine collected for 3h. (Hirom, P.C., unpublished data).

\+ Urine mainly contained conjugates of the Phase I metabolite of phenacetin, i.e. 4-acetamidophenol. (Adapted from Hirom et al., 1977; data from Capel et al., 1972, 1974a).

female rats, the conjugation is strongly biased towards glucuronide formation, whereas in male rats, in urines collected one hour after administration of the phenol, there are approximately equal amounts of sulphate and glucuronide conjugates. (Miller et al., 1974). The other exceptions are phenolphthalein and morphine, both of which are conjugated entirely with glucuronic acid, no sulphate having been detected (Smith et al., 1973).

With some xenobiotics containing a carboxyl group, where glucuronic acid conjugation may compete with amino acid conjugation, the pattern of conjugation of several arylacetic acids in both the rat and the cat is known (see Hirom et al., 1977). Thus, in both species, glucuronides of phenylacetic, 4-chlorophenylacetic, 4-nitrophenylacetic and indol-3-ylacetic acids were not detected, these acids being conjugated with various amino acids. With 1-naphthylacetic acid, however, in the rat the major conjugate is glucuronide, whereas in the cat, it is conjugated with amino acids rather than glucuronic acid. The cat and the rat, on the other hand, both form substantial amounts of the glucuronides of α-methylphenylacetic acid and diphenylacetic acids.

Thus, as the above observations suggest, although the cat is poor at forming glucuronides with a number of compounds, the glucuronidation mechanism appears to be important for certain substrates. There are also other examples in the literature that certain xenobiotics and endogenous compounds are excreted as glucuronides in the cat, particularly in the bile. Table 1.17 summarises the compounds that have or have not been reported as being conjugated with glucuronic acid in the cat, together with the mode of excretion and references.

Studies conducted in other members of the superfamily Feloidea, which includes the cat, show that in the hyaena (Family Hyaenidae), the civet and genet (Family Viverridae), and the lion (Family Felidae), only trace amounts of either

Table 1.17: Glucuronide Formation In The Cat

COMPOUNDS REPORTED AS NOT FORMING GLUCURONIDES SUBSTANTIALLY (0-2%)	COMPOUNDS REPORTED AS FORMING GLUCURONIDES (>2%)
A.COMPOUNDS EXCRETED IN URINE:	
p-Nitrophenol, o-aminophenol, menthol, 1-naphthol, amphetamine, salicylamides .....(1)*	Lorazepam ..... (11)
Benzoic acid .....(2)	Chloramphenicol .....(12)
Chlorobenzene , phenol .....(3)	Metanephrine .....(13)
2,6-dimethoxyphenol, quinol .....(4)	Phenolphthalein .....(10)
4-methylumbelliferone,borneol,phenol, 2-amino-4-nitrophenol, 8-hydroxyquinoline, 2-methoxybenzoic acid .....(5)	
Sulphadimethoxine .....(6)	
Phenylacetic acid, 4-chlorophenylacetic acid, 4-nitrophenylacetic acid .....(7)	
Indol-3-ylacetic acid .....(8)	
1-Naphthylacetic acid .....(9)	

*Numbers refer to References (Page 75)

(continued)

Table 1.17: (continued)

COMPOUNDS REPORTED AS NOT FORMING GLUCURONIDES SUBSTANTIALLY (0-2%)	COMPOUNDS REPORTED AS FORMING GLUCURONIDES (> 2%)
1-Naphthol, morphine, phenacetin ..... (10)	
B. COMPOUNDS EXCRETED IN BILE	
	Phenolphthalein ..... (16)
	Corticosterone, projesterone ..... (17)
	Testosterone ..... (18)
	Thyroxine ..... (19)
	Tyropanoic acid, iodopanoic acid , bunamiodyl ..... (20)
	Bilirubin ..... (21)
	2-Acetamidofluorene ..... (22)
C. COMPOUNDS STUDIED in vitro	
Phenolphthalein ..... (1)	p-Nitrophenol ..... (23)
	Oestrone, 17β-oestradiol ..... (24)
	Bilirubin ..... (25), (26)

References (Table 1.17):

(1) Dutton, 1966
(2) Bridges et al., 1970
(3) French, 1970
(4) Miller et al., 1973
(5) Robinson and Williams, 1958
(6) Adamson et al., 1970b
(7) Idle, 1976
(8) Bridges et al., 1974
(9) Dixon et al., 1974
(10) Capel et al., 1974a
(11) Schillings et al., 1975
(12) Dill et al., 1960
(13) Axelrod et al., 1959
(14) Dixon, 1976
(15) Mulder and Bleeker, 1975
(16) Abou-El-Makarem, 1967
(17) Taylor, 1971
(18) Archer et al., 1965
(19) Myant, 1966
(20) McChesney, 1964
(21) Lathe and Walker, 1958
(22) Weisburger et al., 1964
(23) Mowat and Arias, 1970b
(24) Heath and Dingell, 1974
(25) Jansen and Henderson, 1972
(26) Potrepka and Spratt, 1971

phenol, benzoic acid or 1-naphthylacetic acid are conjugated with glucuronic acid, except in the hyaena where about 40% of the 24 hour urinary excretion with 1-naphthylacetic acid is recovered as the glucuronide conjugate. The major portion of a dose of phenol in these animals, as in the cat, is conjugated with sulphate, while benzoic acid and 1-naphthylacetic acid are conjugated with amino acids (Caldwell et al., 1975b; French et al., 1974; Hirom et al., 1977). These findings, therefore, suggest that the carnivores which belong to the superfamily Feloidea may as a rule have a low glucuronidating capacity with certain phenols as in the cat. But, on account of the small number of species (five out of total 114) from this superfamily that have beeninvestigated with only a small number of glucuronidating substrates, it would be difficult to establish a general pattern of conjugation in these group of animals.

In the cat, the deficiency with regard to forming glucuronides with certain xenobiotics appears to lie in the enzyme UDP-glucuronyltransferase, since UDPGA and UDP-glucose dehydrogenase have been found to be at levels expected in cat liver, and no inhibitor has been found beyond the usual nucleotide-inactivating enzymes (Dutton, 1966). Neither the kidney nor the intestine in the cat contain measurable glucuronyltransferase activities. Effects of a few xenobiotics, namely 3 -methylcholanthrene, phenobarbital and diethylnitrosamine, which have been shown to enhance glucuronyltransferase activities in the rat, have also been examined in cats. Thus, 3-methylcholanthrene caused no increase in enzyme activity (Hollman and Touster, 1962), and phenobarbital, which enhanced bilirubin glucuronidation, did not induce p-nitrophenol glucuronidation (Jansen and Henderson, 1972). Similarly, diethylnitrosamine when added to liver particulate fractions did not increase o-aminophenol glucuronidation in the cat (Mowat and Arias, 1970b).

Despite the differences that have been observed for the glucuronidation of certain xenobiotics in the cat, the glucuronidation mechanism is important for certain xenobiotics. Therefore, at present it has been suggested that due to the belief that there may exist several forms of the enzyme, UDP-glucuronyltransferase, the low glucuronidating capacity for certain xenobiotics in the cat could perhaps be explained in terms of deficiency in one or more of these enzymes. (Capel et al., 1974a).

The other striking instances where glucuronide conjugation is at a low level or absent are certain hereditary manifestations of low transferase levels, so far only encountered in the rat and man. Rats of Gunn strain (Gunn, 1938) exhibit acholuric jaundice; excretion of bilirubin glucuronide, retention of free pigment and levels of UDPGA and UDP-glucose dehydrogenase are all normal, the condition probably being due to deficient UDP-glucuronyltransferase activity. (Dutton, 1966). This deficiency is incompletely dominant, heterozygotes still having subnormal enzyme levels. The extent of the abnormality varies with the aglycone substrate. Thus, in homozygous Gunn rats, there is no detectable activity of bilirubin UDP-glucuronyltransferase and abnormally low rates of glucuronidation with o-aminophenol, o-aminobenzoate, umbelliferone, and menthol, though the rate of p-nitrophenol glucuronidation is only decreased slightly as compared with normal rats. (Schmid, 1972; Zakim et al., 1973d). p-Nitrophenol glucuronidation in homozygous Gunn rats has been shown to be enhanced by phenobarbital, though not that of bilirubin (Jansen and Henderson, 1972), and similarly, diethylnitrosamine has also been shown to activate o-aminophenol (Stevenson et al., 1968) and p-nitrophenol (Nakata et al., 1975, 1976) glucuronidating activities. Recent studies by Nakata et al., (1975, 1976) and Zakim et al. (1973d) have shown that the activity of UDP-glucuronyltransferase from homozygous Gunn rats are

insensitive to treatments which modify the lipid phase of the membrane, and that the p-nitrophenol UDP-glucuronyltransferase from Gunn rats as compared with normal rats has decreased affinity for the UDP - portion of UDPGA. The defect in the function of UDP-glucuronyltransferase in homozygous Gunn rats therefore includes abnormal regulatory properties as well as abnormal rates of catalysis.

In man, there are various hepatic dysfunctions involving lowered UDP-glucuronyltransferase (Kalow, 1963; Brown, 1962; Schmid, 1972). The two main groups seem to be the Crigler-Najjar syndrome and Gilbert's disease. The former certainly has a defective liver UDP-glucuronyltransferase activity with bilirubin, salicylates, N-acetyl-p-aminophenol, menthol and tetrahydrocortisone (Childs et al., 1959). The Gilbert's syndrome, on the other hand, is probably represented by a heterogenous group of benign disorders, or may be a result of several unrelated disturbances (Arias, 1960). Deficient enzyme levels in Gilbert's disease have also been reported (Black and Billing, 1969).

Conjugation of Phenols in Primates.

A major aim of modern toxicology is to determine the possible basis for the logical prediction of toxicity in man. Such predictions must depend upon a proper choice of the correct animal species in terms of the metabolic fate of the chemical under study. Thus, Brodie (1964) has stated:

'The pinnacle of success for the researcher in drug metabolism would be the discovery of an animal species that is close to man in metabolising drugs.'

It might be suggested that animals belonging to the same zoological order are likely to have similar tissue enzymes (Williams, 1974), and some support for this view comes from the study of blood groups in primates which show that there are similarities between the serum proteins of different

primate species and man (Goodman, 1963). It could, therefore, be argued that the animals nearest to man on the phylogenetic scale are more likely to metabolise xenobiotics like man than an animal far removed from man such as rat, rabbit, or dog (Williams, 1974). On the other hand, it is well known that the subhuman primates are not like humans in every respect. As Brodie (1964) has put it,

' ..... monkeys are closely related to man in many things, but in drug enzymes they have gone their separate way.'

But then he adds further,

'I would not go so far as to discount the possibility of a miracle; perhaps a particular primate species, close to man in drug metabolism, really exists.'

Therefore, it would appear that there is a need for more metabolic research among the different primate species. According to Napier and Napier (1967), primates can be classified into two sub-orders namely, the Prosimii and the Anthropoeidae, each of these consisting of six Families. Man belongs to the sub-order Anthropoeidae and in this group are also included the apes, Old and New World monkeys, and lemurs. These primates can be put on a scale based on increasing complexity of structural and behavioural organisation with man at the top and the tree shrew at the bottom (Table 1.18). So far primates have been used for the pharmacological and toxicological testing of many xenobiotics, though only a small number of species (15) compared with the large number of species (215) that exist, have been used for such purposes, the rhesus monkey being the most extensively studied species. Recently, Smith and Caldwell (1977) have reviewed the occurrence among primate species of the main reactions involved in xenobiotic metabolism.

Like man, primate species so far investigated appear to form glucuronic acid conjugates with a number of xenobiotics (Table 1.19). For these compounds most information is

Table 1.18: Classification of Primates (Napier and Napier, 1967; See also Smith&Caldwell, 1977)

SUB-ORDER	FAMILIES		NUMBER OF SPECIES	SPECIES USED IN XENOBIOTIC METABOLISM	
	SYSTEMIC NAME	TRIVIAL NAME		SYSTEMIC NAME	TRIVIAL NAME
Prosimii	Tupaiidae	Tree shrews	19	Tupaia glis	Tree shrew
	Tarsiidae	Tarsiers	3	-	-
	Lemuridae Indriidae Daubentonidae	Malagasy Lemurs	19	--	--
	Lorisidae	Lorises, Galagos	14	(i) Nycticebus coucang (ii) Galago senegalis	(i) Slow loris (ii) Bushbaby
Anthropoidae	A Callitrichidae B Cebidae	New World Monkeys	70	A (i) Saguinus oedipus B (i) Cebus albifrons (ii) Saimiri sciureus (iii) Aotus trivirgatus	A (i) Tamarin B (i) Capuchin (ii) Squirrel monkey (iii) Night Monkey
	Cercopithecidae	Old World Monkeys	77	(i) Cercopithecus aethiops (ii) Erythrocebus patas patas (iii) Papio anubis (iv) Macaca mulatta (v) M. nemestrina (vi) M. arctiodes (vii) M. Fascicularis	(i) Green monkey (ii) Patas monkey (iii) Baboon (iv) Rhesus monkey (v) Pigtail monkey (vi) Stumptail monkey (vii) Cynomolgus monkey
	Hylobatidae	Lesser Apes	7	-	-
	Pongidae	Great Apes	4	Pantroglodytes	Chimpanzee
	Hominidae	Man	1	Homosapiens	Man

INCREASING COMPLEXITY OF STRUCTURAL AND BEHAVIOURAL ORGANISATION

TABLE 1.19: Glucuronic Acid Conjugation of Other Xenobiotics By Primates

Conjugate Type	Aglycone	Primate Species	Reference
Ester	Diphenylacetic acid 1-Naphthylacetic acid	Man, Rhesus, cynomolgus, squirrel, capuchin, marmoset, bushbaby	Dixon (1976)
	Hydratropic acid	Man, Rhesus monkey	Dixon (1976)
	Indomethacin	Man, Rhesus monkey	Hucker et al. (1966)
	Myalex	Pig-tailed, talpoin monkey	Foulkes (1970)
	p-(Cyclopropylcarbonyl)-phenylacetic acid	Rhesus Monkey	Lan et al. (1975)
Alcoholic Ether	1-(p-Chlorophenyl)-2,2-dimethyl-1,3-propane-diol carbamate	Man, Rhesus Monkey	Hadley et al. (1971)

(Continued)

TABLE 1.19 (contd.): Glucuronic Acid Conjugation of Other Xenobiotics By Primates

Alcoholic Ether	1-(p-Chlorophenyl)-2,2-dimethyl-1,3-propane-diol	Man, Rhesus Monkey	Hadley et al., (1971)
Hydroxylaminic	N-Hydroxychloro-phentermine	Man, Rhesus Monkey	Caldwell et al. (1975a)
N-1-Glucuronide	Sulphadimethoxine	Man, Rhesus, green, olive, baboon, squirrel, capuchin, bushbaby, slow loris, tree shrew	Adamson et al. (1970b)
	Sulphamethomidine	Man, Rhesus Monkey	Bridges et al. (1969b)
	Sulphadimethoxy-pyrimidine	Man, Rhesus Monkey	Bridges et al. (1969a)

(From Smith & Caldwell (1977)

available in the Rhesus monkey, though the data does show that several species of the New and the Old World monkeys and prosimians are able to form ester and N-1-glucuronides. The sulphate conjugation has not been well investigated among primate species in vivo, and the only examples of xenobiotics studied so far are phenol and 4-hydroxyamphetamine (Table 1.20).

Thus, both phenol and 4-hydroxyamphetamine are conjugated with glucuronide as well as sulphate. It is of interest to note that in the case of phenol, man and the Old World monkeys, conjugate it mainly with glucuronide. This suggests that man and the closely related Old World monkeys may utilize the sulphate mechanism more extensively than glucuronic acid conjugation for the metabolism of simple phenols, the reverse being true for the New World monkeys. (Smith and Williams, 1974). With 4-hydroxyamphetamine, however, in man and the other primates examined, the sulphate conjugate is the major metabolite, except in the cynomolgus monkey, where a large amount of the compound is excreted unchanged, though the amount conjugated with sulphate being greater than that with glucuronic acid.

The phenolic metabolites derived from chlorobenzene, namely, p-chlorophenol and chlorocatechol, are also conjugated with both sulphate and glucuronic acid in man, the Rhesus, squirrel and capuchin monkeys, though here the relative proportions of the two conjugates are similar (French, 1970). The only other observation that one could make about these results (Table 1.21) is that the two New World monkeys which were seen to form extensive amounts of phenyl glucuronide form very much less glucuronide with chlorobenzene.

In vitro studies with phenol have shown a number of adult human tissues, namely the liver, adrenals, jejunal mucosa, kidneys, ovaries and the spleen as having sulphating ability

TABLE 1.20: Conjugation of 4-Hydroxyamphetamine and Phenols in Some Primates

COMPOUND	PRIMATE SPECIES	% OF DOSE IN URINE IN 24h.	% OF 24h. URINARY EXCRETION AS:		
			GLUCURONIDE	SULPHATE	UNCHANGED
PHENOL *	Man	95	16	78	0
	Rhesus Monkey	43	35	65	0
	Squirrel Monkey	31	93	7	0
	Capuchin Monkey	73	86	14	0
4-Hydroxy-amphetamine +	Man**	93	-	70	10
	Rhesus Monkey	72	2	93	4
	Cynomolgus Monkey	60	3	12.6	84.6
	Squirrel Monkey	21	1	92	7
	Capuchin Monkey	65	1	97	2

* Dose 25 mg/Kg orally, except for Man (0.01mg/Kg) and Rhesus monkey (50 mg/Kg).
\+ Dose 0.01 mg/Kg orally.

Data are taken from Capel et al. (1972) and Capel (1973).
** Data from Sever et al. (1976). Dose 0.43 mg/Kg orally.

Table 1.21: Relative Amounts of Total Sulphate and Glucuronide Conjugates in Man and some Primates after Administration of Chlorobenzene (French, 1970)

SPECIES.	% OF URINARY ^{14}C ACTIVITY AS:	
	GLUCURONIDE	SULPHATE
Man	45	36
Rhesus monkey	31	30
Squirrel monkey	34	22
Capuchin monkey	19	40

Dose: 100 mg/kg chlorobenzene orally

(Bostrom and Wengle, 1967). In this study, the glucuronide conjugation was not examined. The human liver has been reported as forming only the glucuronide conjugate *in vitro* with 4-hydroxy-3-methoxyphenylethanol, a metabolite of Dopa (3, 4-dihydroxyphenylalanine), no sulphate having been detected (Wong, 1976a), whereas another study has shown that a sulphate conjugate of the alcohol predominates in human urine (Karoum *et al.*, 1973). The sulphation of tyramine has also been shown to occur in man (Mullen and Smith, 1971) and the cynomolgus monkey (Wong, 1976b). Tyramine is metabolised by both sulphoconjugation and oxidative deamination. The *in vitro* studies in the monkey liver and intestine suggest that the intestine is more important than the liver for sulphate synthesis. *In vivo* observations in man show that greater amounts of tyramine sulphate are excreted when the xenobiotic is administered orally than when it is given intravenously (Mullen and Smith, 1971; Tacker *et al.*, 1972). Thus, it would appear that for tyramine, in man and the Old World monkey, similar mechanisms for sulphoconjugation are present.

Factors Affecting the Relative extents of conjugation With Glucuronic Acid and Sulphate

The underlying mechanism by which interspecies variations in xenobiotic metabolism may be manifested are complex, and such variations may originate in differences in one or more factors, namely, the rate of absorption of the xenobiotic, binding to plasma proteins and other tissue macromolecules, mode of excretion, pattern of metabolism (rate and route) and the nature of the receptor (Gillette, 1977). Of these, one of the most important in the metabolic factor, i.e. differences in pathways or in rates of metabolism. Since this metabolism is dependent on enzymes, these variations could be due to differences in the following:

(i) The absolute amounts or the specific activity of

the enzyme. Thus, there may be species differences in the primary structure and conformation of a given enzyme that affects the affinity of enzyme for the xenobiotic and hence, its relative importance in the metabolism of the foreign compound may depend upon the dose.

(ii) The amount or the nature of natural inhibitors or activators of the enzyme. Just as there are species differences in the metabolism of xenobiotics, there are also species variations in the metabolism of the inhibitors and these could therefore cause differences in the duration of the action of these inhibitors *in vivo*. For example, desipramine inhibits the metabolism of tremorine in rats but not in mice (Sjoqvist *et al.*, 1968) probably because desipramine is metabolised considerably more rapidly in mice than in rats (Dingle *et al.*, 1964).

(iii) The activity of the enzymes catalysing the reverse reaction; or

(iv) The activity of enzymic reactions competing for the same substrate. Many of these xenobiotic metabolising reactions may be catalysed by several different enzymes, each one of these having its own substrate specificity and its own kinetic parameters. Thus, species variations in the metabolism of a xenobiotic may depend on the number of enzymes that catalyse a given reaction, and on the relative proportions of these enzymes. The cofactor supply may also be rate-limiting.

The kinetics of metabolism of most xenobiotics in the body are in general first-order since their concentration at enzyme sites are usually sub-optimal. Some metabolic reactions are relatively easily saturated particularly by an excess of substrate and when this occurs, two consequences follow:

(i) the reaction kinetics change from a first-order process to that of zero-order; and

(ii) the compound may be metabolised by a secondary pathway.

Saturation of a particular metabolic mechanism may therefore be associated with qualitative and quantitative changes in the pattern of metabolism of a compound in a given species and this in turn may be accompanied by an alteration in the pattern of renaland hepatic elimination of the drug.

As we have seen for the metabolism of phenols in certain primate species and the rat, these compounds can be conjugated with both glucuronide and sulphate. Thus, the enzymes for both sulphation and glucuronidation would be competing for the same substrate in a given animal, and where one of these pathways may be deficient, as for example, in the cat, the other may be expected to completely take over, and compensate for the deficiency, though this may be dependent upon the availability of the enzyme and the cofactor in the appropriate reaction.

Glucuronic acid conjugation appears to have a large capacity and the rate of conjugation of many xenobiotics follows first-order kinetics even at high doses. Sulphate conjugation, however, is a reaction of limited capacity and readily saturable by suitable substrates (i.e. zero-order). The reason for this is that glucuronic acid is readily available from various carbohydrate sources in the body whereas the availability of sulphate is limited (Bray et al., 1952a,b,c,d). Therefore, the cat, which has a low glucuronidating capacity for certain phenols, may be expected to develop its sulphating mechanism to a very high level. This, nevertheless, would be more readily saturable due to cofactor availability, and hence, the possible reason for the toxicity of certain phenols in cats. Table 1.22 compares the toxicity of several phenols to the cat with that to the rat, rabbit and the guinea pig which are known to have normal UDP-glucuronyltransferase activities. It shows that all these phenols are toxic to the cat at very much lower dose levels than the non-carnivorous

Table 1.22 : The Toxicity of Several Phenols to the Cat Compared with some Non-carnivourous Species. (From Hirom et al., 1977)

COMPOUND	SPECIES	ROUTE OF ADMINISTRATION	TOXICITY	DOSE (mg/Kg)
Phenol	Cat Guinea pig	Subcutaneous	MLD	80 450-500
2-Methylphenol (o-Cresol)	Cat Rabbit Rat Guinea pig	Subcutaneous	LD	55 450-500 650 350-400
3-Methylphenol (m-Cresol)	Cat Rabbit Rat Guinea pig	Subcutaneous	LD	180 500-600 900 300-400
4-Methylphenol (p-Cresol)	Cat Rabbit Rat Guinea pig	Subcutaneous	LD	80 300-400 500 200-300

(continued)

Table 1.22 (continued):

COMPOUND	SPECIES	ROUTE OF ADMINISTRATION	TOXICITY	DOSE (mg/KG)
2-Nitrophenol	Cat Rabbit	Subcutaneous	LD	600 1700
4-Nitrophenol	Cat Rabbit	Subcutaneous	MLD	197 600
1-Naphthol	Cat Rabbit	Oral	LD	100-150 9000
2-Naphthol	Cat Rabbit	Oral	LD	100-150 3800

Data taken from Spector (1956)
LD means lethal dose, the amount that kills an animal.
MLD means minimum lethal dose, the smallest of several doses that kills one of a group of test animals.

species. The mechanism of such toxicity in cats could be as follows.

Miller et al. (1973) showed that following an intravenous administration of 20mg/kg 2,6-dimethoxyphenol in the cat, 93% of the radioactivity in the urine was identified as the sulphate conjugate with small amounts of the parent phenol (5%) and glucuronide conjugate (2%). With the dose of 40mg/kg, similar results were obtained except that 3% of the radioactivity was identified as 2,6-dimethoxyquinol. While sulphate conjugation is the major metabolic process from the administration of phenols to cats, this detoxication process may become saturated when higher doses of phenols are given, and the urine might contain more parent phenols together with their oxidation products, quinols. The formation of quinones from quinols may provide an explanation for the toxic effects of phenols in cats, since quinones have been shown to be potent inhibitors of mitochondrial respiration (see below). This toxicity can possibly be prevented by sulphydryl groups as shown by Loveless and Maxwell (1975) who studied the toxic effects of the anaesthetic, (-)-2,6-dimethoxyphenyl-2-morpholinopropionate hydrochloride (M & B 16,573) in the cat. This compound produces a delayed toxicity in the cat, and it was postulated that this was due to the formation of 2,6-dimethoxyquinol and 2,6-dimethoxybenzoquinone during the metabolism of M & B 16,573. The toxicity of these metabolites could be prevented by cysteine and N-acetyl cysteine.

The quinones exert their inhibitory action on the mitochondrial electron transport system by forming addition compounds with substrates containing thiol groups (Redfearn, 1965; Smith and Lester, 1961). Thus, sulphydryl groups may react with the quinones thereby preventing their interaction with thiol- containing substrates in the mitochondrial electron transport chain. (Loveless and Maxwell, 1975). In the rat,

on the other hand, the question of toxicity does not arise except at extremely high doses since both phenol and 2,6-dimethoxyphenol are readily detoxicated by both glucuronide and sulphate conjugation(Capel et al., 1972; Miller et al., 1974).

In a species where the administered compound is excreted both as glucuronide and sulphate, the proportions of the dose appearing in the two forms are complementary (Bray et al., 1952c). The proportion excreted as that conjugate formed by a first-order reaction (glucuronidation) increases with increasing dose level while that formed by a zero-order reaction (sulphation) decreases. As examples, variation at the extent of sulphate formation in the rabbit at different dose levels was recorded for phenol by Williams (1938) and for p-hydroxybenzenesulphonamides by Sammons et al. (1941), both showing that as the dose of the xenobiotic increased so the amount conjugated with sulphate decreased (Table 1.23).

Since it was pointed out by Bray et al. (1952d) that the formation of arylsulphoconjugates in rabbits was rate-limited by the endogenous level of sulphate, there have been further studies indicating this to be true. Thus, Levy and Matsuzawa (1967) showed in man that the fraction of the dose excreted as salicylamide sulphate decreased markedly as the oral dose of salicylamide was increased from 75 to 2,000 mg., and furthermore, that the co-administration of L-cysteine restored the conjugation of salicylamide even at high doses, indicating that the capacity was limited by the availability of sulphate. Similarly, Bennett et al. (1975) employing dogs as experimental animals have established that competition for the available sulphate in the gut wall occurs when salicylamide and isoprenaline are administered orally at the same time, leading to considerable increase in the amount of unconjugated isoprenaline reaching the circulation; L-cysteine feeding abolishes this competition.

Table 1.23: Variation in Sulphate excretion by Rabbits After Administration of Phenol and p-Hydroxybenzene-sulphonamide at Different Dose Levels

(After Bray et al., 1952d)

COMPOUND	DOSE LEVEL	% DOSE EXCRETED AS SULPHATE
Phenol	25	41
	50	37
	100	18
	200	18
	500	19
p-hydroxy-benzene-sulphonamide	100	53
	250	44
	500	33
	750	27

Saturation of a pathway can lead not only to a change in the pattern of metabolism of a compound, but also to a change in the route of elimination. Glucuronic acid conjugates with molecular weights greater than about 350 are rapidly excreted in the bile of rats (Millburn, 1976), while in general, sulphate conjugates are rather poorly excreted via this route, being mainly excreted in urine. Studies conducted by Mulder et al. (1974) have shown that with increasing aglycone supply, glucuronidation of harmol by rats is increased relative to sulphation, the glucuronide being more rapidly excreted in bile than the sulphate conjugate. It has been further shown (Mulder and Bleeker, 1975) that species differences between the rabbit, rat, mouse, guinea pig and cat occur both in the pattern and excretory pathways of harmol conjugation, and it has been suggested that this may be due to species variation in pharmacokinetic behaviour of harmol.

Similar observations have been made with the conjugation of 1-naphthylacetic acid in the rat at different dose levels. (Dixon, 1976). This compound is conjugated both with glycine, a readily saturable pathway, and glucuronic acid. At low doses, it is largely metabolised with glycine and largely eliminated in the urine. However, at high doses, the importance of the glycine conjugating pathway declines with a large concomitant increase in the glucuronic acid conjugation, the main route of elimination being via the bile. The reason for a change in the route of elimination in the above two cases is that the saturation of one pathway results in a greater use of an alternative one, producing metabolites with molecular parameters favouring biliary excretion. Thus, the sulphate and glycine conjugates of the two xenobiotics discussed here, have molecular weights below threshold necessary for appreciable biliary excretion, the reverse being true for the glucuronide conjugates (For a review see Millburn, 1976).

It is necessary, where two reactions may be competing for the same substrate, to know the extent to which this occurs especially in the *in vivo* situation. These types of studies would help in the understanding of pharmacological as well as toxic actions of drugs, thereby, giving a better insight into working out dosage regimes. Such studies are, of course, very important clinically where two drugs, which may compete for the same reactions are administered simultaneously. For example, interactions between sympathomimetics (e.g. isoprenaline) which can be inactivated by conjugation with sulphate, and other drugs (e.g. paracetamol) which are readily available to the public and also similarly metabolised, could occur and be potentially dangerous (Bennett *et al.*, 1975). Viewed from a different aspect, competition between substances which are metabolised, for example, by conversion to sulphate or any other rate-limiting pathway, might be used to enhance the availability and therapeutic usefulness of a drug which would otherwise be readily inactivated by the same detoxication mechanism.

Aims of the Present Study

As the fore-going review has shown, glucuronic acid and sulphate conjugations are very important and widely occurring detoxication mechanisms, and the relative extent of conjugation of a phenolic compound with the respective conjugation mechanism is dependent on the species of animal and the nature of xenobiotic being investigated. A few of the many factors which may be involved in determining these variations are as follows:

(1) The availability of the two co-substrates, UDPGA and PAPS, and the relative proportions of the two enzymes, UDP-glucuronyltransferase and phenolsulphotransferase in various species may be different.

(2) The specificities of these two enzyme systems for a given xenobiotic may differ in different species.

(3) In the case of UDP-glucuronyltransferase, the microsomal membrane plays a major role in the regulation of enzyme activity, and it is possible that species variation in enzyme activity may be caused by differences in membrane structure and fluidity. The physico-chemical properties of the xenobiotic substrate may also influence the regulation of enzyme activity at the membrane level.

The studies that have so far been conducted with phenolic substrates in certain species, namely the rat, cat and some primates, have been qualitative in that they only reveal the pattern of conjugation of these phenols in a given animal at an arbitrarily chosen dose level. Since xenobiotic metabolism is a dynamic process, i.e. metabolites are continuously formed and eliminated, any factor which influences the metabolism of a xenobiotic, will also influence its elimination and hence, its toxicity. Defining the quantitative aspects of xenobiotic metabolism is therefore as important as defining the qualitative aspects of the various reactions. Thus, to obtain a greater insight into quantitative aspects of glucuronidation and sulphation, and the way in which these two conjugation mechanisms may be controlled at the enzymic level in some species, the following investigations have been conducted:

(1) the effect of dose on the *in vivo* conjugation of 1-naphthol in the rat, cat and the guinea pig;

(2) the effect of dose on the *in vivo* conjugation of 1-naphthol and phenol in some primate species;

(3) *in vitro* measurement of UDP-glucuronyltransferase and phenolsulphotransferase activities towards 1-naphthol and phenolphthalein in various tissues in the rat and cat, and in livers from the guinea pig, pig and certain primates; and

(4) bi-substrate kinetic analysis for the rat and cat UDP-glucuronyltransferases with 1-naphthol and phenolphthalein as substrates, and studies into the nature of the micro-environment of the enzyme in these species.

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Materials and Methods

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Materials

Radioactive Chemicals

[1-^{14}C]1-Naphthol (specific activity, 19.2mCi/mmol; The Radiochemical Centre, Amersham, Bucks.), [U-^{14}C]-phenol (33.5 mCi/mmol; The Radiochemical Centre,Amersham), and 3'-phosphoadenosine-5'-phosphosulphate, tetrasodium salt, [^{35}S] (2.55 Ci/mmol; New England Nuclear, U.S.A.) were purchased. [^{14}C]-phenolphthalein (0.3μCi/mg) was prepared in this department by R. J. Parker (see Parker, 1977). Figure 2.1 illustrates the structures of these various xenobiotics, indicating the positions of the radioactive labels. [^{14}C]-phenol is uniformly-labelled on all the carbon-atoms of the benzene ring.

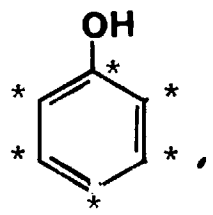
Chemicals and Solvents

All the solvents used were purchased (BDH Chemicals Limited, Poole, Dorset) being of A.R. (analytical reagent) grade. All the organic salts, namely, ethylenediaminetetracetic acid (EDTA), tris (hydroxymethyl)-methylamine, phenol (M.P.42^{o}C),quinol (M.P.173^{o}C) and resorcinol (M.P.110^{o}C) were also bought from BDH Chemicals Limited and were of 'AnalaR' grade.

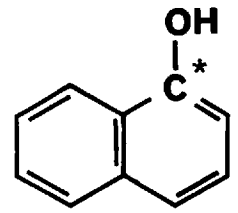
Phenyl-β-D-glucuronide, 1-naphthyl-β-D-glucuronide (sodium salt), and Triton X-100 (p-isooctylphenoxypolyethoxyethanol) were obtained from Koch-Light Laboratories Limited, Colnbrook, Bucks. Catechol (M.P.103^{o}C) and 1-naphthol (M.P. 94^{o}C) were purchased from Hopkin and Williams Limited, Chadwell Heath, Essex. 1-Amino-2-naphthol-4-sulphonic acid (Grade III), phenolphthalein-β-D-glucuronide, phenolphthalein disulphate (potassium salt), phenolphthalein, 1-naphthylsulphate (potassium salt), uridine-5'-diphospho-N-acetylglucosamine (sodium salt; Grade I), ATP (disodium salt),

Figure 2.1: Structures of the Various Radio-actively Labelled Compounds Used In The Present Studies.

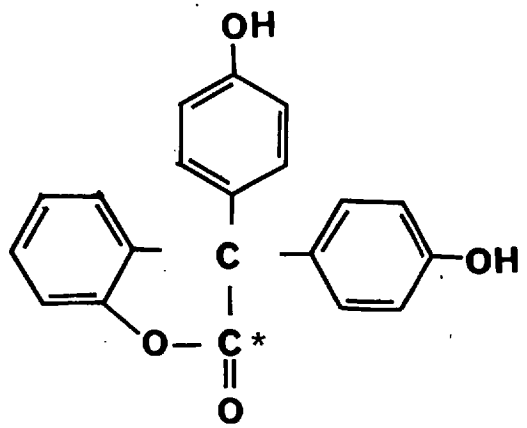
[U-^{14}C] Phenol



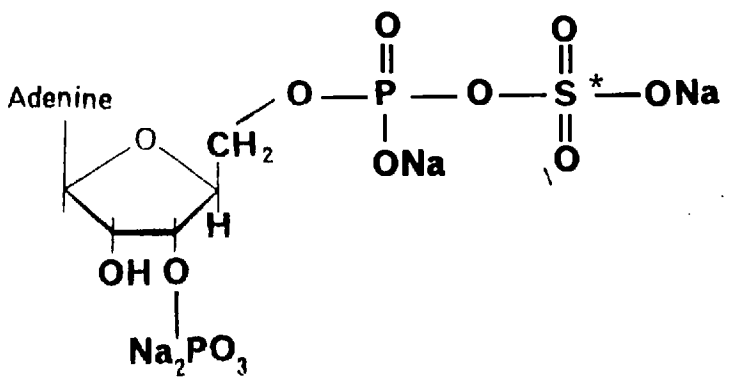
[1-^{14}C] 1-Naphthol



[Carbonyl-^{14}C] Phenolphthalein



3'-Phosphoadenosine-5'-Phosphosulphate, Tetrasodium salt, [^{35}S]



bilirubin (from gallstones; crystalline), bovine serum albumin (crystallised and lyophlised), saccharic acid-1,4-lactone, and standard fatty acid methyl esters were all purchased from the Sigma (London) Chemical Company, Kingston-upon-Thames, Surrey. Phenyl sulphate was synthesized.

Methods

Synthesis of Phenyl Sulphate (Potassium salt)

Phenyl sulphate was synthesized by the method of Feigenbaum and Neuberg (1941), the reaction sequence being as follows:

$$ClSO_3H + 2C_5H_5N \longrightarrow SO_3O_5H_5N + C_5H_5N.HCl$$

$$SO_3C_5H_5N + C_6H_5OH \longrightarrow C_6H_5O.SO_3H + C_5H_5N$$

$$C_6H_5O.SO_3H + KOH \longrightarrow C_6H_5OSO_3K^{+} + H_2O.$$

10g. of phenol (C_6H_5OH) were dissolved in 50mls. of pyridine (C_2H_5N), and this mixture was kept in ice. While being continuously stirred at 0-4°C, 8.5 mls. of chlorosulphonic acid ($ClSO_3H$; technical grade) were added dropwise. When the addition of the latter was complete, the mixture was stirred at 0-4°C for 1 hour, after which 50mls. of 40% aqueous potassium hydroxide (KOH) were added, the mixture still being kept cool. This resulted in a precipitate (potassium salt of phenyl sulphate) which was filtered. The precipitate was washed with ether, and extracted with approximately 200mls. of 99% hot ethanol. The ethanol extract was evaporated to a very small volume and left to cool overnight whereupon , whitish-yellow crystals of potassium phenyl sulphate appeared. These were filtered and the phenyl sulphate was not further purified since on chromatography in 1-butanol: aqueous ammonia (specific gravity, 0.88): water (10:1:1), it was found to be free of impurities. (Yield was 2.8g. ; approximately 9%).

Tests to Determine the Authenticity of Phenyl Sulphate

(1) 1M Barium chloride was added to the solution of the

crystalline solid obtained above, both before and after hydrolysis with 2N-HCl at 90°C for 30 min. A white precipitate of barium sulphate (a test for sulphates) was obtained only after hydrolysis with 2N HCl, suggesting that the compound was a sulphate, possibly of phenol.

(2) Small amounts of the dissolved test compound were spotted onto filter papers both before and after hydrolysis with 2N HCl. These were then sprayed with Gibb's reagent (1% 2,6-dichloroquinonechlorimide in ethanol, followed by saturated sodium bicarbonate). A blue colour for phenols was obtained with this reagent only after acid hydrolysis, showing that the synthesized compound was a phenyl salt.

(3) Chromatography in various solvent systems for the separation of phenol and its metabolites (see later) gave Rfs for phenyl sulphate identical to the published values.

In vivo Techniques

Animals used in Metabolic Studies

The details of the animals used are given in Table 2.1.

Xenobiotic Administration and Collection of Urines.

The appropriate doses of 1-naphthol or phenol dissolved in propylene glycol, together with carrier [1-^{14}C]-1-naphthol or [U-^{14}C]- phenol, were administered intramuscularly into primate species, and intraperitoneally, into rats, cats and guinea pigs. The details of dose levels and the amount of radioactivity given to each animal are listed in Tables of results.

During the experiments, the animals were kept in suitable metabolism cages, and maintained on their normal diets, and allowed water ad libitum. The urines were collected for upto 48 hours. For the primates and cats, these were collected by filtration through a small glass wool plug, whereas for rats and guinea pigs, the design of the all-glass metabolism

TABLE 2.1: ANIMALS USED IN METABOLIC STUDIES

ANIMAL	ORDER	FAMILY	SPECIES	STRAIN OR BREED	WEIGHT (Kg)	SEX	SUPPLIERS *
Rat	Rodentia	Muridae	Rattus norvegious	Wistar Albino	0.18-0.21	F	1
Guinea pig	Rodentia	Muridae	Mus musculus	Dunkin-Hartley	0.45-0.50	F	2
Cat	Carnivora	Felidae	Felis catus	Mongrel	3-3.5	M,F	3
Rhesus Monkey	Primate	Cercopithecidae	Macaca mulatta	-	4-5	M,F	5
Capuchin Monkey	Primate	Cebidae	Cebus albifrons	-	2-2.5	F	6

(continued)

TABLE 2.1: (continued)

ANIMAL	ORDER	FAMILY	SPECIES	STRAIN OR BREED	WEIGHT (Kg)	SEX	SUPPLIERS *
Patas Monkey	Primate	Cercopithecidae	Erythrocebus patas patas	-	-	F	5
Cynomolgus Monkey	Primate	Cercopithecidae	Macaca fascicularis	-	3.2	F	6
Tamarin Monkey	Primate	Callitrichidae	Saguinus oedipus	-	0.5	M	6
Bushbaby	Primate	Lorisidae	Galago senegalis	-	0.6	**	6
Treeshrew	Primate	Tupaiidae	Tupaia glis	-	0.2	**	6

(continued)

Table 2.1 (Contd.)

* Animal Suppliers

1. Anglia Laboratory of Animals,
   Huntingdon, Cambridgeshire.
2. Redfern Animal Breeders,
   Brenchley, Kent.
3. Gamestock Kennels,
   Ripley, Surrey.
5. Studied at the University of Ibadan,
   Nigeria.
6. A.S.L. Farms Ltd.,
   High Road, North Finchley,
   London, N.12.

** Sex not known.

cages ('Metabowls'; Jencons Scientific Ltd., Hemel Hampstead, U.K.) allowed the collection of urines separately from the faeces.

Except for the experiments with cats and the patas monkeys, the latter having been conducted at the University of Ibadan, Nigeria, the urines were collected directly into vessels surrounded with dry ice ('Drikold') in insulated polystyrene boxes. This meant that in most cases, rapid freezing of the urines occurred, thus, hopefully preventing both chemical and bacterial hydrolysis of conjugates. After each 24 hour collection, cages and funnels were washed thoroughly with distilled water, and the washings added to the urines. Urines were stored at -20°C until required for analysis.

Determination of Recovery

Recovery of ^{14}C in urine was determined by scintillation counting. Urine (0.1 to 1.0ml) was counted in duplicate in vials containing a dioxan-based scintillation fluid (5-20 mls; Bridges et al., 1967) using Packard Tri-carb Liquid Scintillation Spectrophotometers, Models 3214 and 3320. Quench correction was performed by the 'channels ratio' method (Kobayashi and Maudsley, 1974).

Determination of Conjugated Metabolites

Percentage ^{14}C in urine as various conjugates was determined by thin layer chromatography (t.l.c.), using aluminium-backed silica gel plates (0.2mm thickness; E. Merck, Darmstadt, W. Germany: Kielselgel 60F 254). Urine was applied as a band to a strip of plate (20 x 4 cm) and the chromatogram developed at room temperature with a suitable solvent (see below) which was allowed to run 15 cm. Plates were dried with cold air and then taped onto glass plates (20 x 5 cm) before scanning to locate peaks of radioactivity using a Radiochromatogram Scanner, Packard Model 7200.

Radioactive peaks were identified by co-chromatography with appropriate standards in at least two solvent systems. Where standards were not available, the metabolite was identified by various hydrolytic procedures (see below) and chromatographically examining the aglycone. For quantitation of the metabolites, the areas on the plates corresponding to the various radioactive peaks were carefully cut out and the aluminium and the silica gel transferred to scintillation vials for counting.

In cases where the radioactivity in urine was too small ($<10^5$ d.p.m per ml) to allow t.l.c. and radiochromatogram scanning of urine, ^{14}C - metabolites were extracted from urine using a column of Amberlite XAD-2 resin (BDH Chemicals Ltd., Poole, Dorset) as described by Mulé et. al. (1971). The methanol eluate from the column was concentrated by evaporation of methanol at 30-35°C. The concentrated methanol extract was then chromatographed as described above and the various metabolites identified.

Solvent Systems for Thin-layer Chromatography

The Rfs for 1-naphthol and its metabolites in two different solvent systems are given in Table 2.2.

The Rfs for phenol and its metabolites, namely phenyl sulphate, phenyl-β-D-glucuronide, and the oxidation products of phenol, i.e. quinol, resorcinol and catechol are given in Table 2.3. These oxidation products of phenol can be further conjugated with glucuronic acid and sulphate. The standards for these conjugates were not available, and were, therefore, identified by the hydrolytic procedures described below, with subsequent chromatography of the aglycones produced in solvent system 'F'. Quantitation of the quinol metabolites was achieved by counting the areas corresponding to the radioactive peaks obtained on chromatography of both the neat and

TABLE 2.2: THIN-LAYER CHROMATOGRAPHY OF 1-NAPHTHOL AND ITS METABOLITES, AND PHENOLPHTHALEIN AND ITS GLUCURONIDE.

Compound	Rf in Solvent System		
	A	B	E
1-Naphthol	0.85	0.72	-
1-Naphthylsulphate	0.47	0.28	-
1-Naphthylglucuronide	0.19	0.0	-
Phenolphthalein	-	-	0.77
Phenolphthalein glucuronide	-	-	0.21

The Rf values were determined in three solvent systems. Compounds were applied to silica-gel plates (0.2mm thickness; E. Merck, Darmstadt, Germany: Kieselgel 60F 254), and the plates were developed at room temperature. The spots were visualised under U.V. light (254nm) from a Hanovia Chromatolite lamp.

Solvent Systems: A, Benzene: Glacial Acetic Acid : Acetone (2 :1: 2 by vol.):

B, Butan-1-ol: aq. Ammonia(sp. gr. 0.88) : Water (4:1:5 by vol.).The upper organic phase was used.

E, 1-Propanol: aq. Ammonia(sp. gr. 0.88) : (7:3 by vol.).

TABLE 2.3: THIN-LAYER CHROMATOGRAPHY OF PHENOL AND ITS METABOLITES

COMPOUND	Rf IN SOLVENT SYSTEM			
	C	D	E	F
Phenol	0.75	0.76	0.76	0.45
Quinol	0.61	0.62	S*	0.17
Resorcinol	0.73	0.59	0.60	0.22
Catechol	0.72	0.38	S*	0.30
Phenyl-βD-glucuronide	0.23	0.10	0.28	0
Phenyl sulphate	0.45	0.32	0.50	0

* S means streaks

Chromatography was carried out as described in the legend to Table 2.2.

Solvent Systems: C, Butan-1-ol: Benzene: Pyridine: Water (5:1:3:2 by volume).

D, Butan-1-ol: aq.Ammonia (sp.gr.0.88): Water (10:1:1 by volume).

E, 1-Propanol: aq.Ammonia (sp.gr.0.88) (7:3 by volume).

F, Ethyl Acetate: Cyclohexane (3:7 by volume).

hydrolysed urines.

Hydrolyses of Urines

The nature of the conjugates was further confirmed or identified by enzymic and acid hydrolysis of urines.

(a) Enzymic Hydrolysis

This was carried out for identifying the glucuronide conjugates. Urine samples (1 ml) were adjusted to pH 5 with 0.2M acetate buffer, (0.5ml), and β-glucuronidase (0.5ml; Ketodase, 5000 units per ml; Warner-Chilcott Laboratories, Eastleigh, Hants) was added. The mixtures were incubated at 37°C for 22-24 hours. At the end of the incubation period, the mixture was neutralised with 1N - NaOH, and was chromatographed as previously described.

Control tubes were set up using phenolphthalein-β-D-glucuronide as substrate, and saccharic acid -1, 4- lactone as an inhibitor of β-glucuronidase. Thus, a metabolite was identified as a glucuronide if it was hydrolysed in the absence of saccharic acid -1, 4- lactone and not hydrolysed in its presence.

(b) Acid Hydrolysis

This was carried out for the identification of sulphate conjugates. To 1ml of urine samples was added 1ml of 2N-HCl, and the mixture incubated at 80-90°C for 30 minutes. At the end of the incubation period, the tube was cooled in an ice-bath, the sample was neutralised with 1N-NaOH, and chromatographed. Control tubes, here, contained 1ml of distilled water instead of 2N-HCl, and were treated as for test incubations. Thus, a metabolite was identified as a sulphate conjugate only if it was hydrolysed in the presence of the acid.

In Vitro Techniques

Animals and Tissues

Rats and guinea pigs of the same stock as shown in Table 2.1 were killed by cervical dislocation. The various tissues were removed and used immediately.

The same female cats (n=6) as shown in Table 2.1 were anaesthetized and then killed. The tissues from these cats were removed immediately, and stored at -20°C until use.

Postmortem samples of the lion and certain primate livers (see Results section for details) used in the present studies were kindly provided by Dr. Rivers of the Nuffield Institute of Comparative Medicine, Zoological Society of London, Regent's Park, London.

Enzyme Preparation

For enzyme preparation, liver, whole kidney and intestinal mucosa were used. The latter was obtained in the following manner. The duodenal and jejunal parts of the small intestine were separated from the rest of the intestine, which were then washed thoroughly with ice-cold 0.154M KCl to remove the intestinal contents. The duodenal and jejunal parts were then cut open and the mucosa scrapped off with a spatula. The mucosa was weighed and treated as for other tissues.

5-20% homogenates of the various tissues were prepared by homogenising in ice-cold 0.154M KCl with an 'Ultra-Turrax' homogeniser for 2-3 minutes. The homogenates were then centrifuged at 600g for 10 minutes in an MSE Highspeed 18 centrifuge at 4°C. The postnuclear (600g) supernatant contained both the phenolsulphotransferase and UDP-glucuronyltransferase activities, and was therefore, used for the assay of both enzyme activities. Enzyme activities thus obtained are presented as per mg. of tissue.

For the effect of various membrane perturbants on rat and cat UDP-glucuronyltransferase activities, microsomal enzyme preparations were used and these were prepared as follows. A 20% liver homogenate in 0.154M KCl was centrifuged at 10,000g for 30 minutes in an MSE Highspeed 18 centrifuge at 4°C. The supernatant thus obtained was then centrifuged at 100,000g for 1 hour in an MSE Highspeed 65 centrifuge at 4°C. The supernatant was discarded and the microsomal pellet, was resuspended by homogenising in 0.154M KCl. The microsomal preparation was then used for assaying the enzyme activity. Protein determination was carried out by the Biuret method (Gornall et al., 1949). The UDP-glucuronyltransferase activities thus obtained are presented as per mg. of microsomal protein.

Co-substrates

The co-substrate for UDP-glucuronyltransferase, UDP-glucuronic acid (UDPGA; Sigma (London) Chemical Company), and that for phenolsulphotransferase, [^{35}S]-labelled 3'-phosphoadenosine-5'-phosphosulphate (PAPS) were purchased. Cold PAPS was synthesised.

PAPS Biosynthesis

PAPS was prepared by the method of van Kempen and Jansen (1972). A 20% rat liver homogenate in 0.154M KCl was prepared with an 'Ultra-Turrax' homogeniser. After centrifuging for 1 hour at 44,000g in an MSE Highspeed 65 centrifuge at 4°C, the supernatant was lyophilised, and the dry powder containing the PAPS-synthesising activity was stored at -20°C.

For the synthesis of PAPS, the preincubation mixture consisted of 40mg. of the lyophilised powder, 8mls. of 0.4M tris-HCl buffer, pH 7.4, 1ml of 20mM $MgCl_2$ - 452 mM K_2SO_4 (in water), and 24.2mg of ATP. The incubation was carried out at 37°C for 1 hour. The reaction was terminated by boiling

for 1 minute. This was cooled immediately by immersing in an ice-bath, and while cooling, 0.53ml. of 1M $BaCl_2$ was added to remove the excess inorganic sulphate. The barium sulphate precipitate and the coagulated protein were removed by centrifugation at 1,000 r.p.m. for 10 minutes. The supernatant was used as the PAPS solution in subsequent incubations for assaying the phenolsulphotransferase activity.

Determination of PAPS Concentration

For accurate kinetic analysis of an enzyme, it is necessary to know the concentrations of the reactants and products. When a component is not readily available in a pure form and has to be synthesized, as for the cosubstrate, PAPS, a method is needed for the determination of its concentration. So far a number of methods have been described (Roy and Trudinger, 1970 ;van Kempen and Jansen, 1973; Stanley et al., 1975). However, all of these have certain disadvantages; for example, they are either time consuming, or the reversibility of the reaction has to be taken into account. These are discussed in detail later.

The method described herein tries to overcome many of the problems associated with PAPS determination. It utilizes the enzymic transfer of the sulphate group to 1-naphthol, and is based on the isotope-dilution principle, i.e. analysis for a component of a mixture based on the addition to the mixture of a known amount of labelled component of known specific activity, followed by isolation of a quantity of the pure component and measurement of the specific activity of that sample. Isotope-dilution methods and variations thereof represent most valuable quantitative procedures in biochemistry and analytical chemistry, and have been used successfully for the simultaneous determination of a number of xenobiotic metabolites in complex mixtures(Parke, 1965).

Procedure:

Incubation mixtures consisting of 0.6μCi of [^{35}S]-PAPS dissolved in ethanol: water (1:1), 20mg. of rat liver 600g supernatant, 1.92mls of the PAPS solution, 80 nmoles of 1-naphthol dissolved in methanol: 0.25M tris-HCl buffer, pH 7.4 (1:100; final concentration of substrate = 20μM), 0.8ml of 0.75M tris-HCl buffer, pH 7.4, containing 75mM EDTA and 94 mM KH_2PO_4, and 0.6ml of 0.25M tris-HCl buffer, pH 7.4, in a total volume of 4mls were set up in duplicate. Control incubations, one omitting only the enzyme, and the other omitting both the enzyme and cosubstrate([^{35}S]-PAPS and PAPS solution) were also set up.

Incubations were carried out at 37°C. 1ml samples were removed after 20 minutes into tubes containing 1ml of methanol to stop the reaction. The precipitated protein was removed by centrifugation in an MSE Major centrifuge at 1,000 r.p.m. for 5 minutes. The samples were analysed for the amount of sulphate transfer from PAPS to 1-naphthol by both fluorimetric and isotopic analyses.

Fluorimetric Analysis

1ml. incubation samples in 1ml of methanol were made alkaline to pH 11 with 2.0mls of 0.5N NaOH. The enzymic disappearance of 1-naphthol was determined by measuring the difference in fluorescene obtained between the test sample, and the control without PAPS or enzyme. The resultant values were expressed in nmoles of 1-naphthol per ml by comparison with a standard curve derived from varying concentration of 1-naphthol in the presence of methanol and 0.5N NaOH.

Fluorescence was measured in 10x10x45mm fused silica cuvettes on an MSE Spectro-Plus (MSE Scientific Instruments, Crawley, Sussex) set up in the fluorescence mode. The excitation wavelength used was 345mμ while emission was measured

at 445 mμwith a cut-off filter. When authentic 1-naphthyl sulphate at pH 11 was measured at these wavelengths, an insignificant fluorescence reading was obtained. It was possible therefore to detect 1-naphthol in the presence of 1-naphthyl sulphate at these wavelengths.

That the enzymic disappearance of 1-naphthol was only due to its sulphation was checked by running appropriate controls as mentioned above, and further confirmed by running a concommitant incubation using [1-^{14}C]-1-naphthol as carrier substrate and by specific measurement of [1-^{14}C]-1-naphthyl sulphate formed (for further details see the section for kinetic measurements for phenolsulphotransferase activity). Thus, Table 2.4 compares the amount of 1-naphthol converted to its sulphate, as determined by fluorimetric analysis and by specific measurement of [1-^{14}C]-1-naphthyl sulphate.

Isotopic Analysis

The amount of ^{35}S transferred to 1-naphthol from [^{35}S] - PAPS was determined by chromatographic separation of 1-naphthyl-[^{35}S]-sulphate from the unreacted [^{35}S]-PAPS in two solvent systems, each utilizing different stationary phases.

(a) Thin-layer Chromatography

Samples (20μl) were applied to strips (20 x 4cm) of aluminium-backed silica-gel plates (Merck, Darmstadt, W. Germany: Kieselgel 60F 254) and the chromatograms developed with methanol. Radioactive areas were located by radiochromatogram scanning and quantitated by scintillation counting in the same way as ^{14}C (see *in vivo* techniques).

Radioactive peaks were identified by first, co- chromatography with authentic 1-naphthyl sulphate, and secondly, running and scanning chromatograms to which only [^{35}S]-PAPS had been applied. The Rfs for [^{35}S]-PAPS and 1-naphthyl

TABLE2.4: The Amount of 1-Naphthylsulphate formed As Determined By Flourimetric Analysis and By Measurement of [1-^{14}C]1-Naphthylsulphate.

METHOD OF DETERMINATION	AMOUNT OF 1-NAPHTHOL CONVERTED TO 1-NAPHTHYL SULPHATE (nmoles/min./g. liver)
Flourimetry	90 ± 9.02
[1-^{14}C]1-Naphthyl Sulphate Determination	91.3 ± 6.6

See text for details of methods used.
Results are presented as means ± S.E.M.
The number of determinations is 3 in each case, each determination on enzyme preparation from three pooled livers.

sulphate are given in Table 2.5. This system does not separate [^{35}S]-PAPS from its degradation products, APS and inorganic sulphate, which the following system can do.

(b) Paper Chromatography

Samples (20µl) were applied to Whatman paper Chromedia DE-81 and chromatograms were developed by decending chromatography with 0.1M phosphate buffer, pH 6.0, as described by Denner and Stokes (1972). A sufficient separation of all the compounds could be achieved by running the chromatograms for a distance of approximately 30cm. (running time about 3hrs.). The radioactive peaks were located, identified and quantitated as for thin-layer chromatography.

Table 2.5 gives Rfs for [^{35}S]-PAPS, [^{35}S]-APS, inorganic-[^{35}S]-sulphate and 1-naphthyl-[^{35}S]-sulphate in this chromatographic system. The standards for [^{35}S]-APS and inorganic [^{35}S]-sulphate were not available but these were tentatively identified by comparison with those Rfs given by Denner and Stokes (1972).

Routinely, the thin-layer chromatographic system was used. Radiochromatograms obtained by both systems are illustrated in Figures 2.2 and 2.3. That both chromatographic procedures yielded similar results is shown in Table 2.6.

Calculation For Obtaining PAPS Concentration In the Lyophilised Preparation

Based on the principle of isotope-dilution analysis, the amount of PAPS in the assay mixture can be obtained as follows:

$$\text{Concentration of PAPS (nmoles/ml)} = \frac{x}{(y/z).v}, \quad \text{(Equation 2.1)}$$

where x = Specific activity, in µCi/nmole, of [^{35}S]-PAPS added.

TABLE 2.5: Chromatographic Separation of [^{35}S]-PAPS and its Degradation Products, and 1-Naphthylsulphate.

Compound	Rf in Solvent System	
	G	H
[^{35}S]- PAPS	0.09	0.0
[^{35}S]- APS	-	0.25
Inorganic [^{35}S]-sulphate	-	0.73
1-Naphthylsulphate	0.72	0.96

T.L.C.:

Samples were applied to aluminium-backed silica-gel plates (E.Merck, Darmstadt, Germany; Kieselgel 60F 254). 1-naphthylsulphate was visualised under U.V. light, while the Rf for [^{35}S]-PAPS was obtained by radiochromatogram scanning.

Solvent System G: Methanol.

Paper Chromatography:

Samples were applied to Whatman Paper Chromomedia DE-81. Rfs were obtained by radiochromatogram scanning.

Solvent System H: 0.1M Phosphate Buffer, pH 6.0.

Figure 2.2: Separation of [^{35}S]-PAPS and Its Degradation Products From 1-Naphthyl-[^{35}S]Sulphate By Thin-Layer Chromatography.

(See Text For Details)

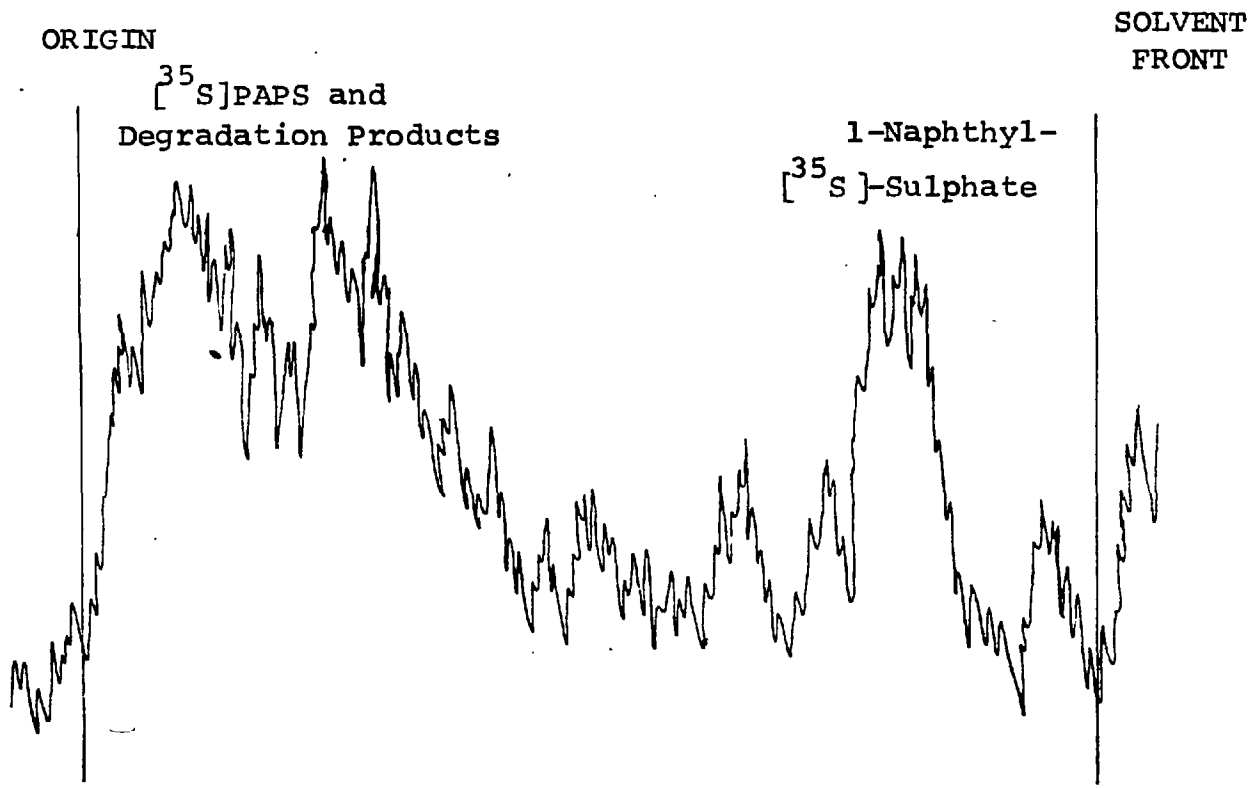


Figure 2.3:Separation of [^{35}S]PAPS, [^{35}S]APS, Inorganic-[^{35}S]Sulphate and 1-Naphthyl-[^{35}S]Sulphate By Paper Chromatography.

(See Text For Details)

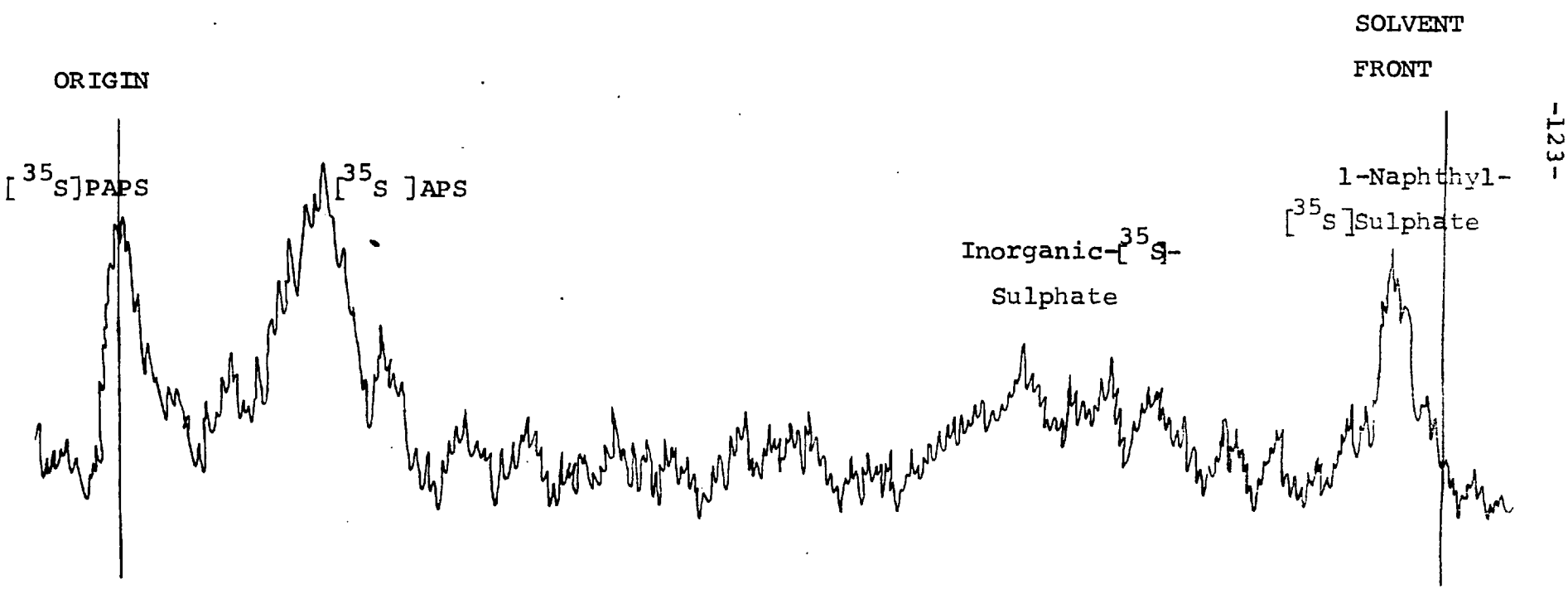


TABLE 2.6: The Percentage of ^{35}S As 1-Naphthyl Sulphate As Determined by Thin-layer and Paper Chromatography

CHROMATOGRAPHIC SYSTEM	% ^{35}S DETERMINED AS 1-NAPHTHYL SULPHATE
Thin-layer	25.2 ± 6.03
Paper	24.1 ± 5.66

See text for details of methods used.
Results are presented as means ± S.E.M.
The number of determinations is 3 in each case, each determination on enzyme preparation from three pooled livers.

y = μCi/litre of 1-naphthyl-[^{35}S]-sulphate generated (determined by isotopic analysis).

z = nmoles/litre of 1-naphthylsulphate formed (determined by fluorimetric analysis).

y/z = Specific activity, in μCi/nmole, of 1-naphthyl-[^{35}S]-sulphate formed.

v = Volume of PAPS solution added.

As determined by this method, the concentration of PAPS in the lyophilised preparation was found to be in the range 85-140 μmoles/litre, for at least three different lyophilised preparations, each prepared from three rat livers. Such a wide variation obtained for the concentration of PAPS is not experimental, but is due to the use of different lyophilised preparations, as well as the determination of concentration on the same lyophilised preparation after different periods of storage; a lyophilised preparation gradually loses PAPS-synthesizing activity during storage at -20^{o}C. This wide variation also indicates that it is necessary to know the concentration of 'active sulphate' generated by, for example, a lyophilised preparation, when such a system is being used to study the phenolsulphotransferase reaction.

<u>The Present Method For the Determination of PAPS Concentration Versus Other Existing Methods</u>

Previous methods of assay for 'active sulphate' have been reviewed by Roy and Trudinger (1970), the methods being determination of the transfer of the sulphuryl group from PAPS to a suitable acceptor, 2-naphthol or p-nitrophenol, through the action of phenolsulphotransferase. When using 2-naphthol as substrate, the 2-naphthyl sulphate formed is determined as a methylene blue salt, this determination being rather time consuming and very much dependent upon the quality

of the methylene blue reagent. When using p-nitrophenol as substrate, one has to take into account the reversibility of the transferase reaction owing to the partial anhydride character of the p-nitrophenylsulphate formed.

Another more rapid and sensitive method utilizing the fluorogenic substrate, 4-methylumbelliferone, (van Kempen and Jansen, 1973) has also been described. This method involves the separation and then hydrolysis of the reaction product, 4-methylumbelliferone sulphate, to release the aglycone which is measured fluorimetrically. This method depends upon the construction of a standard curve using chemically-synthesized PAPS, and then obtaining the concentration of PAPS generated by a crude sulphate-activating system from this standard curve.

Crude sulphate-activating and phenolsulphotransferase systems, however, contain PAPS-degrading enzymes (Dodgson and Rose, 1975; Figure 1.4), and as shown by the present studies, the degradation of [^{35}S]-PAPS to [^{35}S]-APS and inorganic-[^{35}S]- sulphate by both the 600g supernatant used as the enzyme preparation, and the PAPS solution obtained from the lyophilised preparation does occur (Figure 2.3).

Therefore, if the concentration of PAPS generated by a crude sulphate-activating system is obtained from a standard curve constructed using chemically-synthesized PAPS, the values obtained for PAPS concentration are likely to be erraneous since in such a system, the effects of PAPS degrading enzymes present in the crude sulphate-activating systems will not have been taken into account. Those present in the enzyme preparation, though, may have been allowed for as the same enzyme preparation was used for the assay of standard PAPS as the biosynthetic PAPS. The other disadvantages of the method of van Kempen and Jansen (1973) are that it uses a column chromatographic procedure for the separation of the

reaction product from the aglycone which may be time consuming, and also that it requires the hydrolysis of the aglycone sulphate before it can be determined; this means that in order to avoid errors, one has to be certain that complete hydrolysis of the sulphate has taken place which may not always occur in a uniform manner.

More recently, a bioluminescence technique for determining PAPS in biological materials by hydrolysing it first to PAP has been described (Stanley et al., 1975). PAP, in the reaction, produces light proportional to its concentration in the bioluminescence system of Renilla reniformis. The assay is sensitive and specific for PAPS provided appropriate controls are included to assay the endogenous PAPS prior to acid hydrolysis. The bioluminescence system of Renilla reniformis, however, is not easily available. This method is different from other existing methods in that it does not utilize the phenolsulphotransferase system for assaying the co-substrate.

The method for the determination of PAPS devised here, takes into account PAPS degrading enzymes in both the enzyme and the crude PAPS preparations in the following way. This PAPS assay is dependent upon the presence of both the standard [^{35}S]-PAPS and the biosynthetically derived PAPS simultaneously. Therefore, some decomposition of the PAPS by whatever mechanism will be immaterial because both the labelled and unlabelled molecules will be equally affected and their ratio will remain the same, thus making no difference to the calculated value of PAPS concentration (Equation 2.1). The other advantages of this assay system are the rapid and sensitive fluorimetric assay of 1-naphthol (concentrations of 1-naphthol as low as 1μM can be assayed) in a complex mixture, and rapid chromatographic procedures with excellent resolution, one of which, if desired, can be used to quantitate the degradation products of PAPS as well. The commercial

availability of [^{35}S]-PAPS makes this technique for determining PAPS concentration in crude systems even more attractive. Furthermore, this method does not have to be dependent upon 1-naphthol as the sulphate acceptor, and can be used with any substrate that can be assayed easily by non-radioactive as well as radioactive means.

Assay of Enzyme Activity

(a) UDP-glucuronyltransferase activity

Enzyme activity towards 1-naphthol was measured in incubation media containing 1.5mM UDPGA (final concentration) in 0.67M tris-HCl buffer, pH 7.4, with 20mM $MgCl_2$, aqueous [1-^{14}C]-1-naphthol (upto 0.5μCi per incubate), and sufficient amounts of cold 1-naphthol dissolved in methanol: 0.25M tris-HCl buffer, pH7.4 (1:100) to give final substrate concentrations in the range 0.01mM to 5mM. The total volume was made upto 1ml with 0.25M tris-HCl buffer, pH7.4. Enzyme activity towards phenolphthalein was measured as above using [^{14}C]-phenolphthalein, dissolved in water: ethanol (1:1), as the substrate.

The kinetic parameters for UDP-glucuronyltransferase with UDPGA as the varying substrate were measured in a similar manner as above except that 1-naphthol concentration was kept constant and the total incubation volume was made upto 1ml. by adding varying amounts of 0.67M tris-HCl buffer, pH 7.4, and 30 mM UDPGA in 0.67M tris-HCl buffer, pH 7.4, containing 20mM $MgCl_2$, to give the final UDPGA concentrations in the range 0.275-6.0 mM.

The amount of enzyme used varied from 5-40 mg. of tissue per incubate. To ensure linearity of the reaction with time, appropriate incubation periods (5-60 min) were chosen so that not more than 20% of the acceptor substrate would be con-

verted during that period. In each case, incubations were carried out at 37°C in duplicate for each substrate concentration, and suitable controls, omitting the enzyme, were included.

For bisubstrate kinetic analysis, initial rates of UDP-glucuronyltransferase activity were studied as a function of the concentration of UDPGA at several different fixed concentrations of 1-naphthol or phenolphthalein, and as a function of varying concentrations of 1-naphthol or phenolphthalein at several fixed concentrations of UDPGA. The substrate concentrations used are indicated with the appropriate figures in the Results sections.

Effects of various membrane perturbants on UDP-glucuronyltransferase activity.

The incubation media were essentially as above with 1.5 mM UDPGA (final concentration), aqueous [1-^{14}C]1-naphthol (0.2μCi per incubate) and sufficient amounts of cold 1-naphthol to give final concentrations of 20μM(rat) or 1000μM (cat). Each incubation medium (total volume 1ml) contained either 0.3mg. (rat) or 0.6 mg. (cat) of microsomal protein. Reaction rates were determined when product formation was linear with time.

For the effect of Triton X-100, varying amounts of Triton X-100 were added to the assay system prior to the addition of the enzyme to give the Triton X-100: microsomal protein ratios in the region 0.033 to 0.33.

For the effect of bilirubin, varying amounts were added to the assay system to give the final concentrations in the range 0.1 to 2.0 mM. Bilirubin solution was freshly prepared by dissolving in 0.07 N NaOH. The control incubations contained equal amounts of alkali as the test incubations. The amount of base added was insufficient to alter the pH of the incubation mixtures.

For the effect of phospholipases A and C, the microsomes were preincubated with the two phospholipases as follows. Rat and cat liver microsomal preparations were treated with phospholipase A (Lecithinase A, 2-acyl-hydrolase; E.C.3.1.1.4; from Naja Naja venom; Sigma (London) Chemical Company) at a microsomal to phospholipase A protein ratio of 100:1 in 12.5 mM tris-HCl, pH 8.0 and 2.5mM calcium chloride, at 23°C. The microsomal preparations were treated with phospholipase C (Lecithinase C from Cl. welchii; Type I; Sigma (London) Chemical Company) as above except that a microsomal to phospholipase C protein ratio of 16:1 was used. Aliquots were removed from each at 0.5, 3, 5, 10, 15 and 20 minutes for rat microsomes and for upto 60 minutes for cat microsomes, and assayed at 37°C for UDP-glucuronyltransferase activity. The assay tubes in this case contained 3mM EDTA (final concentration) to inhibit the further action of the two phospholipases. Control incubations were carried out which were assayed with microsomal preparations treated in exactly the same way as those for phospholipase-treated microsomes except that the two phospholipases were not included in the preincubation mixtures.

Effect of UDP-N-acetylglucosamine on UDP-glucuronyltransferase activity.

This was determined by adding to the incubation mediums, UDP-N-acetylglucosamine at final concentrations of 1,2 and 4 mM for both rat and cat microsomes, and assaying for UDP-glucuronyltransferase activities as before.

(b) Phenolsulphotransferase activity

The incubation medium contained sufficient volumes of the PAPS solution to give various concentrations of PAPS (15-75μM; final concentrations; see Results section), 0.2 ml of 0.75M tris-HCl buffer, pH7.4, with 75mM EDTA and 94 mM KH_2PO_4, and aqueous [1-^{14}C]-1-naphthol (upto 0.5μCi per in-

cubate) to give final substrate concentrations ranging from 0.5-15μM. The total volume was 1ml. and the amount of enzyme varied from 1-25 mg of tissue per incubate. The incubation times (5-10 minutes) were chosen as stated for UDP-glucuronyltransferase activity. Again appropriate controls were included.

For bisubstrate kinetic analysis, initial rates of phenolsulphotransferase activity were studied as for UDP-glucuronyltransferase activity except as a function of the concentration of PAPS at several fixed concentrations of the aglycone substrate.

Termination of the Reaction

Both for UDP-glucuronyltransferase and phenolsulphotransferase activities, to terminate the reaction, at the end of each incubation period, 0.1 ml samples were removed into tubes containing 0.1 ml of methanol with the respective authentic aglycone (i.e. either 1-naphthol or phenolphthalein), its glucuronide and sulphate. The samples were then centrifuged at 1,000 r.p.m. for 10 minutes in an MSE Major centrifuge to precipitate the coagulated protein, and then chromatographed for analysis.

Thin-layer Chromatography and Quantitation of Metabolites in The Incubated Samples

For the analysis of incubation samples, 20μl samples were applied to pieces (100 x 25 mm) of aluminium-backed silica gel plates (0.2 mm thickness; E. Merck, Darmstadt, W. Germany; Kieselgel 60F254) and the plates developed with the appropriate solvent system for 10-30 minutes at room temperature. For 1-naphthol and its metabolites, solvent system 'A' was used, while solvent 'E' was used for the separation of phenolphthalein and its glucuronide (Table 2.2).

The incubated samples already contained the standard

aglycone substrates and their metabolites so that when the plates were dry, the spots could be visualized under U.V. light. The bands corresponding to the various metabolites and the aglycone substrates were cut up and the ^{14}C -label in the appropriate pieces determined by counting in either a dioxan-based scintillation fluid, or a toluene-based scintillation fluid containing Triton X-100 (1 volume of Triton X-100 to 2 volumes of the scintillant).

Effect of Triton X-100 on Microsomal Protein and Phospholipid concentrations, and on the Distribution of the ^{14}C-labelled Aglycone Substrate.

This was determined for both the rat and cat microsomal preparations by first treating the microsomal preparations with various amounts of Triton X-100 to give similar protein to Triton X-100 ratios as those used for determining the effect of the detergent on UDP-glucuronyltransferase activity. The treated microsomes, also containing the ^{14}C-labelled aglycone substrate ([1-^{14}C]1-naphthol) and sufficient amounts of cold material to give the same final substrate concentration as those used for determining the effect of the detergent on enzyme activity, were left at 4°C for 15 minutes. The samples were then centrifuged at 80,000g for 30 minutes in an MSE Superspeed 65 centrifuge at 4°C. At the end of centrifugation, the solubilised protein and phospholipid were left in the supernatant, while the unsolubilised components were brought down in a pellet. These were separated and the pellet was resuspended in 0.154M KCl in a similar volume as that of the supernatant. The solubilised and unsolubilised preparations were then determined for protein by the Biuret method (Gornall et al. 1949), for phospholipid (see below), and for [1-^{14}C]-1-naphthol by scintillation counting.

Determination of Phospholipid Phosphate

This was determined by the method of Fiske and SubbaRow

(1925).

Reagents:

0.22% Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$)

1mM Standard sodium phosphate ($Na_3PO_4.10H_2O$)

70% Perchloric acid (Sp.gr. 1.54; BDH Chemicals Limited)

Fiske-SubbaRow reagent prepared as follows.

Preparation of the Fiske-SubbaRow Reagent:

This was always freshly prepared. 30g. of sodium meta-bisulphite were dissolved in distilled water to a final volume of 200 ml. When the solution was clear, 0.5g. of 1-amino-2-naphthol-4-sulphonic acid was added which turned the solution milky. 1g. of anhydrous sodium sulphite was added to clear the solution. This was then used as the Fiske-SubbaRow reagent.

Procedure for Phospholipid Determination:

(1) 0.1 ml samples were pipetted into test-tubes. For the callibration curves, volumes of standard phosphate were made upto 0.1 ml to give concentrations in the range 0.2-1.0 mM. For blanks, 0.1 ml. samples of 0.9% NaCl were used.

(2) All samples were evaporated to dryness in an oven set at 120°C.

(3) After complete evaporation had taken place, 0.5 ml of perchloric acid was added to each sample and mixed well.

(4) Tubes were placed in a tube-heating block (Grant Instruments Limited, Cambridge, U.K.) set at 120°C for 1 hour, and then cooled.

(5) 4.6 mls of ammonium molybdate were added followed by 0.2 ml of Fiske-SubbaRow reagent, and mixed well.

(6) All tubes were covered with glass marbles and placed at 100°C in the tube-heating block for 8 minutes.

(7) When cool, the samples were read at 830 nm. in an SP 600 (Unicam Instruments, Cambridge, U.K.) against NaCl blanks.

N.B. Assay tubes were always soaked in chromic acid and thoroughly washed with distilled water before use because clean-

ing detergents contain phosphates which interfere with the phospholipid determination of samples.

Cholesterol Determination

Cholesterol in rat and cat microsomes was determined by conversion to a light absorbing substance by the Liebermann Burchard reaction in which the addition of sulphuric acid in the presence of acetic anhydride gives a green chromophore. All the reagents used were purchased in form of a Biochemica Test Combination (Boehringer-Mannheim, W. Germany) for β-Lipoproteins.

To 50µl samples of microsomes (protein concentration 30 mg/ml) were added 2.5 mls. of a 7.0 M acetic acid - 6.5M acetic anhydride (Colour Reagent). The tubes were mixed, allowed to stand in a water-bath at 20-25°C for 5 minutes and then 0.5ml. of concentrated sulphuric acid was added with vigourous mixing. The tubes were allowed to stand in a water-bath at 25°C for 10 minutes, followed by the measurement of optical density at 570 nm. For blanks and standards, 0.05 ml of distilled water and 0.05 ml of the cholesterol standard provided, were treated as described for the microsomes. The concentration of cholesterol in microsomes was obtained by comparison with the standard.

Microsomal Phospholipid and Fatty Acid Extraction and Fatty acid Analysis

(a) Extraction of Microsomal Phospholipids

Extraction of the microsomal phospholipids was carried out by the Bligh and Dyer extraction (1959). Freshly prepared microsomes were homogenised in 17 volumes of chloroform: methanol (2:1), and filtered through a sintered funnel. To the filtrate was added 0.2 volumes of 0.75% KCl, and the mixture was shaken for 5 minutes. It was centrifuged at approximately 800 r.p.m. for 10 minutes in an MSE Major centrifuge.

After centrifugation, the top layer was aspirated off and discarded. The lower layer was washed once with chloroform: methanol: water (1:16:16) containing 0.74% KCl. The top layer was removed and the bottom layer evaporated to dryness under nitrogen. To this was added 10 mls. of acetone saturated with $MgCl_2$, and the mixture was left at -20°C for 16 hours. After storage, it was centrifuged and the precipitate dissolved in 5 mls. of chloroform: methanol (1:1).

(b) Fatty Acid Extraction and Methylation

This was carried out by alkaline hydrolysis of the phospholipids followed by methylation with boron trifluoride.

2 mls. of the solution obtained above were taken and evaporated to dryness under nitrogen. 4mls. of 0.5N methanolic NaOH were added and the solution heated for 5 minutes on a steam bath, after which it was acidified with 2 mls. of 1N - HCl in methanol. To this were added 2mls. of 14% boron trifluoride in methanol and the solution heated for 2 minutes. After cooling, 5 mls. of saturated NaCl were added together with 6 mls. of n-hexane. The mixture was shaken and centrifuged. The top layer was transferred to a flask and dried with anhydrous Na_2SO_4. The fluid layer was decanted and evaporated to a small volume (0.5-1.0 ml) for fatty acid analysis.

(c) Analysis of Fatty Acid Methyl Esters

The methylated fatty acids were analysed by gas-liquid chromatography (G.L.C.) on an F. and M. Scientific 402 High Efficiency Gas Chromatograph (Hewlett-Packard Model 17503A) with a hydrogen flame ionisation detector. The samples were separated on a 3mm. x 300 cm. column packed with 3% diethyl glycol succinate (Distributors: K. J. Clarke, Glemsford, Suffolk) on chromosorb W-H.P. operated at 180°C with nitrogen as the carrier gas (flow rate, 30 ml/min.).

The microsomal fatty acids were identified by comparison with retention times obtained for authentic samples of various

fatty acid methyl esters, and quantitated by extrapolation from callibration curves constructed using known quantities of the standard fatty acid methyl esters.

PART I - In Vivo STUDIES

CHAPTER THREE

Conjugation of 1-Naphthol In The Rat, Guinea pig and Cat

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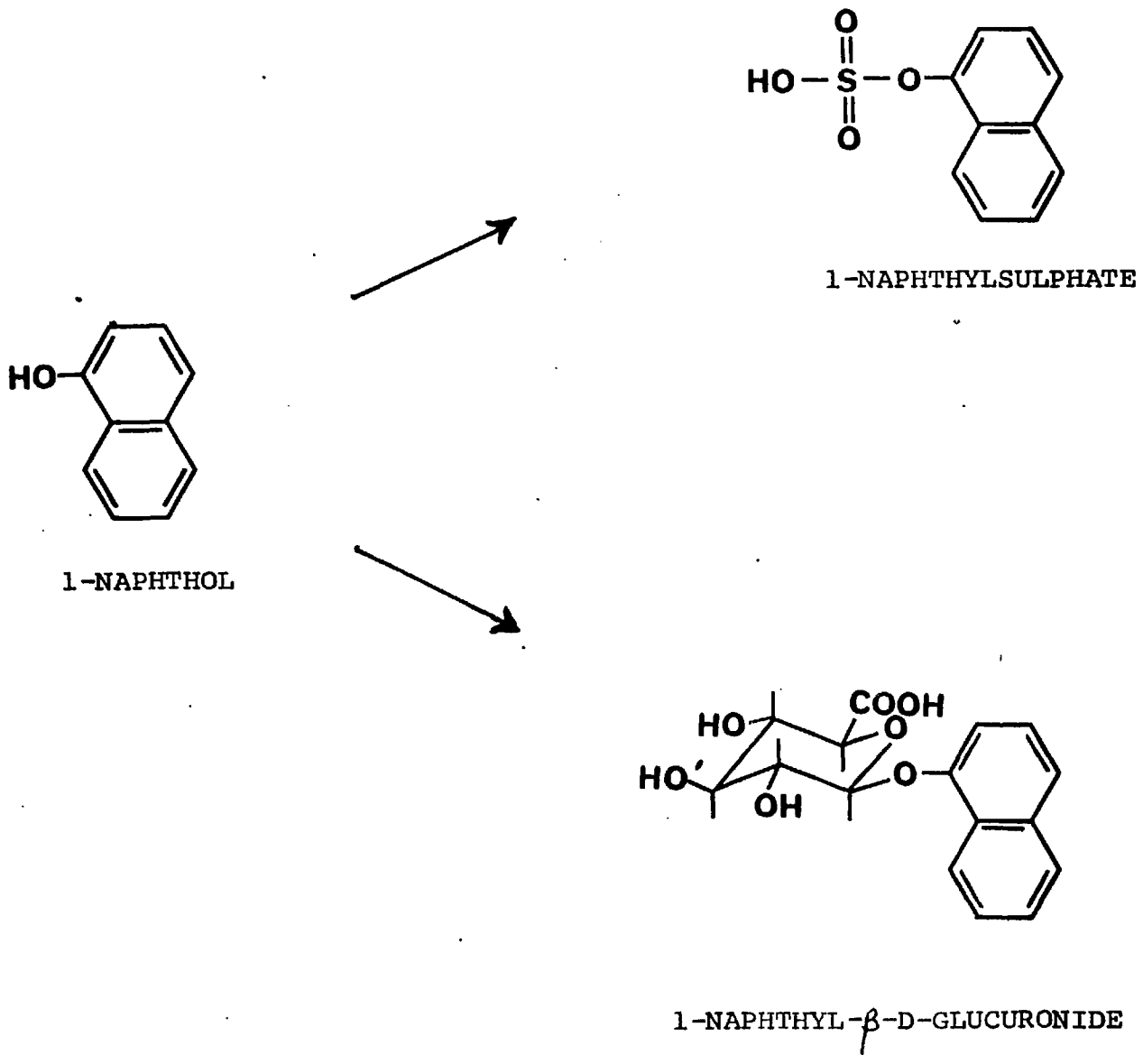
Introduction

1-Naphthol undergoes the typical metabolism of phenols in most mammalian species studied, and is conjugated with either glucuronic acid or sulphate, or both. (Figure 3.1).

Figure 3.1



Thus, the rat has been shown to excrete about 50-60% of a 100mg/Kg dose of 1-naphthol as 1-naphthyl-β-D-glucuronide and about 20% as the sulphate (Berenbaum and Young, 1951). Capel et al. (1974a) have also shown that for an intraperitoneally administered dose of 25mg/Kg, about equal quantities of both the glucuronide and sulphate conjugates of 1-naphthol are excreted by the rat. In fact, for a number of phenols studied in the rat (Table 1.16), both glucuronide and sulphate conjugates are formed, except for phenolphthalein which is excreted in the bile entirely as the glucuronide conjugate. In vivo studies in a number of rodent species (rat, mouse, gerbil, jerboa and lemming; Capel et al., 1972) have indicated that all of these species utilize both glucuronic acid and sulphate for the conjugation of phenol (dose: 25mg/Kg) in similar proportions, the exceptions being the hamster and the guinea pig which excrete a much greater proportion of the glucuronide conjugate (approximately 80%) compared with that of sulphate (about 20%). The cat, on the other hand, is known to have a low glucuronidating capacity for a number of phenolic compounds, 1-naphthol included, though with certain phenols, for example, phenolphthalein, significant amounts of the glucuronide are formed.

Thus, the above observations in the three species, used in the present study namely the rat, guinea pig and cat, illustrate that although a number of different species may use identical pathways of metabolism for a given compound, quantitative differences may occur in the metabolic fate of that compound. These quantitative differences in metabolism may be caused by several factors (see Chapter 1), the dose of the xenobiotic administered being one of them. In the past, comparisons of the species differences in conjugation of phenols (Capel et al., 1972, 1974a) have involved only one dose of the phenolic compound in a given species. Therefore, in order to

determine whether variation in the dose of a phenol could alter the pattern of metabolism in the rat, cat and guinea pig, the metabolism of 1-naphthol in these species at a number of dose levels was investigated.

Results

Rat

When [1-^{14}C] -1-naphthol together with cold 1-naphthol was injected intraperitoneally into rats at various dose levels, most of the radioactivity (90-98%) was excreted in 24 hours, in the urine. (see Table 3.1.). When samples of urine were chromatographed and scanned, two peaks of radioactivity corresponding to 1-naphthyl sulphate and 1-naphthyl-β-D-glucuronide were obtained at each dose. Figure 3.2 illustrates typical radiochromatogram scans obtained from urine of three species studied at the 25mg/Kg dose, indicating the distinctive pattern of excreted metabolites seen in each species.

On quantitation of the metabolites in the rat at the two lower doses of 0.07 mg/Kg and 1mg/Kg, about 38% of the 24 hour excretion was found as glucuronide and about 62% as the sulphate conjugates (Figure 3.3). At the 25mg/Kg and 100mg/Kg dose levels, about 50% of each of the conjugates were found. Unchanged 1-naphthol was not detected at any of the dose levels investigated.

Guinea pig

The conjugation of 1-naphthol in the guinea pig was tested only at two doses (Figure 3.4). An attempt was made to study the dose at 100mg/Kg but death ensued within two hours after administration. No excretion had occurred during that period.

In the guinea pigs (n=3 at each dose level), the excretion of 1-naphthol was lower than in the rat, 70-80% of ^{14}C having been excreted in the first 24 hours, and very little being

Table 3.1

Aqueous [1-^{14}C]1-naphthol was administered intraperitoneally (2μCi/animal for rats and guinea pigs and 10μCi/animal for cats) together with sufficient carrier 1-naphthol dissolved in propylene glycol to give the indicated dose levels. For details of animals used see Materials and Methods, Table 2.1. Urine was collected for upto 48h. ^{14}C in urine was determined by scintillation counting.

Results are presented as means ± S.E.M.

* n = number of animals

TABLE 3.1: URINARY EXCRETION OF [1-^{14}C]-1-NAPHTHOL IN RAT, GUINEA PIG AND CAT

SPECIES	n*	DOSE (mg/Kg)	^{14}C EXCRETED (% DOSE) IN	
			24h	48h
RAT	6	0.07	93.9 ± 4.7	-
		1.0	89.6 ± 5.6	-
		25.0	97.7 ± 2.02	-
		100.0	94 ± 4.3	-
GUINEA PIG	3	0.03	71.7 ±12.9	72.5 ± 12.6
		25.0	79 ± 2.9	79.9 ± 2.8
CAT	3	1.0	7.6 ± 3.9	-
		5.0	45.3 ± 9.9	58.5 ± 5.8
		10.0	42 ± 5.3	48.5 ± 4.5
	6	25.0	70.3 ± 8.7	98.1 ± 4.9

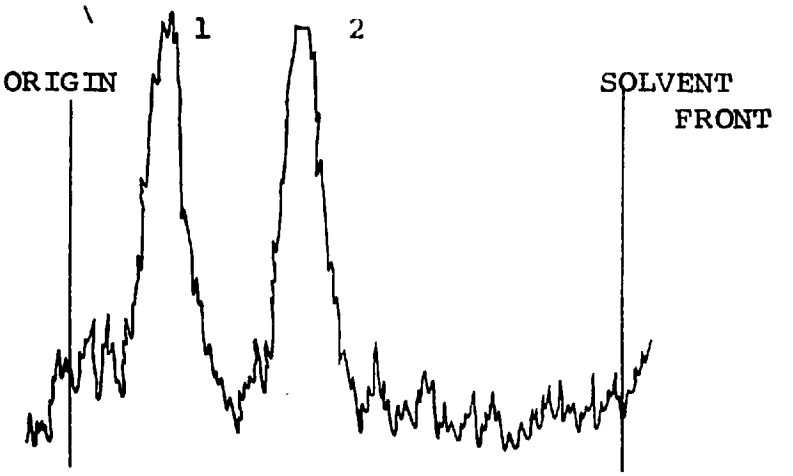
Figure 3.2

Urines (24h.) were chromatographed as described in legend to Table 2.2. Solvent A was used.

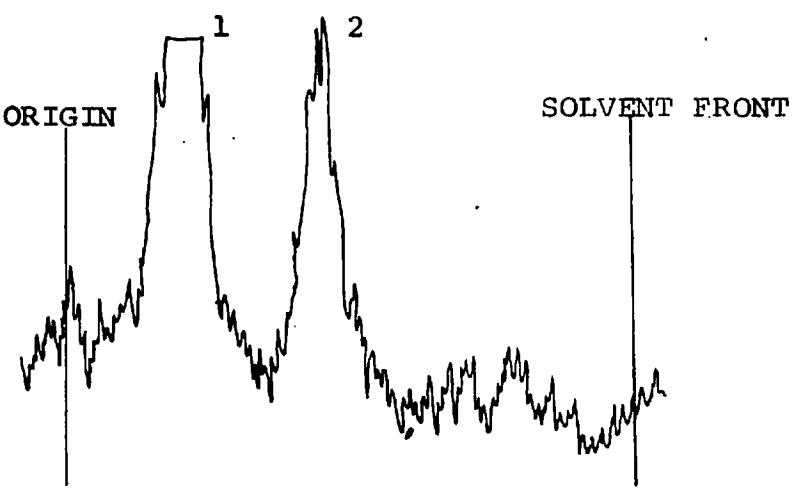
Peak 1	1-Naphthylglucuronide
Peak 2	1-Naphthylsulphate

Figure 3.2: Patterns of Excreted Metabolites of 1-Naphthol in Different Species at 25 mg/Kg Dose Level.

RAT



GUINEA PIG



CAT

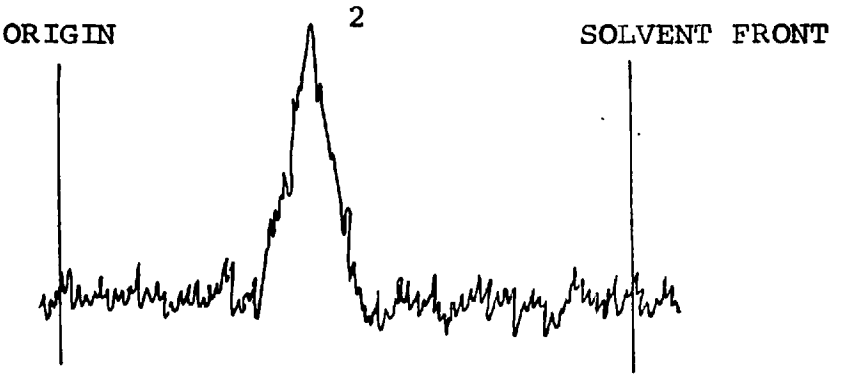


Figure 3.3

For details of animals , and urine collection and analysis, see legend to Table 3.1 and Materials and Methods, Chapter 2. Results are presented as mean ± S.E.M. Bars indicate S.E.M. n, the number of animals =6.

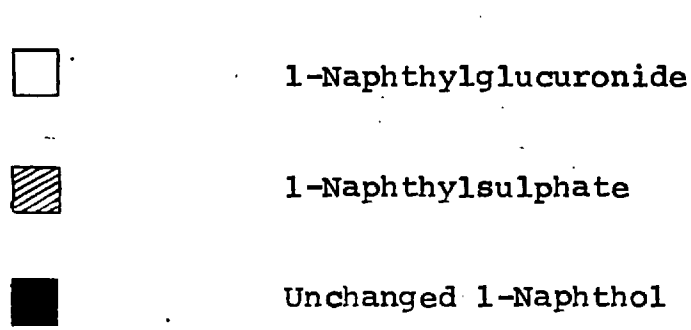


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Figure 3.3: Urinary Metabolites of 1-Naphthol at Various Dose Levels in the Rat.

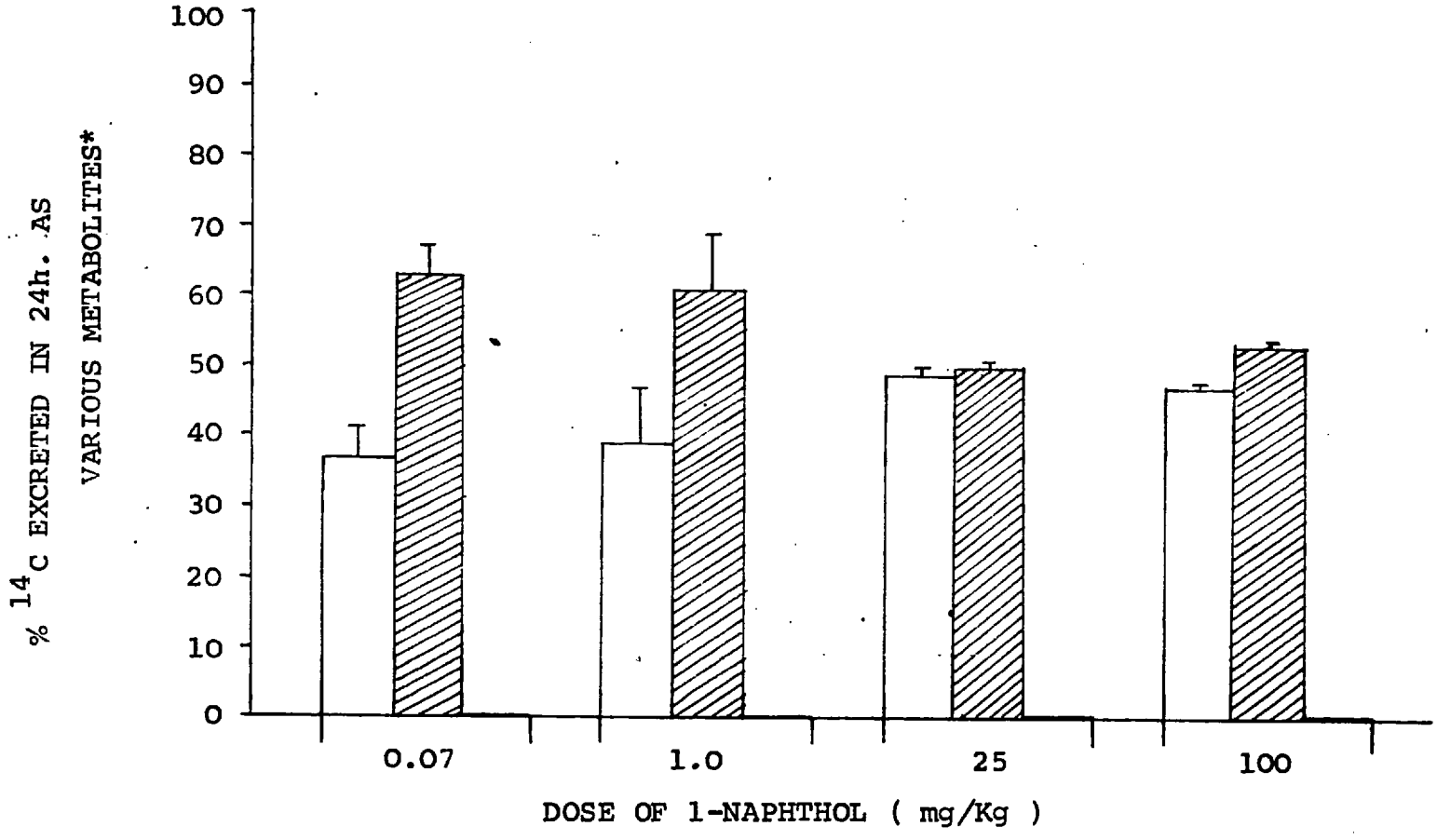

Figure 3.4

For details of animals,and urine collection and analysis, see legend to Table 3.1 and Materials and Methods, Chapter 2. Results are presented as mean ± S.E.M. Bars indicate S.E.M. n, the number of animals = 3.

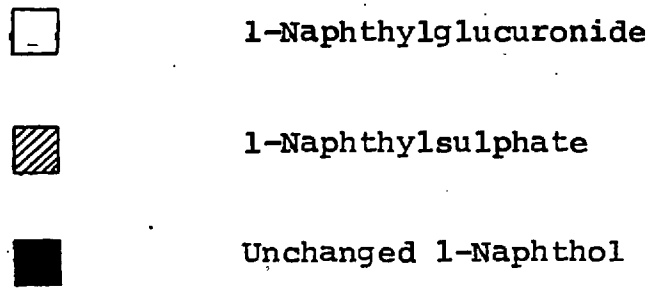

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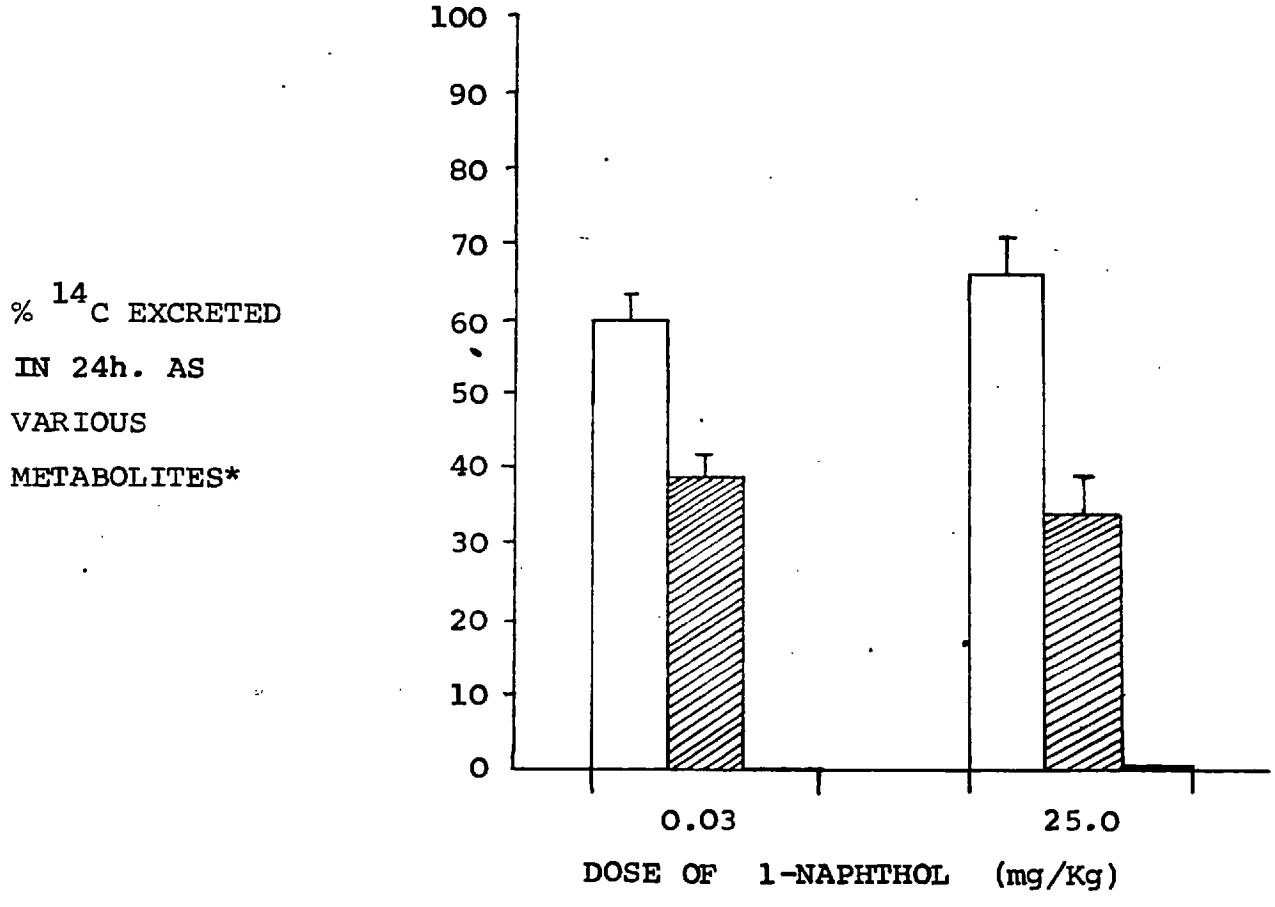
Figure 3.4: Urinary Metabolites of 1-Naphthol at Two Dose Levels in the Guinea Pig.



excreted during the next 24 hours. When 24 hour urines were analysed for the conjugates of 1-naphthol (Figure 3.4), at the lower dose of 0.03mg/Kg , 60% of the ^{14}C was recovered as the glucuronide conjugate and about 40% as the sulphate. At 25 mg/Kg dose, about 66% was found to be conjugated with glucuronic acid and approximately 34% with sulphate. A very small amount (about 0.9% of the dose) of the unchanged compound was excreted at the 25mg/Kg dose level.

Cat

The conjugation of 1-naphthol in the cat was determined at four dose levels. (Figure 3.5). For the lowest dose (1.0 mg/Kg)very small amounts of the radioactivity (2-15%) were excreted in 24 hours, and therefore, only one of the urines was analysed for the conjugated metabolites as this could only be achieved after preliminary column chromatography of the urine on XAD-2 resin.

On analysis of the urines (Figure 3.5), only traces of 1-naphthyl-β-D-glucuronide (0.2-1.5%) were detected at 5 and 10 mg/Kg doses, while at 25mg/Kg dose, there was a significant amount of the glucuronide conjugate (3-7%) present. At all doses, large amounts of the sulphate conjugate were found. Unchanged 1-naphthol was found to occur to some extent especially at the 1mg/Kg dose level (about 13% of excreted ^{14}C). This relatively large quantity may be an artefact produced by conjugate hydrolysis occuring during the prolonged analytical procedure.

Discussion

Figure 3.6 compares the amount of 1-naphthol conjugated with glucuronic acid to that conjugated with sulphate (glucuronide to sulphate ratio) in all three species at the various dose levels studied. It indicates that at all doses of 1-naphthol, the rat and guinea pig utilize both glucuronic acid

Figure 3.5

For details of animals, and urine collection and analysis, see legend to Table 3.1 and Materials and Methods ,Chapter 2. Results are presented as mean ± S.E.M. Bars indicate S.E.M. n is the number of animals.

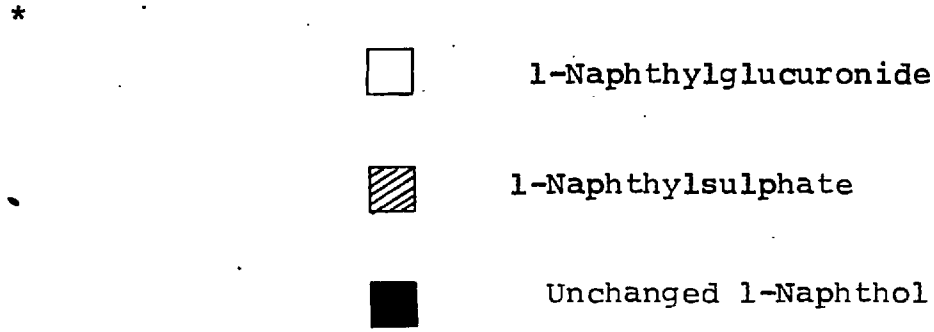


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Figure 3.5 : Urinary Metabolites of 1-Naphthol at Various Dose Levels in the Cat.

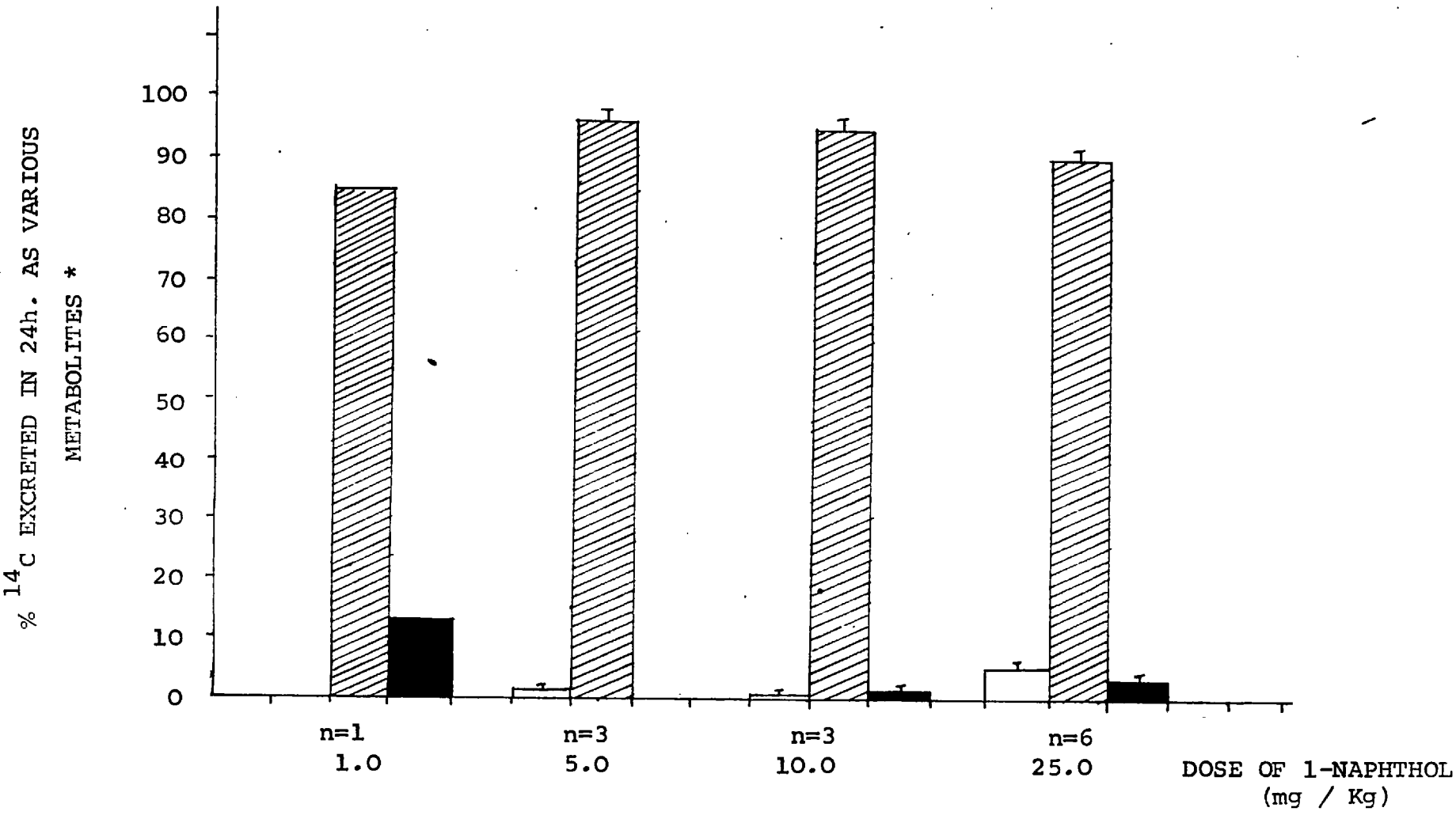
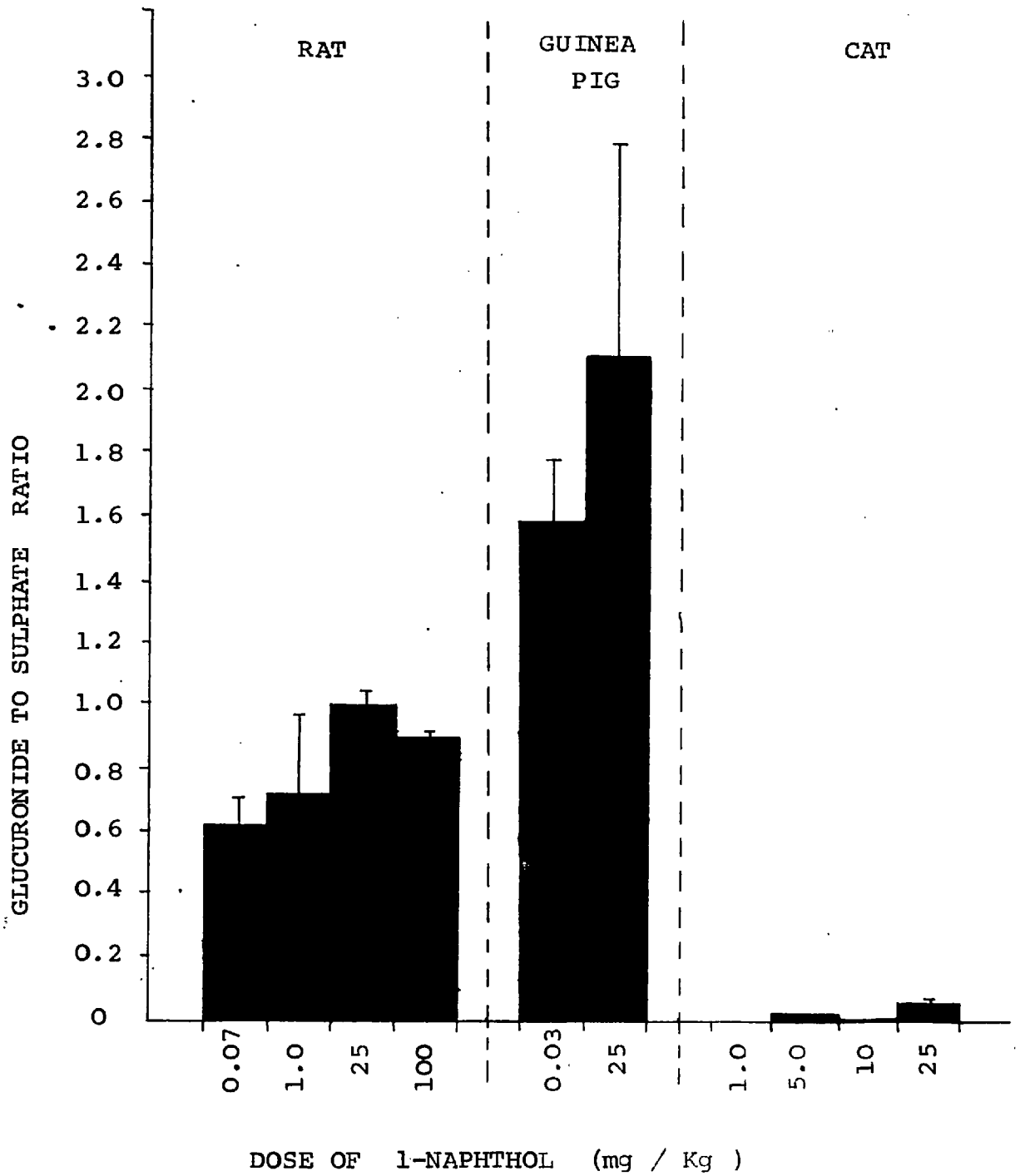


Figure 3.6: Glucuronide to Sulphate Ratios In Different Species At Various Doses Of 1-Naphthol.



and sulphate for conjugation but that in the guinea pig the inclination towards glucuronidation (glucuronide to sulphate ratio in the region 1.6-2.7) is greater than in the rat (glucuronide to sulphate ratio: 0.5-1.0).

The cat, on the other hand, exhibits a striking difference in the amount of 1-naphthol conjugated by the two mechanisms, the glucuronide to sulphate ratios being very much lower (0.005-0.015) at all doses. Thus, in the cat, this phenol is conjugated almost entirely with sulphate, small but definite amounts of glucuronide being formed at 5, 10 and 25mg/Kg dose levels.

The results, at the 25mg/Kg dose of 1-naphthol, are therefore in agreement with those obtained by Capel et al. (1972, 1974a) for 1-naphthol in the cat and the rat, and similar to those they obtained for phenol in all three species.

Bray et al. (1952(b), (c)) have studied the kinetics of glucuronic acid and sulphate conjugations in the rabbit using a number of phenols, and they found that the rate of glucuronide formation is proportional to the 'body level' of the phenol, i.e. first-order, but the rate of conjugation with sulphate at small doses of a phenol is proportional to the 'body level' of the phenol, while for large doses the rate is constant and equal to the maximum rate of supply of the sulphate ion. (i.e. zero-order). The percentage of ethereal sulphate formed can in fact be increased by including in the animals' diet sulphate precursors such as L-cysteine or sodium sulphate (Bray et al. 1952(c); Levy and Matsuzawa, 1967; Bennett et al., 1975). Moreover, when an administered compound is excreted only as two conjugates, as in the case of phenols, the proportions of the dose appearing in the two forms are complementary; the proportion excreted as that conjugate formed only by a first-order reaction (glucuronidation) increases with increasing dose level, while that formed by a zero-order reaction (ethereal sulphate)

decreases. The extent to which this can occur will, of course, be dependent upon the 'body level' of the sulphate ion as well as the relative activities of the enzymes involved in the two conjugation mechanisms. Both these factors may differ in different species. This is quite well illustrated by the glucuronide and sulphate ratios obtained for the metabolism of 1-naphthol in the three species used in the present studies.

The glucuronide to sulphate ratios vary quite markedly when comparisons are made between the three species at all doses. Within a particular species, there appears to be a tendency towards an increase in the amount conjugated with glucuronic acid and a concommitant decrease in that conjugated with sulphate with increasing doses of 1-naphthol. Thus, in the rat, when the dose of 1-naphthol is varied from 0.07 to 25mg/Kg, the glucuronide to sulphate ratio appears to increase with the dose, though at 100mg/Kg of 1-naphthol, the amount conjugated with glucuronic acid decreases compared with that at 25mg/Kg dose, On statistical analysis of the data, a significant increase in the percentage conjugated with glucuronic acid and a significant decrease in that conjugated with sulphate (Student's t distribution; $P \leq 0.05$) was found between the dose levels of 0.07mg/Kg and 25mg/Kg, and between 1mg/Kg and 25mg/Kg. However, an insignificant difference in the percentage conjugated by either mechanism ($P > 0.05$) was found between the doses of 0.07 mg/Kg and 1.0mg/Kg, 0.07 and 100mg/Kg, 1.0 and 100mg/Kg, and 25 and 100mg/Kg dose levels.

Similar analysis on the data obtained from the guinea pig showed that the differences in the conjugation patterns observed at the two doses of 1-naphthol studied were not significant ($P > 0.05$). In the cat, however, there was a significant increase in the amount of 1-naphthol conjugated with glucuronic acid and a consequent decrease in that conjugated with sulphate ($P \leq 0.05$) when the dose of 1-naphthol was in-

creased from either 5mg/Kg or 10mg/Kg to 25mg/Kg, but not when it was increased from 5mg/Kg to 10mg/Kg.

Therefore, in the rat and guinea pig, although there may be a slight tendency towards saturation of the sulphation mechanism at higher dose levels, this effect is not very marked suggesting that the rat, at least, has a reasonably good capacity to utilize both glucuronic acid and sulphate for the conjugation of 1-naphthol. In the guinea pig, however, at the lowest dose (0.03mg/Kg) that could be studied due to practical reasons, there is quite a high proportion of the glucuronide conjugate (60%), compared with the rat at the same dose level, suggesting that at this dose of phenol, the sulphating mechanism may have already become saturated, and that in this species, the threshold for the saturation of this mechanism is comparatively low. In the cat, on the other hand, conjugation with sulphate predominates at all doses of 1-naphthol, though at the highest dose used, there is a definite increase in the amount conjugated as glucuronide showing that the cat has a very high capacity for the sulphating mechanism, which is nevertheless, more readily saturable than glucuronide conjugation.

Previous *in vivo* studies with a number of aglycones, for example, p-nitrophenol (Gessner, 1974), harmol and harmalol (Mulder and Hagedoorn, 1974), phenol (Capel *et al.*, 1972) and 2,6-dimethoxyphenol (Miller *et al.*, 1974), have raised no doubt that the rat can utilize both the glucuronide and sulphate conjugation mechanisms. With respect to the relative proportions ofthe various aglycones conjugated by the two mechanisms, comparisons cannot be made since most of them were studied at different single dose levels. For 2,6-dimethoxyphenol, which was studied at three dose levels in the rat (Miller *et al*, 1974), the pattern of conjugation was essentially the same at all doses of the phenol. Thus, the effect of dose on the metabolism of 2,6-dimethoxyphenol is similar to that observed for 1-naphthol, where marked differences in the relative amounts

conjugated by the two mechanisms did not occur with increasing doses of the phenol.

However, when Miller et al. (1973) studied 2,6-dimethoxyphenol at 2 dose levels in the cat, the sulphate conjugate predominated at both doses but at the higher dose, there was also some dimethoxyquinol (an oxidation product of the parent phenol) present. In the same study, similar observations were made when phenol was injected into cats at 20mg/Kg and 40mg/Kg doses.

Therefore, it would appear from the studies reported in the past as well as the present study, that whilst sulphation is a major mechanism of conjugation in the cat, this mechanism can become readily saturated when higher doses of a phenol are administered, and that in such a situation, an alternative pathway, if available, may take over the metabolic process. Thus, phenol and dimethoxyphenol are excreted either unchanged, or as the parent quinol and its metabolites, while for 1-naphthol, the cat uses the very low glucuronidating capacity that it has for detoxication, some unchanged 1-naphthol being excreted too. This alternative pathway, in the cat, however, may not always be able to complete the process of detoxication due to it being either readily saturable or not very readily available, and hence, perhaps the reason for the high toxicity of phenols in cats (Table 1.22).

CHAPTER FOUR

Conjugation of 1-Naphthol and Phenol in Several Species of Primates

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Introduction

For activity and safety evaluation of drugs and other xenobiotics used by man, an animal is needed whose mode of xenobiotic metabolism is similar to that of man. Since primates are nearest to man on the phylogenetic scale, they might be expected to metabolise xenobiotics more like man than an animal far removed from man such as the rat or rabbit.

However, among the primates, a large number of species exist (215), only fifteen of which, probably mainly due to practical availability, have been studied (Table 1.18). It is important to know which of these fifteen species comes nearest to man so that results of tests in this species can be extrapolated to man; thus the reasons for studying interspecies variation among primates. Furthermore, such studies are of academic interest in that they can uncover metabolic reactions which are either absent or predominate in several species, and may aid in considerations of primate taxonomy and constitute a new application of metabolic studies (Smith and Caldwell, 1977; Williams, 1977).

So far the conjugation of phenols in primates has not been well studied, the only examples being phenol (Capel et al., 1972; Table 1.20), and the phenolic metabolites of chlorobenzene (p-chlorophenol and chlorocatechol; French, 1970; Table 1.21) though glucuronic acid conjugation of some drugs by primates has been studied (Table 1.19). In the study involving phenol, the metabolism in various primate species was compared at different single dose levels, and for chlorobenzene, where the phenols were metabolically derived, it would be difficult to estimate the true dose of the parent phenols.

Therefore, here the metabolism of phenol and 1-naphthol has been studied at one dose level in several primates (3 Old World and 2 Prosimian species), and to get some insight into quantitative aspects of glucuronidation and sulphation, three of the species have been studied at various doses of the phenols.

The metabolic fate of phenol in mammalian species is summarised in Figure 4.1. Phenol itself is an oxidation product of benzene, \and besides being conjugated with glucuronic acid and sulphate, can be further hydroxylated to quinol and, to a lesser extent, to catechol (Parke and Williams, 1953 (a), 1953(b)); these hydroxylated compounds can then be conjugated as for phenol.

Results

The primates investigated will be mentioned here only by their trivial names. For systemic names and details of species used, see Tables 1.18 and 2.1.

Metabolism of 1-naphthol and phenol at 10mg/Kg dose in several primate species

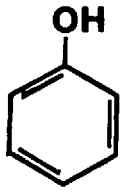
Table 4.1 shows the 24h, and 48h. excretions of ^{14}C, and the amounts of 24h. urinary ^{14}C found as various metabolites for phenol as the aglycone. The comparative data for 1-naphthol are given in Table 4.2. The number of animals used for these studies are very small mainly due to availability; in many cases only one animal has been studied. All the urines were collected over dry ice ('Drikold') except for those from the patas monkeys, these having been investigated only for the metabolism of 1-naphthol at the University of Ibadan, Nigeria, where ideal conditions were not available.

For both phenols, the rates of excretion were similar. Where a low percentage of ^{14}C had been excreted in the first 24h., urines were collected for 48h.

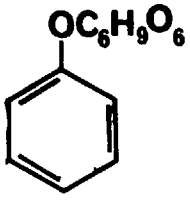
In some cases, four metabolites of phenol were detected, the quinol metabolites usually being excreted less than those of phenol. Neither unchanged phenol nor 1-naphthol were detected except for 1-naphthol in the patas monkeys. The latter could have occurred by hydrolysis of conjugated metabolites due to unfavourable collection conditions. The percentage

Figure 4.1: Phenol and Its Metabolites.

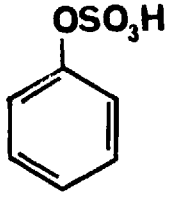
PHENOL



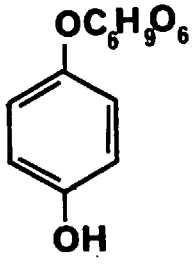
PHENYLGLUCURONIDE



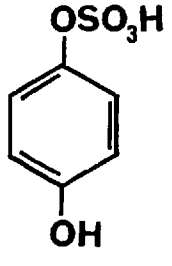
PHENYLSULPHATE



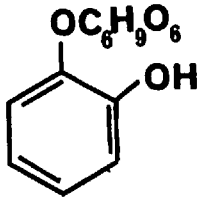
QUINOL MONOGLUCURONIDE



QUINOL MONOSULPHATE



CATECHOL MONOGLUCURONIDE



CATECHOL MONOSULPHATE

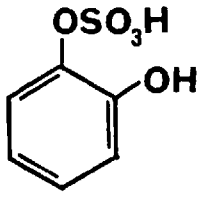


TABLE 4.1

Aqueous [U-^{14}C] - phenol (5-10μCi per animal) together with sufficient amounts of carrier phenol dissolved in propylene glycol (0.5-1.5ml) to give a dose of 10mg/Kg was administered intramuscularly. Urine was collected for upto 48 hours in the presence of dry ice ('Drikold') and chromatographed in at least two solvent systems (Table 2.3). The metabolites were identified and quantitated as described in Materials and Methods (Chapter 2).

n.d. means not detected.

* Number refers to the number of animals investigated; m = male; f = female.

Where two or more animals were studied, the values are given as means with individual values in brackets.

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TABLE 4.1: URINARY METABOLITES OF PHENOL IN PRIMATES

PRIMATE *	% DOSE EXCRETED IN:		% ^{14}C EXCRETED IN 24h FOUND AS:			
	24h	48h	QUINOL SULPHATE	PHENYL SULPHATE	QUINOL GLUCURONIDE	PHENYL GLUCURONIDE
Rhesus (1m,1f)	80.0 (82.4;77.6)	81.3 (82.8,79.7)	n.d.	76.8 (68.0,85.5)	n.d.	22.3 (31.0,13.5)
Cynomolgus (1f)	98.4	100.4	1.2	65.2	0.9	34.0
Capuchin (2f)	39.2 (48.4,29.9)	45.8 (54.5,37.0)	n.d.	41.4 (44.4,38.3)	n.d.	57.7 (55.5,59.8)
Tamarin (1m)	93.1	95.3	n.d.	11.2	10.5	78.1
Bushbaby (1)	60.4	63.5	0.2	76.9	n.d.	22.2
Treeshrew (1)	61.0	78.9	1.0	52.5	n.d.	46.9

Table 4.2

Aqueous [1-^{14}C]1-naphthol (5-10 μCi /animal) together with sufficient amounts of carrier 1-naphthol dissolved in propylene glycol (0.5-1.5ml) to give a dose of 10mg/Kg was administered intramuscularly. Urine was collected for upto 48h. in the presence of dry ice ('Drikold') except for the Patas monkeys and chromatographed in two solvent systems (Table 2.2). The metabolites were identified and quantitated as described in Materials and Methods, Chapter 2.

n.d. means not detected.

\- means not determined.

Where two or more animals were studied, the values are given as means with ranges in brackets.

* See legend to Table 4.1

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TABLE 4.2: URINARY METABOLITES OF 1-NAPHTHOL IN PRIMATES

Primate	% Dose excreted in:		% ^{14}C excreted in 24h found as:		
	24h	48h	1-Naphthyl Sulphate	1-Naphthyl Glucuronide	Unchanged
Rhesus (1m,1f)*	99.7 (98.7,100.6)	100.7 (100.2,101.2)	84.7 (83,86.3)	13.0 (13.6,12.4)	n.d.
Cynomolgus (1f)	97.4	98.9	76.3	22.6	n.d.
Patas (3f)	40.3 (36-43)	-	73.5 (50-87)	16.7 (9-24)	8.8 (0-24)
Capuchin (2f)	29.0 (25.5,32.4)	32.6 (26.8,38.3)	0.83 (1.65,0)	97.8 (96.8,98.7)	n.d.
Tamarin (1m)	83.9	86.9	15.1	83.5	n.d.
Bushbaby (1)	38.3	38.3	72.7	25.2	n.d.
Treeshrew (1)	70.8	77.2	66.4	27.6	n.d.

of dose excreted as glucuronide and sulphate in 24h. for both phenols in all species studied have been compared in Figure 4.2.

Effect of dose on the conjugation of phenol and 1-naphthol in the rhesus, cynomolgus and capuchin monkeys

Table 4.3 shows the 24h. and 48h. excretions obtained for all three species at various doses of the two phenols. Where two animals were investigated, results are presented as means, the individual values having been indicated in brackets. Again the rates of excretion for the two phenols do not differ markedly.

The percentage ^{14}C excreted in 24h. as various metabolites at different dose levels for the three species are indicated for phenol in Figure 4.3, and for 1-naphthol in Figure 4.4. Unchanged phenol was not excreted at any dose level in the three species. Very small amounts of unchanged 1-naphthol (0.5-2.1%) were detected for the rhesus monkey at 0.01 and 1.0mg/Kg doses and for the capuchin monkey, at the 25mg/Kg dose (1.6%).

Discussion

At 10 mg/Kg doses of phenol and 1-naphthol (Figure 4.2), greater proportions of the phenols were conjugated with sulphate than glucuronide in the three Old World species (rhesus, cynomolgus and patas monkeys), and the two Prosimian species (bushbaby and tree shrew). Conversely, there was more glucuronide than sulphate present for both phenols in the New World species (capuchin and tamarin monkeys).

When comparisons are made between the two phenolic substrates, in the tamarin monkey, similar amounts of phenol and 1-naphthol are conjugated with glucuronic acid (88.6% total glucuronide with phenol, and 83.5% glucuronide with 1-naphthol), while in the capuchin monkey, a greater amount of 1-naphthol

Figure 4.2

For experimental conditions and analysis of urines, see legends to Tables 4.1 and 4.2.

* Phenol was not studied in this species.

** SPECIES

R	Rhesus Monkey
Cy	Cynomolgus Monkey
P	Patas Monkey
Ca	Capuchin Monkey
Ta	Tamarin Monkey
B	Bushbaby
Tr	Treeshrew

***

Metabolites of:

1-Naphthol

Phenol and Quinol

Figure 4.2: % Of A 10mg/Kg Dose of Phenol and 1-Naphthol Excreted in 24h. as Glucuronide and Sulphate Conjugates.

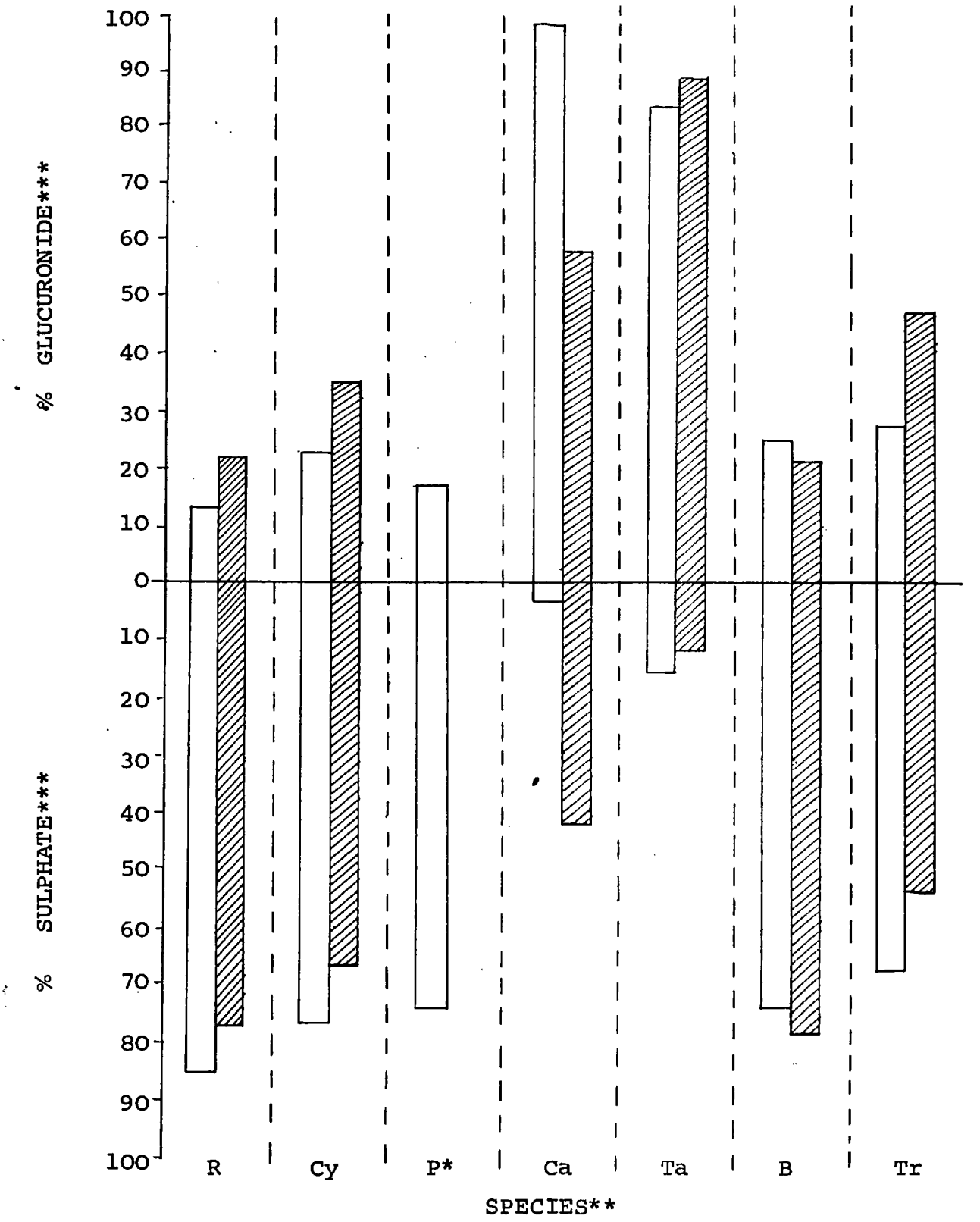


Table 4.3

Aqueous [U-^{14}C]phenol or [1-^{14}C]1-naphthol (5- 10 μCi/animal) together with sufficient amounts of carrier phenol or 1-naphthol dissolved in propylene glycol (0.5-1.5ml) to give doses of 0.01, 1.0, 10.0 and 25.0 mg/Kg were administered intramuscularly. Urines were collected for upto 48h. in the presence of dry ice (Drikold).

Results are presented as means with individual values in brackets.

\+ Two animals except for capuchin monkey at 25mg/Kg 1-naphthol.

* One animal.

-Facing Page 164-

TABLE 4.3: 24h. AND 48h. URINARY EXCRETION OF ^{14}C AT VARIOUS DOSES OF PHENOL AND 1-NAPHTHOL IN DIFFERENT SPECIES

SPECIES	DOSE (mg/Kg)	% ^{14}C OF DOSE EXCRETED WITH			
		PHENOL		1-NAPHTHOL	
		in 24h	in 48h	in 24h	in 48h
RHESUS +	0.01	83.0 (82.7,83.2)	-	99.2 (94.3,104)	-
	1.0	65.6 (62.9,68.3)	67.3 (64.3,70.2)	82.6 (65.1,100.1)	82.5 (82.5, -)
	10.0	80.0 (77.6,82.4)	81.3 (79.7,82.8)	99.7 (100.6,98.7)	100.2 (-,100.2)
	25.0	68.3 (59.4,77.2)	70.6 (62.0,79.2)	82.4 (66.7,98.1)	99.8 (99.8,-)

(continued)

TABLE 4.3: (continued)

CYNOMOLGUS*	0.01	69.1	-	60.7	-
	1.0	70.3	72.1	97.0	98.5
	10.0	98.4	100.4	97.4	98.9
	25.0	64.2	65.5	57.8	70.2
CAPUCHIN+	0.01	30.4 (12.0,48.8)	35.7 (21.4,50.0)	18.9 (12.9,24.8)	-
	1.0	39.1 (36.1,42.1)	46.4 (42.8,50)	54.8 (56.4,53.1)	56.8 (55.6,58)
	10.0	39.2 (29.9,48.4)	45.8 (37.0,54.5)	29.0 (32.4,25.5)	32.6 (38.3,26.8)
	25.0	39.8 (27.1,52.5)	41.6 (29.5,53.6)	46.7	50.8

Figure 4.3

For experimental conditions, see legend to Table 4.3.

For analysis of urines, see Materials and Methods, Chapter 2.

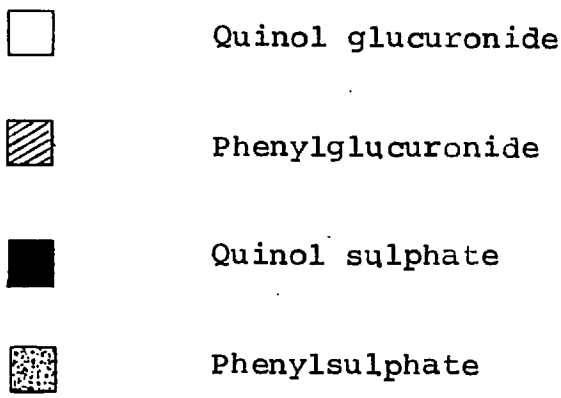


Figure 4.3: Urinary Metabolites of Phenol at Various Dose Levels In Different Species.

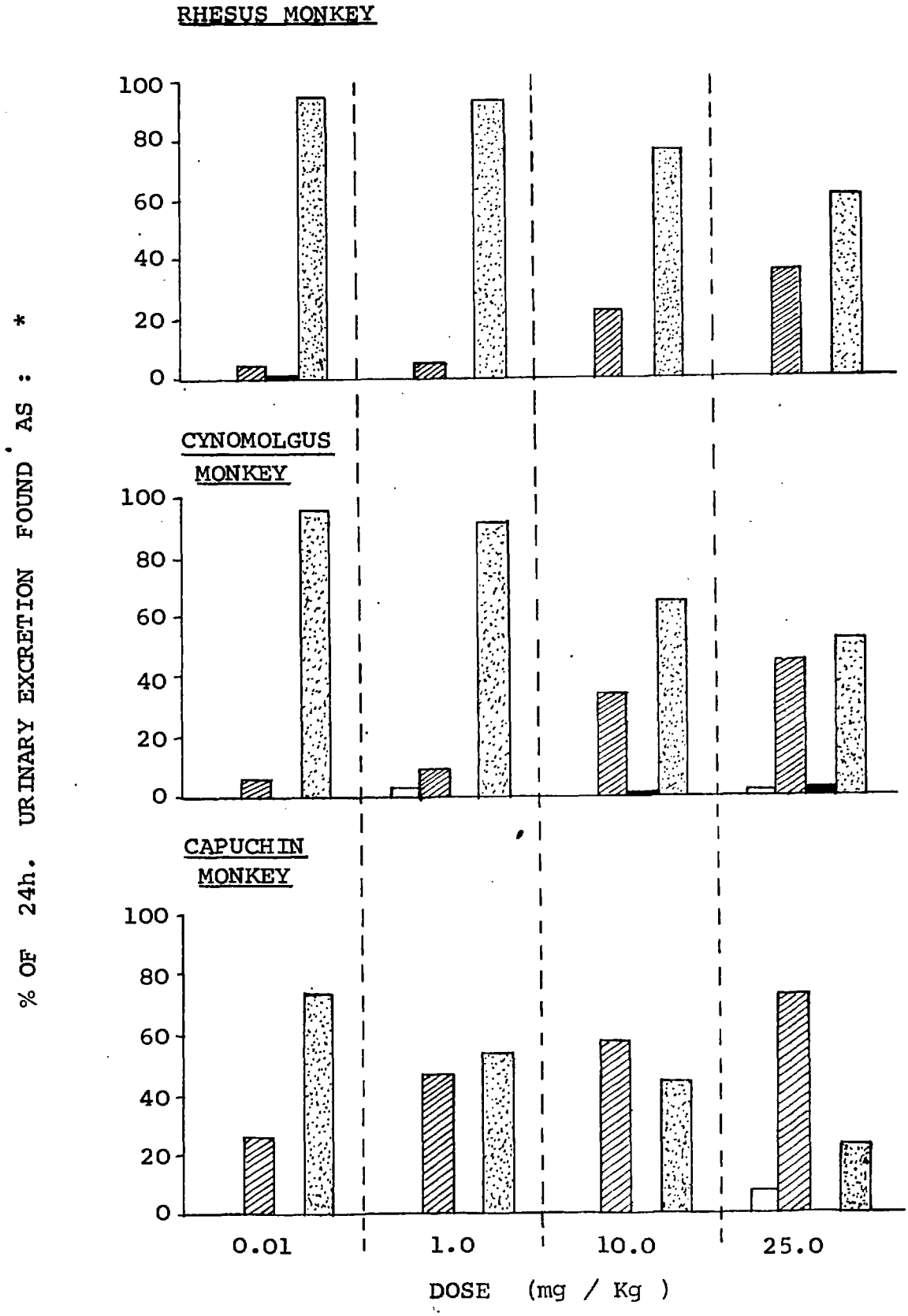


Figure 4.4

For experimental conditions see legend to Table 4.3.

For analysis of urines , see Materials and Methods, Chapter 2.

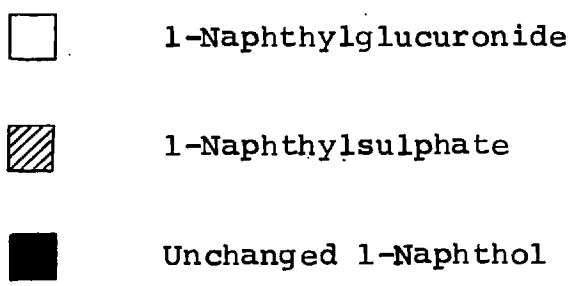
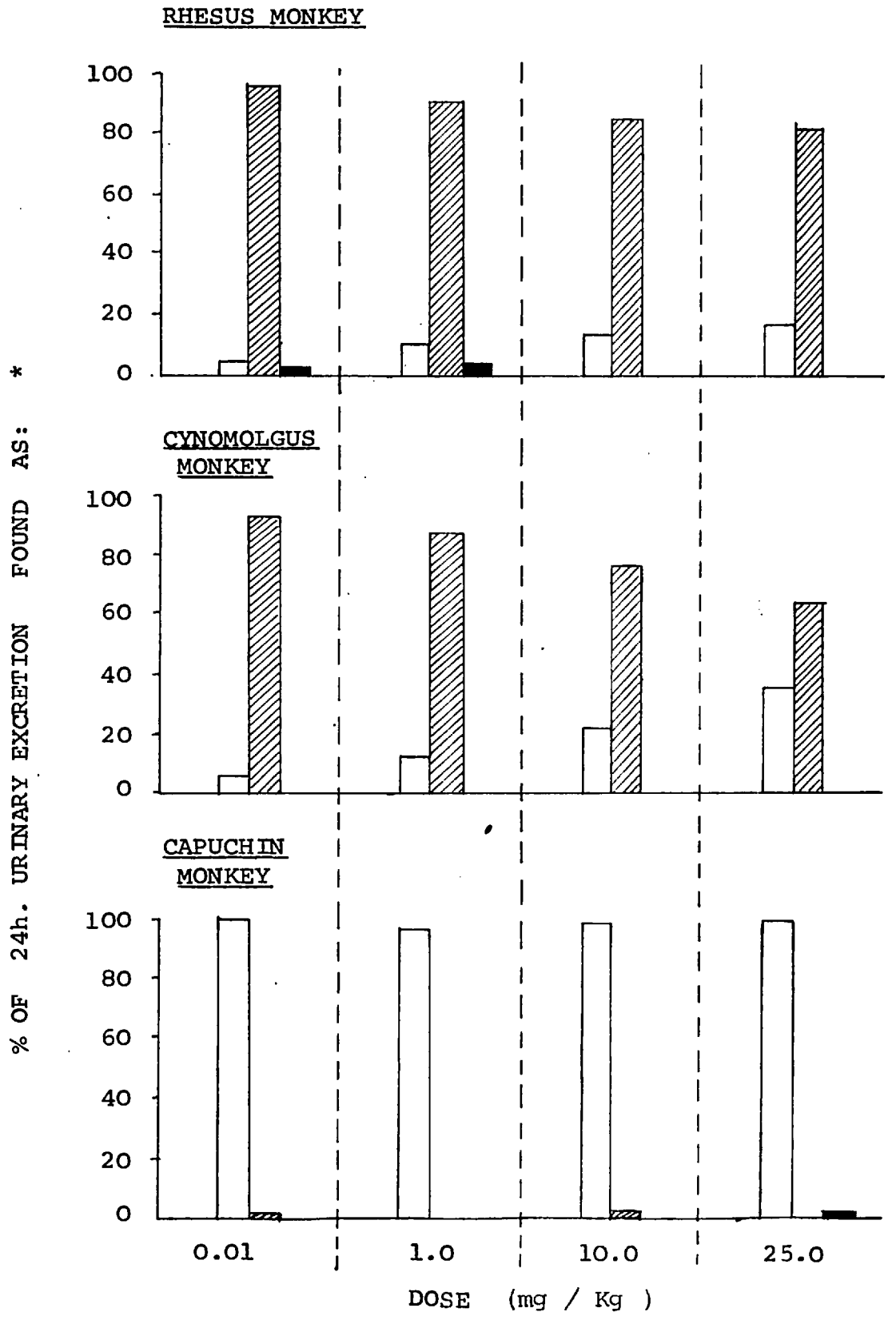


Figure 4.4: Urinary Metabolites of 1-Naphthol At Various Dose Levels In Different Species.



is glucuronidated compared with phenol (only 57.7% phenyl-glucuronide cf. 97.8% 1-naphthylglucuronide). Phenol appears to be a slightly better glucuronogenic substrate than 1-naphthol in the rhesus and the cynomolgus monkeys, and the tree shrew, while in the bushbaby 1-naphthol and phenol are not different with respect to conjugation with glucuronic acid.

In the capuchin monkey which was studied at three other dose levels too, for 1-naphthol, the glucuronide conjugate predominates at all doses, only minor amounts (upto 1.4%) of the sulphate conjugate being detectable at 0.01 and 10mg/Kg dose levels (Figure 4.4). With phenol, however, at the lowest dose (0.01mg/Kg), the sulphate conjugate predominates and this decreases as the dose is increased upto 25mg/Kg, when the glucuronide conjugate predominates. Nevertheless, at all doses of phenol, there is a greater amount of the glucuronide conjugate in the capuchin monkey than in either the rhesus or cynomolgus monkeys at comparative dose levels. With the latter two species the sulphate conjugate predominates at all doses of both phenol and 1-naphthol (Figures 4.3 and 4.4).

Thus, the comparative studies in several primates at 10mg/Kg doses of phenol and 1-naphthol suggest that the New World species investigated here have a poorer sulphating capacity than either the Old World or Prosimian species. The dose level studies, however, show that the pattern of conjugation is dependent on the dose as well as the phenolic substrate.

As discussed in Chapter 3, sulphate conjugation is dependent upon the availability of sulphate and therefore, compared with glucuronidation is a more readily saturable process. While in the rat and guinea pig the decrease in sulphation with increasing dose levels was not very marked, in the primates, for both phenols, there is a definite increase in the proportion conjugated with glucuronic acid and a concommitant decrease in that conjugated with sulphate, when the doses are increased

upto 25mg/Kg. The only exception is the capuchin monkey with 1-naphthol where only minute amounts of the sulphate conjugate are present at all dose levels. With 1-naphthol, this saturation effect with increasing levels of the phenol is more marked in the cynomolgus than the rhesus monkey. With phenol, on the other hand, the effect can be seen at its best in the capuchin monkey where a complete reversal of the pattern of conjugation is observed. Thus, at the lowest dose (0.01 mg/Kg), the sulphate conjugate predominates while at the highest dose (25mg/Kg), the glucuronidation mechanism has taken over. Further comparisons between the two phenols show that in the Old World species, the sulphating mechanism is more readily saturable with phenol than with 1-naphthol.

In the present studies, the primates did not excrete appreciable amounts of quinol metabolites, the only exception being the capuchin monkey which excreted 7% quinol glucuronide at the 25mg/Kg dose. In their studies with phenol (25mg/Kg dose) in the capuchin monkey, Capel et al. (1972) observed 21% of the ^{14}C excreted in 24h. as quinol glucuronide.

No unchanged phenol or quinol were observed in the present studies. Miller et al. (1973) in their studies with 2,6-dimethoxyphenol and phenol in the cat at two dose levels, observed the presence of the parent quinols at the higher dose levels and suggested that this was due to saturation of the pathways for the conjugation of the phenols. No unchanged phenol or quinol, the latter of which can be more toxic than phenol, were detected in the three primates at various doses of phenol presumably because although there are quantitative differences among the Old and the New World species in the conjugation of phenol, all of these species (cf. cat) are able to utilize both glucuronidating and sulphating mechanisms for detoxication of phenol. The absence of the sulphate conjugate of 1-naphthol in amounts greater than 1.4% in the capuchin

monkey, and the comparative results for phenol where the sulphate conjugate is formed, suggest that in this species sulphate conjugation is not absent, though at a lower level than the Old World species, and that the effect observed with 1-naphthol is characteristic of that phenolic substrate.

In the capuchin monkey, even with 1-naphthol, where only one conjugating mechanism is operating at the dose levels examined, there is not present any appreciable amount (<2% at 25mg/Kg dose) of the unchanged compound. This is perhaps a reflection of the observations that glucuronide conjugation is a first-order mechanism dependent upon the dose of the phenol rather than the availability of the conjugating agent, and therefore not, in the least, as readily saturable as sulphation (Bray et al., 1952(b), (c)). Thus, a species having a low capacity for glucuronidation (e.g. the cat) is likely to be at a greater 'disadvantage' than that having a low capacity for sulphation (e.g. the capuchin monkey).

Primate classification has so far been based upon considerations of structural organisation and behaviour. Recently, however, studies have concentrated on comparative biochemical criteria such as serum albumins (Sarich and Wilson, 1966). Further, interest has developed in the possibility of studying xenobiotic metabolism reactions in relation to primate taxonomy since certain reactions are thought to be restricted in their occurrence to primate species and occasionally to particular groups of primates and this may be correlated with evolutionary status (Smith and Caldwell, 1977). Thus, for example, the capacity to form N1-glucuronides with sulphadimethoxine appears to be a well-developed feature of primate species and this reaction is either absent or at a low level in non-primates (Adamson et al., 1970(a)); and the capacity to utilize glutamine for the conjugation of simple arylacetic acids such as phenylacetic acid has previously been thought to

be a metabolic feature of anthropoid apes and monkeys (James et al., 1972), though recently, the utilisation of glutamine for the conjugation of 2-naphthylacetic acid by the rat, rabbit and ferret (Emudianughe et al., 1977), and that for, phenylacetic acid by the ferret (Hirom et al., 1977a) has been reported.

The above differences in occurrence of certain metabolic reactions in primates however, do not take into account other metabolic factors such as absorption, excretion, tissue distribution and rate of metabolism. Although the pattern and extent of metabolism of a xenobiotic may be similar in man and a given animal, other factors may be different. Thus, the drug sulphadimethoxine (Adamson et al., 1970(b)) forms N-1-glucuronides as major urinary metabolites in man and rhesus monkey, but the rates of excretion of the drug differ completely in these two species; in this respect, the man is more comparable to the rat than the rhesus monkey.

When we compare the patterns of phenol and 1-naphthol metabolism, at 10mg/Kg dose, the Old World and the Prosimian species are similar in that these two phenols are conjugated mainly with sulphate whereas the two New World species differ in that they conjugate these phenols mainly with glucuronide. However, when one considers the effect of dose upon the conjugation of phenol, the capuchin monkey, at the lowest dose (0.01 mg/Kg) is similar to the two Old World species in that the major urinary metabolite is the sulphate conjugate. There are also differences in the conjugation patterns between the two phenolic substrates, especially in the capuchin monkey.

Thus, from the above, it would appear that it is unwise to use simply the pattern of biotransformation investigated at an arbitrarily chosen dose level for considerations of primate taxonomy. Such a situation would particularly be applicable to a xenobiotic like phenol which can be metabolised by at least two competing pathwaysand the metabolic pattern

of which would, therefore, be very much dose-dependent. Furthermore, many more primate species from the various groups as well as xenobiotics need to be studied before generalisations can be made.

Another reason for studying interspecies variation in primates is to find a good metabolic model for man so that the data from this animal model can be extrapolated to man. The survey conducted by Smith and Caldwell (1977) shows that for comparisons made purely on the basis of pathways of metabolism, the best metabolic for man is provided by the rhesus monkey (for 17 out of 23 compounds). By contrast the rat provides a good model only on four occassions and other non-primate species (dog, rabbit and guinea pig) only on five occassions.

However, when other metabolic factors (see above) are taken into account, as for example, the rates of excretion for the drugs sulphadimethoxine and sulphadimethoxypyrimidine (Adamson et al., 1970(b); Walker and Williams, 1972), the rat is a better metabolic model for man than the rhesus monkey. For the arylacetic acids as well, the conjugation pattern can vary markedly with chemical structure and the primate species, and in some instances primates may be suitable models while for others, non-primates may be appropriate (Smith and Caldwell, 1977).

The only study where phenol has been investigated in man is that of Capel et al. (1972), Thus, phenol at a dose of 0.01mg/Kg was conjugated mainly with sulphate (78%), only 16% having been found as the glucuronide conjugate. When this data is compared at the same dose level in the three non-human primate species used in the present studies, the pattern of conjugation (i.e. the main urinary metabolite being the sulphate conjugate) in man is similar to that of both the Old World as well as the New World species, while quantitatively, the

capuchin monkey comes nearest to man. However, when comparing these particular studies, the route of administration has also to be taken into account since in man phenol was orally administered while in the present studies, it was given intramuscularly.

Therefore, as suggested by the present studies as well as those in the literature (Smith, 1966; Smith and Williams, 1974; Smith and Caldwell, 1977), it is difficult to predict xenobiotic metabolism in man purely on comparisons of patterns of biotransformation, and other factors such as dose, the type of xenobiotic and route of administration have to be taken into account. Morever, as suggested by the above authors, the search for metabolic models for man should also take into consideration other parameters of xenobiotic metabolism (e.g. rate of metabolism, rate of absorption, rate of excretion), and that certain metabolic reactions are restricted in their occurrence to certain primate species; there would, therefore, appear to be an obvious need for systemic studies of drugs in a much broader sample of primates.

To summarise then, these studies in several species of primates indicate that it is important to consider both the substrate and its dose when making interspecies comparisons. Thus, with 1-naphthol , there is a clear species difference between the Old World, New World and Prosimian species; at the dose levels studied, sulphation is the major pathway of conjugation in the Old World and Prosimian species, while in the New World species, glucuronic acid conjugation predominates. With phenol, however, the species variation is dependent on the dose; thus, at the lowest dose, sulphate conjugation predominates in both the New and Old World species, but at the highest dose, the patterns of conjugation are similar to those for 1-naphthol.

PART II - In Vitro STUDIES

CHAPTER FIVE

UDP-glucuronyltransferase and Phenolsulphotransferase Activities Towards 1-Naphthol and Phenolphthalein in Several Species

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Introduction

The *in vivo* metabolism of 1-naphthol has been compared in the rat, cat and guinea pig in Chapter 3, whereupon species differences in the relative extent of conjugation with glucuronic acid and sulphate have been observed. Therefore, to try to correlate these *in vivo* differences with the activities of the two enzymes catalysing these conjugation reactions, namely UDP-glucuronyltransferase and phenolsulphotranferase, studies have been carried out in different tissues of the rat, cat and guinea pig.

Furthermore, prompted by the *in vivo* findings by Capel *et al.* (1974a) that the cat, which normally has a poor capacity for glucuronide conjugation with phenols, forms 60% glucuronide and 40% sulphate with phenolphthalein (dose:25mg/Kg), the UDP-glucuronyltransferase activity, with phenolphthalein as substrate, in cat tissues has been measured, and compared with that in the rat. Capel *et al.* (1972, 1974a) also found that the pig, in contrast with the cat, is poor at forming sulphate conjugates with a number of phenols, the exception being 1-naphthol which at a dose of 25 mg/Kg, was excreted as glucuronide (66%) as well as sulphate (32%). In order to determine if the enzyme activities in the pig liver were similar as in the rat, the kinetic parameters for the two pig liver enzymes have been measured.

Some postmortem samples of certain primate livers (baboon, patas and night monkeys) and a lion liver were kindly made available by Dr.Rivers of the Nuffield Institute of Comparative Medicine, Zoological Society of London, Regent's Park, London. Therefore, it was of interest to see if the differences already observed in the relative extents of conjugation of 1-naphthol with glucuronic acid and sulphate in certain species of New and Old World monkeys(Chapter 4) were also to be found in these species of primates. Baboon and the patas monkey are Old World species,

and the night monkey, a New World species. The lion liver has also been examined for UDP-glucuronyltransferase and phenolsulphotransferase activities towards 1-naphthol in order to obtain a comparison with the cat. Such a comparison would be interesting since recent studies on the conjugation of a number of xenobiotics (phenol, benzoic acid and 1-naphthylacetic acid) in the species belonging to the Superfamily Feloidea, namely the lion, civet, genet and the hyaena, have shown that none of these species form the glucuronide conjugates, except for the hyaena which excretes about 40% of the dose of 1-naphthylacetic acid as the glucuronide; these studies, therefore, suggest that these species, which belong to the same Superfamily as the cat, may be similar in having a low glucuronidating capacity for most phenols.

The *in vivo* studies in previous chapters have shown that wherever possible, the two conjugating mechanisms, glucuronidation and sulphation, compete for the same substrates. One of these pathways, sulphation, is much more readily saturable than the other. *In vitro* studies in the past have involved either the measurement of UDP-glucuronyltransferase activity, or of phenolsulphotransferase, both being carried out under entirely different conditions. Thus, UDP-glucuronyltransferase activity has usually been measured in microsomal preparations (e.g. Vessey and Zakim, 1971; Halac and Reff, 1967; Bock *et al.*, 1973; Sanchez and Tephley, 1974; Winsnes, 1969), while the phenolsulphotransferase activity in partially purified preparations (e. g. Bannerjee and Roy, 1968; McEvoy and Carroll, 1971; Davis, 1975).

However, the studies by Mulder and his collegues (Mulder *et al.* 1974; Mulder and Hagedoorn, 1974; Mulder and Bleeker, 1975), with harmol and harmalol as substrates, and more recently, by Wong (1976a) with 4-hydroxy-3-methoxyphenylethanol as substrate, have involved measurement of the two enzyme activities

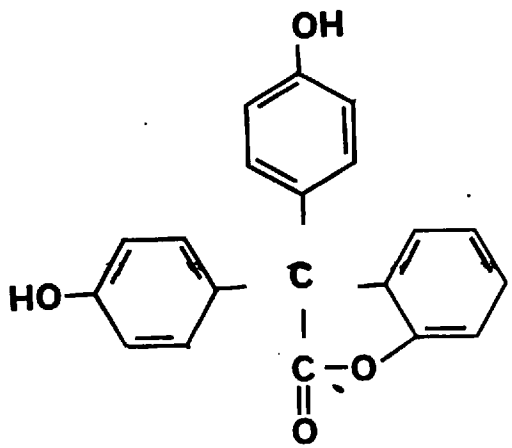
under similar conditions. In fact, Mulder (1975) has described an *in vitro* method for the simultaneous measurement of UDP-glucuronyltransferase and phenolsulphotransferase; it utilizes the 600g supernatant as the enzyme preparation, which contains activities towards both enzymes. In the following studies, the same enzyme preparation has been used so that the relative activities of the two enzymes can be compared. The conjugation of phenolphthalein has been illustrated in Figure 5.1, and that of 1-naphthol in Figure 3.1.

Results

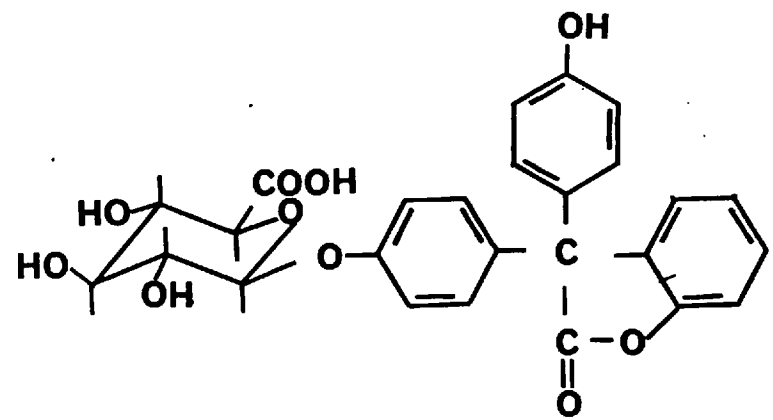
Figure 5.2 illustrates the rates of conversion of 1-naphthol to its glucuronide and sulphate conjugates in the rat, cat, guinea pig and pig livers. In each case, determinations were carried out on 600g. supernatants from three pooled livers except for the pig where liver from only one animal was available. Preliminary studies of the phenolsulphotransferase reaction with 1-naphthol as the substrate in both 600g. and 100,000g. supernatants of the rat liver indicated similar enzyme activities in the two preparations. Identical studies of the glucuronidation reaction in the microsomal and 600g. supernatant preparations showed that the enzyme activities in these two preparations were quite comparable. The 600g. supernatant preparation, therefore, appeared quite a viable preparation for the measurement of the two enzyme activities under similar conditions.

The kinetic parameters obtained for the two enzymes, UDP-glucuronyltransferase and phenolsulphotransferase, in various tissues of the rat and the cat, and in pig liver, with 1-naphthol as the substrate are shown in Table 5.1. The data for UDP-glucuronyltransferase with phenolphthalein as the substrate are also tabulated (Table 5.2). The details of the number of animals used and the number of experiments are stated in the legends to these tables. In the guinea pig tissues, only the rates of conversion of 1-naphthol (concentration 20μM)

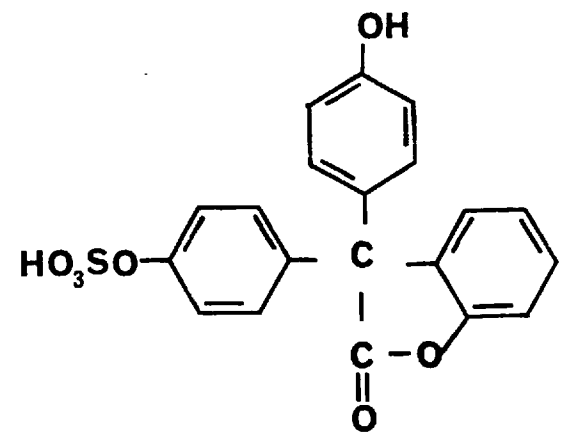
Figure 5.1: Phenolphthalein and its Metabolites



PHENOLPHTHALEIN



PHENOLPHTHALEIN MONOGLUCURONIDE



PHENOLPHTHALEIN MONOSULPHATE

Figure 5.2

The concentration of 1-naphthol in each case was 20 μM in the presence of 1.5mM UDPGA (for glucuronidation) ,or approximately 10 μM PAPS (for sulphation). For details of reaction mixtures, see Materials and Methods (Chapter 2). For each determination , enzyme preparation (600g. supernatant) from three pooled livers was used except for the pig, where liver from only one animal was available. The amount of enzyme varied from 1 mg.of tissue per 1 ml. incubation mixture to 40 mg.. In all cases, female livers were used.

*

▨ Sulphate

□ Glucuronide

Figure 5.2: In Vitro Conjugation of 1-Naphthol in Rat, Cat, Guinea pig and Pig Livers.

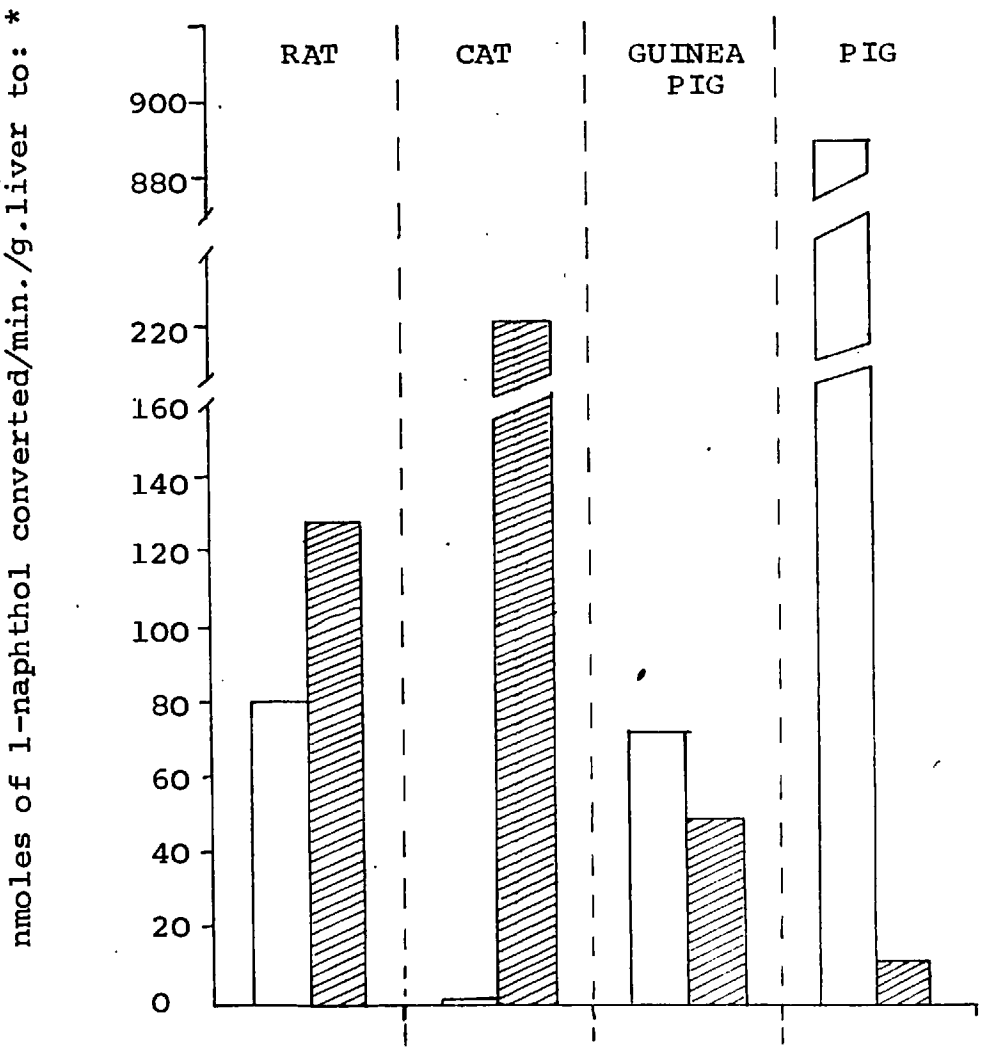


Table 5.1

For details of incubation mixtures, see Materials and Methods, Chapter 2.

\+ Results were obtained with tissues pooled from three animals and with two incubation mixtures at each concentration of substrate. The only exceptions were the determination of cat liver phenolsulphotransferase activity (see legend to Figure 5.4), and the pig, where liver from only one animal was available. In the latter, data are from three experiments.

* Kinetic parameters obtained by Saturation Kinetics (e.g. Figure 5.4)

** r is the Correlation coefficient.

n. d. Activity not detectable.

† Units are nmoles of 1-naphthol converted/min./mg.tissue.

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TABLE 5.1: KINETIC PARAMETERS FOR 1-NAPHTHOL IN VARIOUS TISSUES OF THE RAT AND THE CAT

SPECIES+	ENZYME	TISSUE	APPARENT K_M (μM)	APPARENT V_{max} †	r**
Rat	UDP-glucuronyltransferase	Liver	12.3	0.125	0.97
		Kidney	27.0	0.328	0.95
		Mucosa	20.0	0.227	0.97
	Phenolsulphotransferase	Liver	7.41	0.171	0.98
		Kidney	3.23	0.0114	0.94
		Mucosa	7.69	0.009	0.99
Cat	UDP-glucuronyltransferase	Liver	4.55×10^3	0.156	0.94
		Kidney	n.d.	n.d.	-
		Mucosa	n.d.	n.d.	-
	Phenolsulphotransferase	Liver	0.40	0.0830 *	-
		Kidney	0.53	0.042 *	-
		Mucosa	0.20	0.095 *	-
Pig	UDP-glucuronyltransferase	Liver	8.7	1.21	0.97
	Phenolsulphotransferase	Liver	32.3	0.026	0.98

Table 5.2

Concentrations of phenolphthalein were in the region 80-200 μM in the presence of 1.5mM UDPGA, and 40mg. of tissue. For more details of incubation mixtures, see Materials and Methods, Chapter 2. Data are from tissues pooled from three animals, and with two incubation mixtures at each concentration of substrate.

* Determined by saturation kinetics (e.g. Figure 5.4)

n.d. Activity not detectable.

\+ r is the correlation coefficient.

† Units are nmoles of phenolphthalein converted/min./mg.tissue.

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TABLE 5.2: KINETIC PARAMETERS FOR UDP-GLUCURONYLTRANSFERASE IN VARIOUS TISSUES OF THE RAT AND THE CAT WITH PHENOLPHTHALEIN AS THE SECOND SUBSTRATE

SPECIES	TISSUE	APPARENT K_M (μM)	APPARENT V_{max}†	r +
Rat	Liver	<27	0.047 *	-
	Kidney	<32	0.021 *	-
	Mucosa	<40	0.017 *	-
Cat	Liver	115.6	0.104	0.95
	Kidney	n.d.	n.d.	-
	Mucosa	n.d.	n.d.	-

to glucuronide and sulphate have been determined (Table 5.3).

In most cases, the apparent K_M and V_{max} were determined by plotting the data according to Lineweaver and Burk (1934; e.g., Figure 5.3). The lines were computed using linear regression analysis with a programme devised by Dr. J. R. Idle. The correlation coefficient, r, which gives a measure of linearity of the experimental data has been shown for each determination in the tables. In some cases, it was only possible to determine the kinetic parameters by saturation kinetics (e.g., Figure 5.4). Thus, in the cat tissues, the activities were found to be very high, the enzyme already becoming saturated at about 0.5 μM of the substrate (Figure 5.4). Since concentrations of 1-naphthol below 0.1 μM were beyond the scope of the analytical procedures employed, it was not possible to study only the linear part of the hyperbola. Similarly, for the determination of kinetic parameters for UDP-glucuronyltransferase with phenolphthalein as substrate, due to the very low specific activity of [^{14}C]-phenolphthalein (0.3 μCi/mg), it was not possible to use substrate concentrations below 80μM. Thus, for the rat tissues, only approximate values obtained by saturation kinetics have been indicated (Table 5.2).

The rates of conversion of 1-naphthol to its glucuronide and sulphate in the primate and the lion livers are shown in Figure 5.5. The data are from livers from single animals, and the enzyme activities have been determined with 1-naphthol concentration at 20 μM.

Discussion

Enzyme Activities Towards 1-Naphthol In Rat, Cat, Guinea pig and Pig Livers.

As Figure 5.2 shows, the rates of conversion of 1-naphthol to its glucuronide and sulphate in the rat liver are slightly different, that of sulphation being somewhat higher than

TABLE 5.3: In Vitro Conjugation of 1-Naphthol in the Guinea Pig

TISSUE	RATE OF CONVERSION OF 1-NAPHTHOL TO: (n-moles of 1-naphthol converted/min/mg. tissue)	
	GLUCURONIDE	SULPHATE
Liver	0.072	0.049
Kidney	0.060	0.004
Intestinal Mucosa	0.025	0.039

Rates in different tissues were determined as described in the legend to Figure 5.2.

Figure 5.3

Concentrations of 1-naphthol were in the region 3-15 μM in the presence of approximately 10 μM PAPS. Reaction mixtures contained 0.75M tris-HCl buffer, pH 7.4 , with 75mM EDTA and 94mM KH_2PO_4, 0.25M tris-HCl buffer, pH 7.4, 3mg. of rat liver 600g. supernatant, and incresing amounts of [1-^{14}C]1-naphthol to give the above range of substrate concentrations. Results were obtained with livers pooled from three animals and with two incubation mixtures at each concentration of substrate.

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.

r is the correlation coefficient.

Figure 5.3: Lineweaver-Burk Plot for the Determination of Kinetic Parameters of Rat Liver Phenol-sulphotransferase with 1-Naphthol as the Substrate.

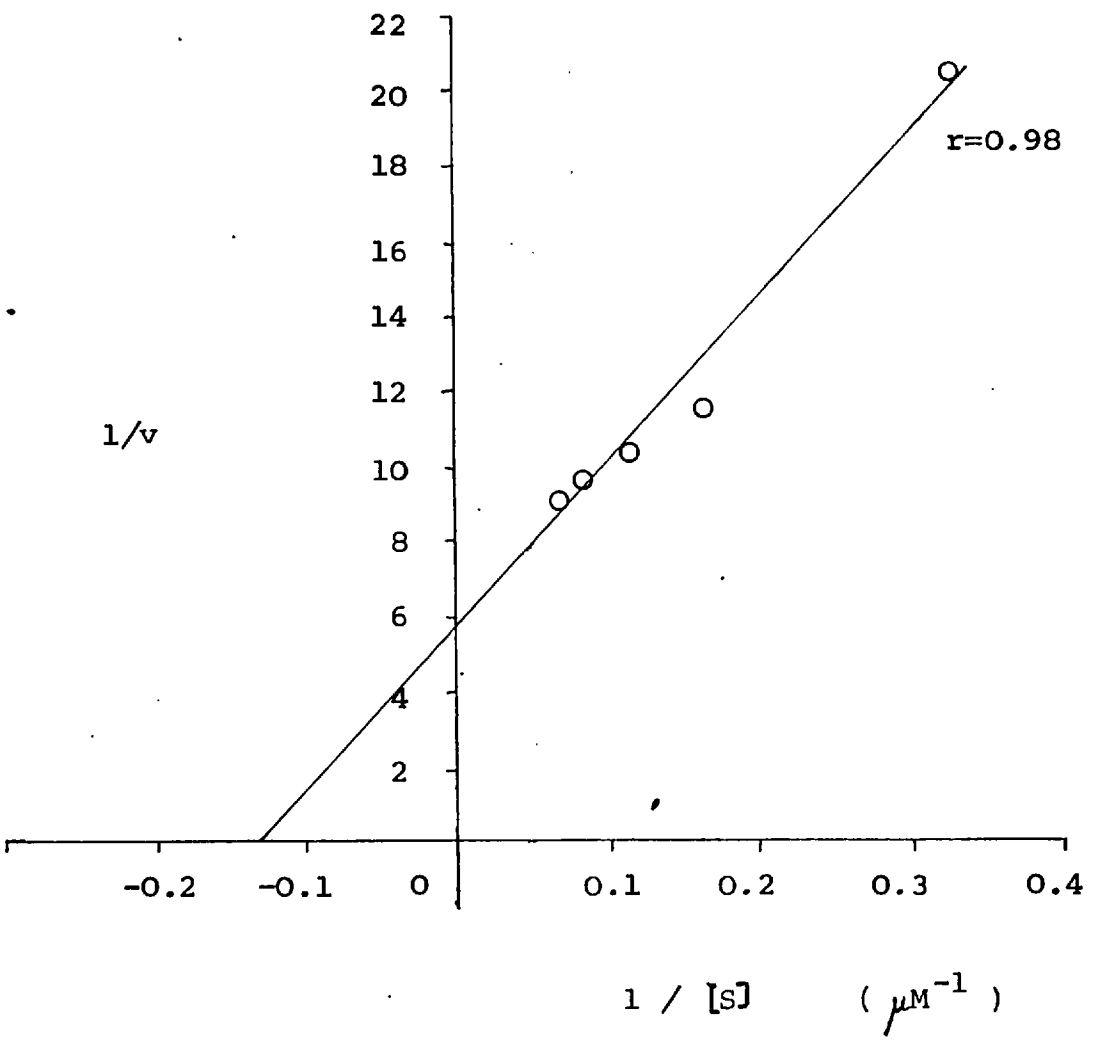

Figure 5.4

Concentrations of 1-naphthol were in the region 0.1-3.4 μM in the presence of approximately 10μM PAPS (100 μl of PAPS solution). Reaction mixtures contained 0.75M tris-HCl buffer, pH 7.4, with 75mM EDTA and 94mM KH_2PO_4, 0.25M tris-HCl buffer, pH 7.4, 1mg. of cat liver 600g. supernatant, and incresing amounts of [1-^{14}C]1-naphthol to give the above range of substrate concentrations. Results are from three experiments determined on a total of six cat livers.

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Figure 5.4: Plot of Velocity as a Function of 1-Naphthol Concentration for the Determination of Kinetic Parameters for Cat Liver Phenolsulphotransferase.

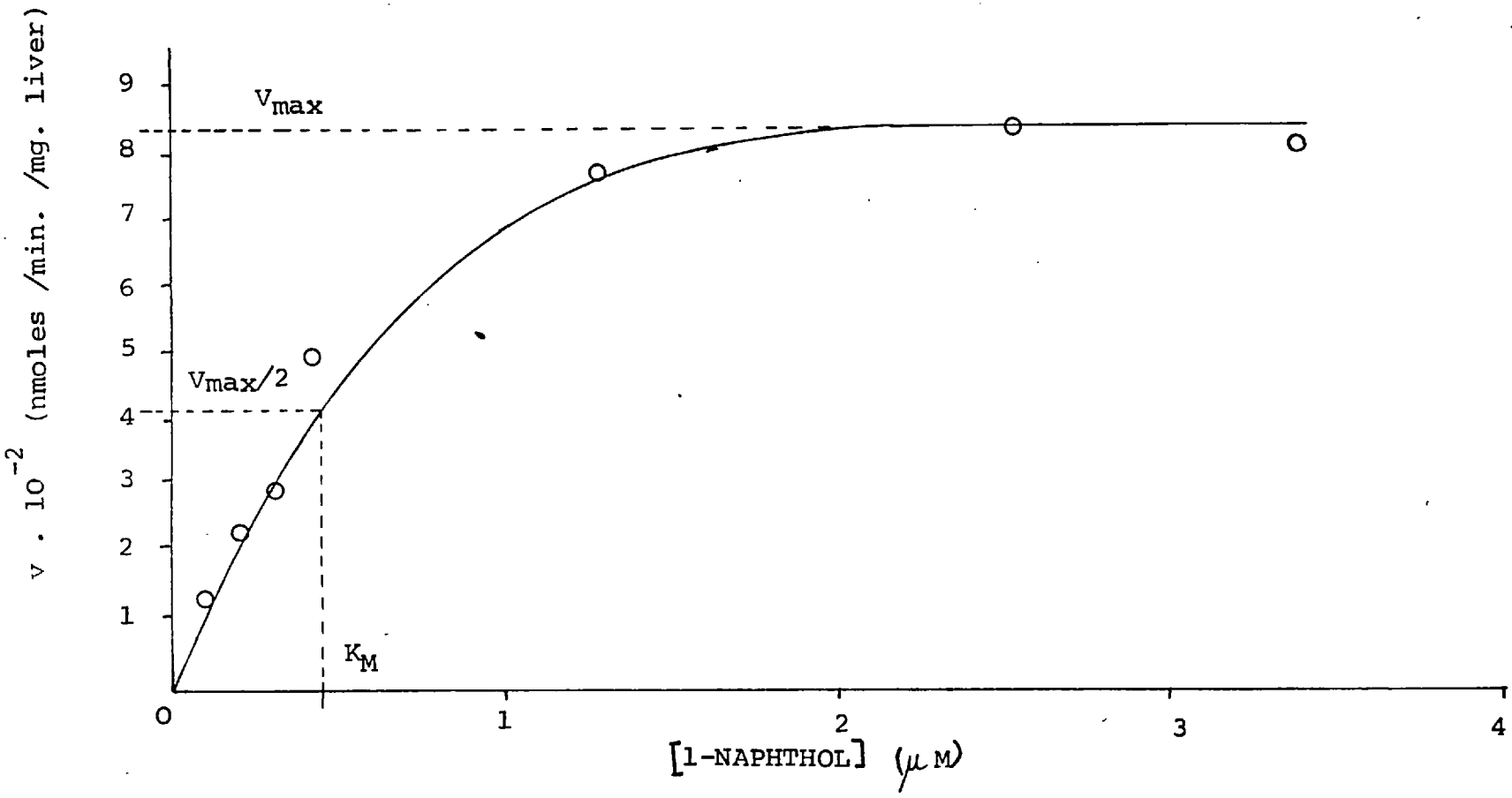


Figure 5.5

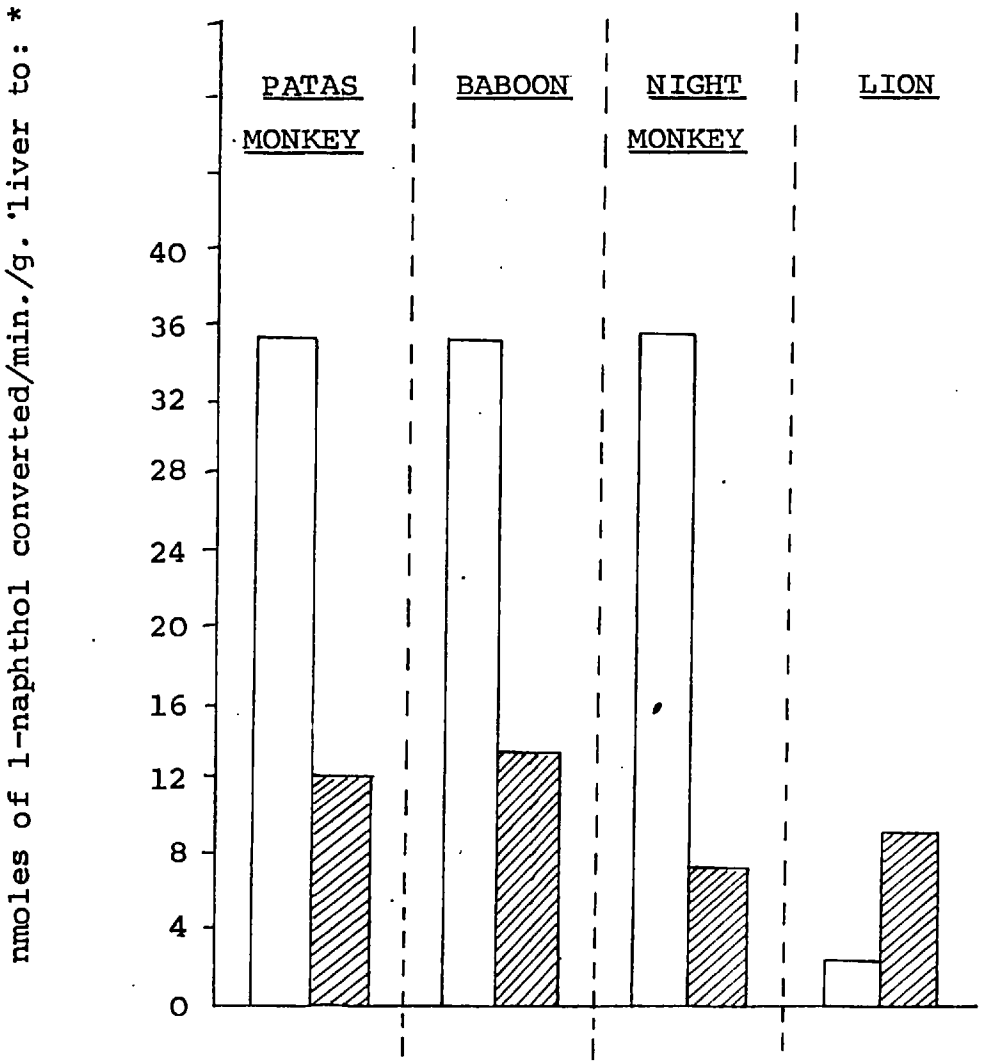
The concentration of 1-naphthol in each case was 20 μM in the presence of 1.5mM UDPGA (for glucuronidation), or approximately 10 μM PAPS (for sulphation), and 20 mg. of liver (600g. supernatant). For details of reaction mixtures, see Materials and Methods (Chapter 2). The data have been obtained with livers from single animals, and with two reaction mixtures for each species. Male primate livers and a female lion liver have been used.

*

▨ Sulphate

□ Glucuronide

Figure 5.5: In Vitro Conjugation of 1-Naphthol in Some Primate and Lion Livers



glucuronide. A similar situation exists in the guinea pig though the glucuronidation rate is slightly higher than sulphation. In contrast, in the cat, very little 1-naphthol is converted to its glucuronide (0.4 nmoles/min./g. liver), the rate of conversion to sulphate being considerably greater (220 nmoles/min./g. liver). An opposite situation to that observed for the cat exists in the pig, where although, 1-naphthol can be conjugated with both glucuronic acid and sulphate, the rate of conversion to the former is markedly higher (898 nmoles/min./g. liver) compared to that of sulphate (12 nmoles/min./g. liver).

These results are in agreement with the _in vivo_ observations. Thus, in the rat, at four dose levels, a greater amount of 1-naphthol was excreted in the urine as the sulphate than the glucuronide conjugate, the reverse being true in the guinea pig at two dose levels of 1-naphthol. In the cat, on the other hand, the major metabolite of 1-naphthol was the sulphate conjugate (see Chapter 3). Capel _et al_. (1974a) have shown that the pig has a low sulphating ability for a number of phenols (phenol, 2-naphthol, 4-hydroxyamphetamine and phenacetin), though with 1-naphthol, substantial amounts (32%; 25mg/Kg dose) of the sulphate conjugate are formed. The _in vitro_ data obtained in the present studies indicate that as expected from the _in vivo_ observations, the pig can conjugate 1-naphthol with both sulphate and glucuronide conjugates; nevertheless, the rate of conversion to glucuronide is considerably greater than that for conversion to sulphate, and therefore, quite different from either the rat or the guinea pig.

The above still applies when one compares the kinetic parameters for UDP-glucuronyltransferase and phenolsulphotransferase with 1-naphthol as the substrate in the rat, cat and pig livers (Table 5.1). Thus, the rat liver phenolsulphotransferase has a slightly higher affinity (lower apparent K_M) and

a higher apparent V_{max} for 1-naphthol than the corresponding glucuronidating enzyme. In contrast, the cat liver UDP-glucuronyltransferase exhibits a very much lower affinity for 1-naphthol than either the same enzyme in the rat, or the cat liver phenolsulphotransferase. Although the rat and cat liver glucuronyltransferases have similar apparent V_{max} the difference between the apparent K_M is approximately 300-fold. Contrary to this, the cat liver phenolsulphotransferase has a considerably greater affinity for the xenobiotic than the corresponding rat liver enzyme. This affinity of the cat liver phenolsulphotransferase also differs to a marked extent from that of the cat liver UDP-glucuronyltransferase (by about 10,000-fold). The pig liver UDP-glucuronyltransferase, in agreement with the *in vivo* findings, has both a higher V_{max} and a lower K_M for 1-naphthol than the phenolsulphotransferase in the same species.

Enzyme Activities Towards Phenolphthalein in the Rat and Cat Livers

The cat can conjugate phenolphthalein, unlike certain other phenols, with glucuronic acid (60%; dose: 25mg/Kg; Capel *et al.*, 1974a). As the data in Table 5.2 shows, the cat liver UDP-glucuronyltransferase has a much greater affinity for phenolphthalein as the aglycone substrate than 1-naphthol; thus, the K_M for phenolphthalein is approximately 35-fold lower than that for 1-naphthol. In the rat liver, the apparent K_M and V_{max} determined are only approximate (see the Results section). However, it can be seen that the K_M is yet again much lower (approximately 3-fold) than that for the cat liver enzyme, suggesting that although the cat can conjugate phenolphthalein with glucuronide, the extent to which this can occur is less than that in the rat. This would perhaps be expected when it is considered that *in vivo*, the rat conjugates most of phenol-

phthalein to its glucuronide (98%), all of this being excreted in the bile (100%; Abou-El-Makarem, 1967; Millburn et al., 1967).

Capel et al.(1974a) also reported that the cat excreted approximately 40% of the phenolphthalein as a sulphate conjugate, probably the monosulphate of phenolphthalein; no sulphate conjugates of this phenol have as yet been detected in the rat. The in vitro sulphation of phenolphthalein in the cat liver was attempted without any success, the reasons for this being unknown.

Enzyme Activities towards 1-naphthol and Phenolphthalein in Different Tissues of Rat, Cat and the Guinea pig

The rat and the cat have been studied in detail with respect to tissue distribution of the two enzyme activities with 1-naphthol and phenolphthalein as the aglycones (Tables 5.1 and 5.2). In the guinea pig tissues, only the rates for conversion of 1-naphthol to its glucuronide and sulphate have been studied (Table 5.4), whereupon it has been found that the liver, compared with the kidney and intestinal mucosa, has higher rates of conversion for both conjugations. The rate of conversion to glucuronide relative to that for sulphate, in all tissues except the intestine is greater; in the latter, the sulphation rate is slightly higher (0.039 compared with 0.025 nmoles/min./mg. tissue) than that for glucuronidation.

In the rat, as well, the liver compared with the kidney and mucosa, exhibits the highest capacity for both glucuronidation and sulphation of 1-naphthol. Thus, the rat liver UDP-glucuronyltransferase has a much lower K_M for 1-naphthol (12.3μM) than either the kidney or intestinal enzymes. Although the rat liver phenolsulphotransferase has similar K_M to that for the intestinal enzyme, the apparent V_{max} for the latter is approximately 18-fold less than that for the liver enzyme. The rat kidney phenolsulphotransferase has a lower K_M (3.23μM)

for 1-naphthol than either the liver or mucosal enzymes; however, the apparent V_{max} though higher than for the intestinal enzyme, is much lower than for the liver phenolsulphotransferase. The apparent K_M values for the rat UDP-glucuronyltransferase in the kidney and intestinal mucosa with phenolphthalein as the substrate are again slightly higher than that for the liver enzyme, and the V_{max} values are also somewhat lower (Table 5.2).

In the cat, no UDP-glucuronyltransferase activity with either 1-naphthol or phenolphthalein as substrates was detectable in either the kidney or the intestinal mucosa. This is despite the findings that the cat is better at glucuronidating phenolphthalein. Therefore, it would appear that in the cat where the glucuronidating capacity for phenols is generally low anyway, the very little UDP-glucuronyltransferase activity that does exist is confined to the liver. The phenolsulphotransferase activity in the cat with 1-naphthol as the aglycone, on the other hand, is similar in all three tissues examined. (Table 5.1).

These enzymic studies in the rat tissues with both 1-naphthol and phenolphthalein indicate that the liver is better at conjugation than the intestinal mucosa; thus, either the apparent K_M for the liver enzyme is lower or the apparent V_{max} is higher, or both occur simultaneously. This is in agreement with the following <u>in vitro</u> studies that have been reported in the literature. The rate of conversion of phenol to its sulphate in adult human liver is greater (2513 nmoles/g.tissue/60 min.) than that in the jejunal mucosa (502 nmoles/g.tissue/60 min.; Bostrom and Wengle, 1967). Similarly, Aitio (1973a) from a number of studies on glucuronide conjugation concluded that the liver was quantitatively the most important organ of glucuronide synthesis, though the gastro-intestinal mucosa does make a significant contribution as well. The biosynthesis

of salicyl glucuronide by tissue slices of various organs from different species has also been studied (Schacter et al., 1959), again with identical conclusions.

Whether the same situation would apply in vivo is difficult to say at the present time. Recently, Powell et al. (1974) have suggested that for orally-ingested phenols, the gut is the major organ for detoxication. It is to be expected that the route of administration of a drug would be important in determining the major first line of defence against toxic effects of a xenobiotic. For example, when the gastro-intestinal tract is by-passed by intravenous administration of free phenol, the liver may be expected to assume importance as a centre of detoxication. Thus, Capel et al. (1974b) have observed that in the hen, the extent to which phenol can be conjugated with either glucuronic acid or sulphate is dependent upon the route of administration. They have made suggestions for these differences found on the basis of the tissue locations of conjugation and the possible concentration of phenol at the sites of conjugation. The latter, of course, is believed to play a major role in determining the relative extents of conjugation with either glucuronic acid or sulphate (see Bray et al., 1952a, b,c,d; also Chapters 3 and 4).

The kinetic parameters obtained for the intestinal enzymes with both 1-naphthol and phenolphthalein also indicate that the intestinal mucosa is better at glucuronidation than sulphation of the two phenols. The studies undertaken in the rat 'in situ' intestinal preparation by Parker (1977) as well as by Bock and Winne (1975) show that in vivo a similar situation may be occurring.

Thus, the investigations conducted by Bock and Winne (1975) have indicated that when the small intestine is perfused with 65 μM 1-naphthol, the phenol is almost exclusively glucuronidated (rate: about 7 nmoles/min./g. intestinal tissue).

Parker (1977) has also found that when the small intestine is perfused with 3.5 μM 1-naphthol, the overall rate of conjugation with glucuronic acid is 27.1 nmoles/min/g. intestinal tissue, while that with sulphate is only 2.78 nmoles/min./g. intestinal tissue. Similarly, for phenolphthalein at the same dose level, no sulphate conjugate has been observed, the rate of conversion to glucuronide being 0.44 nmoles/min./g. intestinal tissue. When the glucuronidation rates for 1-naphthol and phenolphthalein in the '*in situ*' intestinal loops are compared, it can be seen that phenolphthalein is less readily conjugated with glucuronic acid than 1-naphthol. The kinetic parameters for UDP-glucuronyltransferase with 1-naphthol and phenolphthalein as substrates obtained in the present studies agree accordingly; thus, the apparent V_{max} for phenolphthalein is very much lower (0.017 nmoles/min./mg. intestinal mucosa) compared with that for 1-naphthol (0.227 nmoles/min./mg. intestinal mucosa).

Enzyme Activities towards 1-naphthol in Certain Primate and Lion livers

In the primates, two of which are Old World species (patas monkey and baboon) and one, a New World species (night monkey), the rates of conversion of 1-naphthol to its glucuronide are the same (35 nmoles/min./g. liver), while those for conversion to sulphate are very similar (Figure 5.5). In all species, the rates for conversion to sulphate are lower than those for glucuronidation. It is difficult to compare this *in vitro* data with that obtained *in vivo* in several primate species (Chapter 4), since the species used for *in vitro* studies are different except for the patas monkey. Furthermore, the livers were obtained from male primates whereas the *in vivo* studies were mainly conducted in females. However, the observations from the present studies that the UDP-glucuronyltransferase and phenolsulphotransferase activities in the New

World and Old World species are similar, suggest that differences in patterns of conjugation of phenols between the New World and Old World species may not be as clear-cut as thought (see Smith and Williams, 1974; Smith and Caldwell, 1977). This, therefore, further indicates that many more species and number of animals need to be studied before simply the patterns of metabolism are used in considerations of primate taxonomy (see also Chapter 4).

The result in the lion liver shows that as in the cat, the rate of conversion of 1-naphthol to its glucuronide is low (1.8 nmoles/min/g. liver in the lion liver cf.0.4 nmoles/min./g. liver in the cat liver; Figures 5.2 and 5.5). However, the rate for sulphation (9 nmoles/min./g. liver), though higher than that for glucuronidation, is not as high as in the cat (220 nmoles/min/g.liver). French et al. (1974) found that phenol, in vivo, was conjugated only with sulphate in the lion, no glucuronide conjugate having been detected. Whether a similar in vivo situation would be encountered with 1-naphthol is difficult to predict solely from this in vitro finding.

To summarise then, the enzyme activities obtained in livers from the rat, cat, guinea pig and the pig are in accordance with either the in vivo results obtained in the past Chapters, or with the investigations that have been reported in the literature. Thus, in the cat, the UDP-glucuronyltransferase activity towards 1-naphthol is very much lower than in the rat, while the phenolsulphotransferase activity is greater than in the rat. With phenolphthalein as the aglycone, the glucuronidating activity in the cat is only slightly less compared with that in the rat. In the pig liver, the UDP-glucuronyltransferase activity towards 1-naphthol is similar to that in the rat; however, the phenolsulphotransferase activity is somewhat lower in comparison with the rat.

The studies in different tissues of the rat, cat and guinea pig indicate that overall the liver is better at conjugating 1-naphthol and phenolphthalein than either the kidney or the intestinal mucosa. However the role of the intestinal mucosa as a major detoxication site for phenols may be determined by the route of administration (see Powell et al., 1974).

The preliminary studies in certain primate livers, in agreement with the in vivo findings in Chapter 4, suggest that many more New and Old World species have to be studied before patterns of conjugation of phenols are used in considerations of primate taxonomy. The lion liver appears to have a similar glucuronidating activity towards 1-naphthol as in the cat, though the phenolsulphotransferase activity is by no means as high as in the cat.

CHAPTER SIX

Bisubstrate Kinetic Analysis of UDP-glucuronyltransferase and Phenolsulphotransferase from Rat and Cat Livers.

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Introduction

The classical approach to determining the properties of an enzyme is to first prepare purified forms. Unfortunately, this has not been possible in many cases and is an especially difficult problem in the case of membrane-bound enzymes such as UDP-glucuronyltransferase. Thus, a number of attempts to purify this enzyme have been unsuccessful, mainly because all methods now available for separating microsomal enzymes from their attachments to the membrane will alter protein-lipid interactions which are very important in determining the properties of the enzyme (see Chapter 1). However, kinetic investigations with a crude enzyme system have been useful in resolving, for example, many questions concerning the possible multiplicity of hepatic glucose-6-phosphatase (E.C. 3.1.3.9; Arion and Nordlie, 1964; Nordlie et al., 1968, 1971) and also in determining the kinetic mechanism of UDP-glucuronyltransferase (Vessey and Zakim, 1972b; Rao et al., 1976; Sanchez and Tephly, 1975), and in understanding the defective function of UDP-glucuronyltransferase in Gunn rats (Nakata et al., 1976).

The studies in the previous chapter indicated that the apparent K_M's for UDP-glucuronyltransferase with 1-naphthol as the aglycone in the rat and the cat vary widely, suggesting that the low glucuronidating capacity for 1-naphthol in the cat could be due to a reduced affinity of the enzyme for the xenobiotic.However, a possibility exists that the cat liver enzyme may also have a low affinity for the co-substrate, UDPGA and that this could be a reason for the low level of glucuronidation for 1-naphthol in this species.

Furthermore, in the studies in the last chapter, the kinetic parameters were determined at only one concentration of the co-substrate whereas it is widely known that both glucuronidation and sulphation are two substrate reaction pathways. (see Chapter 1; Bannerjee and Roy, 1968; Vessey and

Zakim, 1972b; Sanchez and Tephly, 1965; Rao et al., 1976). Thus, to obtain further information on the nature of this low UDP-glucuronyltransferase activity in the cat, bisubstrate kinetic analysis of the enzyme with 1-naphthol as the aglycone has been carried out, and compared with the activity for phenolphthalein, a substrate which is better glucuronidated by the cat (Chapter 5; Capel et al., 1974a). For comparison between species, the corresponding enzyme for 1-naphthol in the rat has been similarly analysed, and further compared with phenolsulphotransferase in rat liver with 1-naphthol as the phenolic substrate.

UDP-N-acetylglucosamine, a naturally occurring metabolite, activates UDP-glucuronyltransferase in vitro, and it does so by increasing the apparent affinity of the enzyme for UDPGA and decreasing that for UDP, an end-product of the reaction; it may even inhibit UDPGA breakdown by UDPGA pyrophosphatase, or increase selectively the membrane permeability of UDPGA (Pogell and Leloir, 1961; Winsnes, 1969, 1972a; Vessey et al., 1973b; Zakim and Vessey, 1977a). The effects of this activator, which may play an important role for enzyme regulation in vivo (Zakim and Vessey, 1974; 1977a), have also been examined on UDP-glucuronyltransferase activity towards 1-naphthol in the rat and cat livers.

Results

Kinetic Studies

The initial rates of UDP-glucuronyltransferase activity in the rat and cat livers have been studied as a function of the concentration of UDPGA at several fixed concentrations of 1-naphthol, and as a function of varying concentrations of UDPGA. The data have been plotted according to Lineweaver and Burk (1934) in double reciprocal form as in Figures 6.1-6.4. The lines were computed as described in Chapter 5, the

Figure 6.1

Each point is based on initial rate determination of UDP-glucuronyltransferase, carried out in duplicate on livers pooled from at least three animals (see Results Section), as described in Materials and Methods (Chapter 2), with the indicated concentrations of 1-naphthol. The concentrations of UDPGA were:

▲	0.75 mM
■	1.50 mM
△	3.0 mM
○	4.5 mM
□	6.0 mM

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.

r is the correlation coefficient.

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Figure 6.1: Double Reciprocal Plots of Initial Rates of UDP- glucuronyltransferase Activity As a Function of Varying Concentrations 1-Naphthol At Different Fixed Concentrations of UDPGA in 600g. Supernatants from Rat Liver.

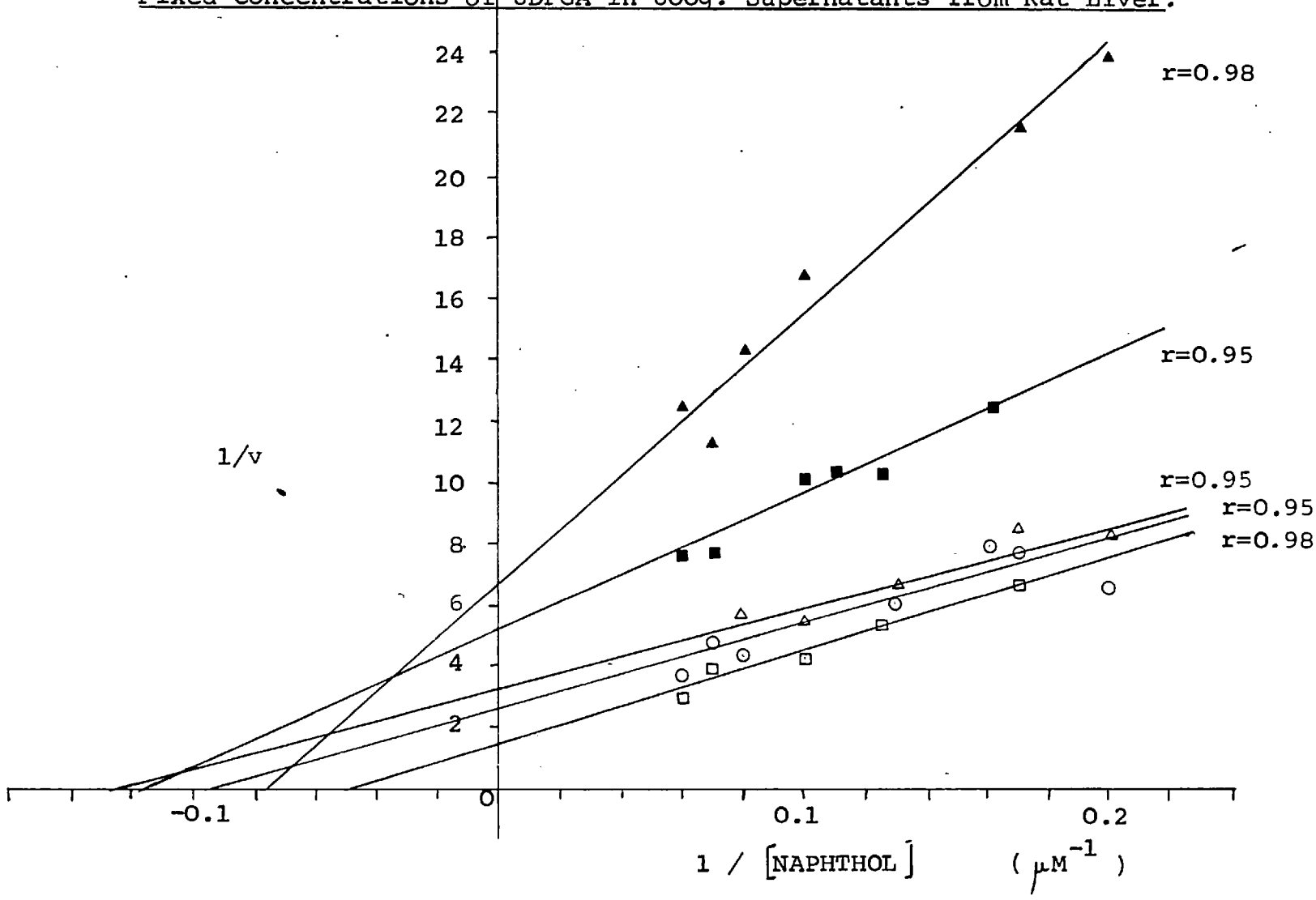

Figure 6.2

Each point is based on initial rate determination of UDP-glucuronyltransferase, carried out in duplicate on livers pooled from at least three animals (see Results Section), as described in Materials and Methods (Chapter 2), with the indicated concentrations of UDPGA. The concentrations of 1-naphthol were:

▲	6 μM
■	8 μM
△	10 μM
○	12 μM
□	16 μM

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.

r is the correlation coefficient.

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Figure 6.2: Double Reciprocal Plots of Initial Rates of UDP-glucuronyltransferase Activity as a Function of Varying Concentrations of UDPGA at Different Fixed Concentrations of 1-Naphthol in 600g. Supernatants from Rat Liver

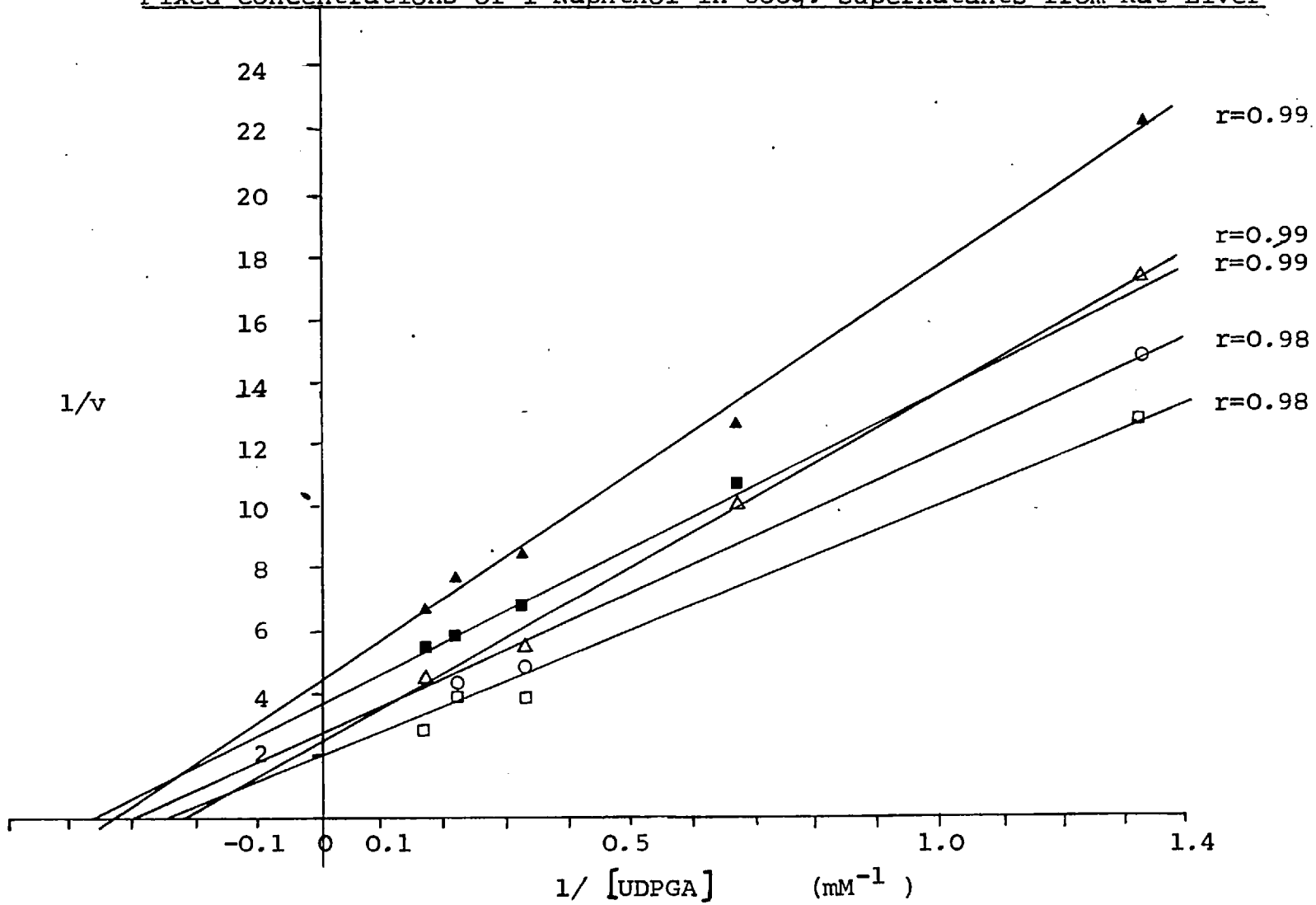

Figure 6.3

Each point is based on initial rate determination of UDP-glucuronyltransferase , carried out at least in duplicate on livers pooled from three animals (see Results Section), as described in Materials and Methods (Chapter 2), with the indicated concentrations of 1-naphthol. The concentrations of UDPGA were:

▲	0.75mM
■	1.50mM
△	3.00mM
○	4.50mM
□	6.00mM

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.

r is the correlation coefficient.

Figure 6.3: Double Reciprocal Plots of Initial Rates of UDP-glucuronyltransferase Activity As a Function of Varying Concentrations of 1-Naphthol at Different Fixed Concentrations of UDPGA in 600g. Supernatants from Cat Liver.

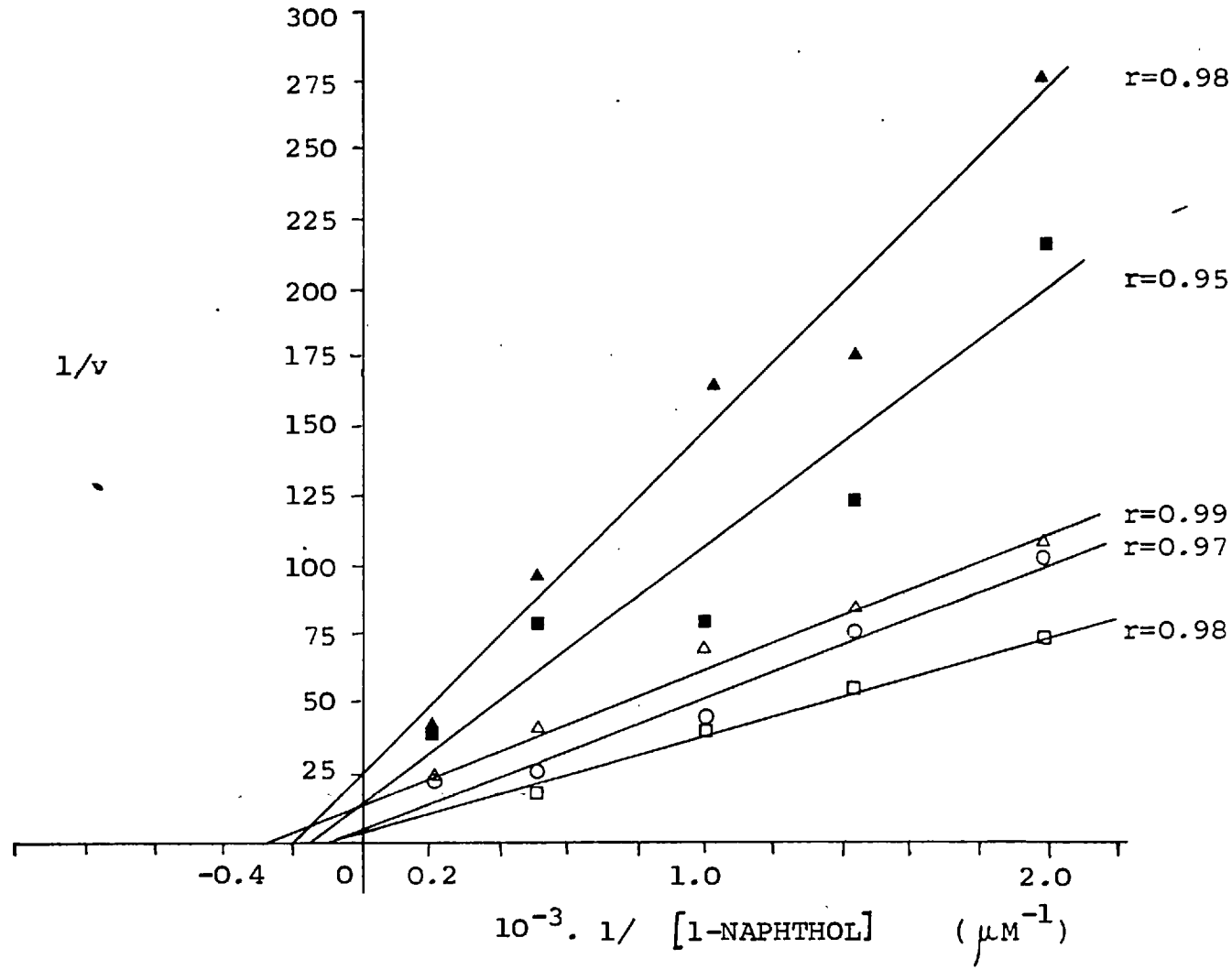

Figure 6.4

Each point is based on initial rate determination of UDP-glucuronyltransferase, carried out at least in duplicate on livers pooled from three animals (see Results Section), as described in Materials and Methods (Chapter 2), with the indicated concentrations of UDPGA. The concentrations of 1-naphthol were:

▲	506.25 μM
■	706.25 μM
△	1006.25 μM
○	2006.25 μM

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.

r is the correlation coefficient.

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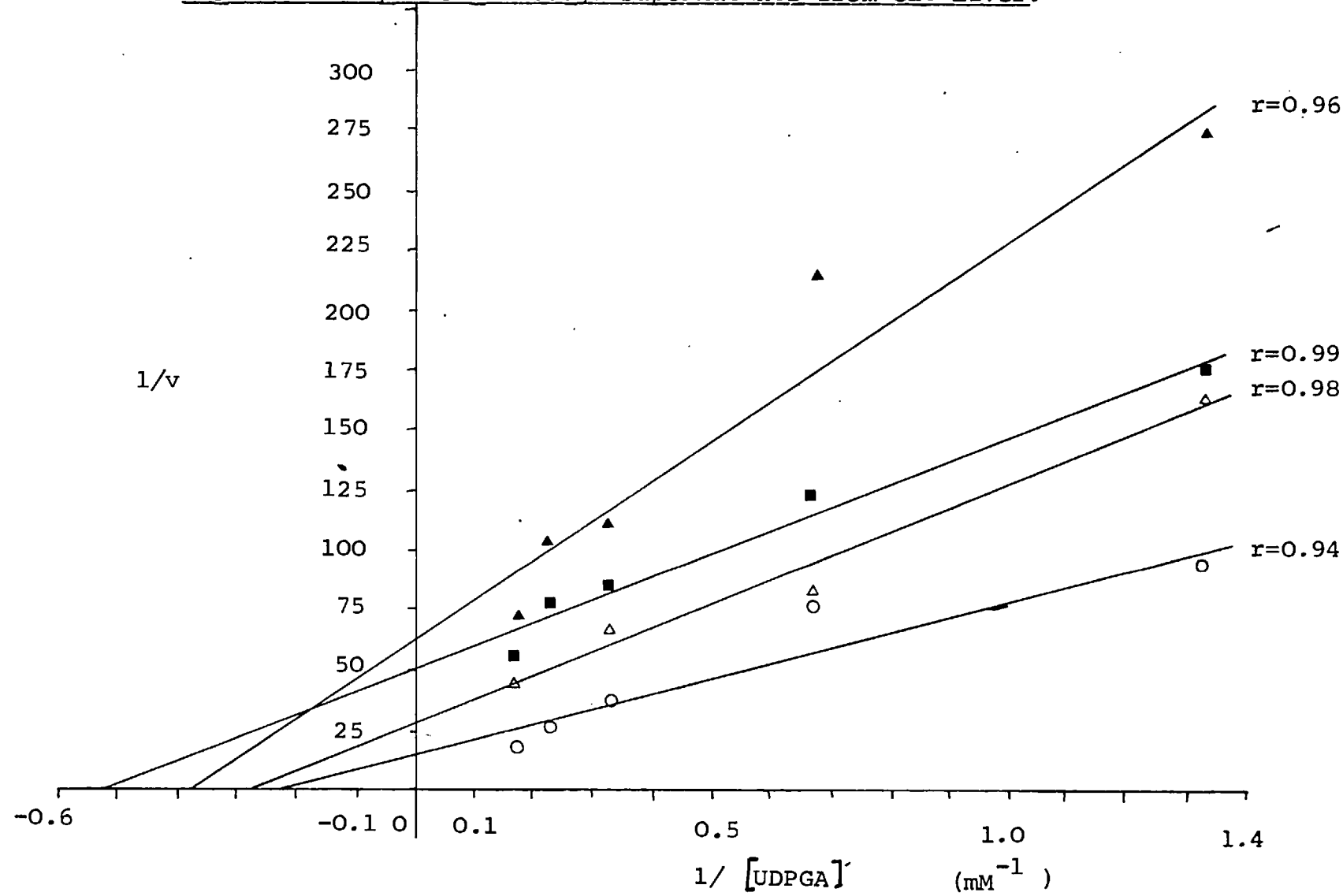
Figure 6.4: Double Reciprocal Plots of Initial Rates of UDP-glucuronyltransferase Activity As a Function of Varying Concentrations of UDPGA at Different Fixed Concentrations of 1-Naphthol in 600g. Supernatants from Cat Liver.



intercepts on the ordinates and abscissae, and the slopes having been calculated by the programme. Experiments were repeated to obtain consistent results and correlation coefficients (r) in the region 0.95-0.99. Secondary plots of the intercepts on the ordinate and the slopes obtained from the double reciprocal plots as in Figures 6.1 and 6.3 were then used to determine the activities at V_{max} (Table 6.1; Figures 6.5 and 6.6).

The kinetic parameters for UDP-glucuronyltransferase with phenolphthalein as the xenobiotic substrate in cat liver, and for phenolsulphotransferase in the rat liver with 1-naphthol as the phenolic substrate have been similarly determined. For the latter, the co-substrate is PAPS, the concentration of this having been determined as described in Materials and Methods (Chapter 2). The various kinetic parameters obtained have been listed in Table 6.1.

The various kinetic parameters can also be determined from replots of the intercepts and slopes obtained from the primary data plotted as a function of the concentration of the co-substrate at several fixed concentrations of 1-naphthol (for example, Figures 6.2 and 6.4). The values thus obtained were somewhat different to those presented in Table 6.1, but these differences were not such as to affect the conclusions drawn from the data. A possible explanation for these discrepancies could be the use of crude enzyme systems to study these conjugation reactions.

The effect of UDP-N-Acetylglucosamine on UDP-glucuronyltransferase Activity

Figure 6.7 illustrates the effect of UDP-N-acetylglucosamine on the microsomal UDP-glucuronyltransferase from rat and cat livers. Determinations have been carried out at four different concentrations of UDP-N-acetylglucosamine.

Figure 6.5

Initial rates of activity were determined as described in legends to Figures 6.1 and 6.3, and Materials and Methods (Chapter 2). The reciprocals of the rates at infinite concentrations of 1-naphthol (the intercepts on the ordinates of the data plotted in Figures 6.1 and 6.3) are plotted as a function of the reciprocals of each fixed concentration of UDPGA. The intercepts on the ordinate are $1/V_{max}$ of the glucuronidation reactions in the two species.

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.

r is the correlation coefficient.

Figure 6.5: Determination of Activity at V_{max} of UDP-glucuronyltransferase in 600g. Supernatants from the Rat and Cat Livers.

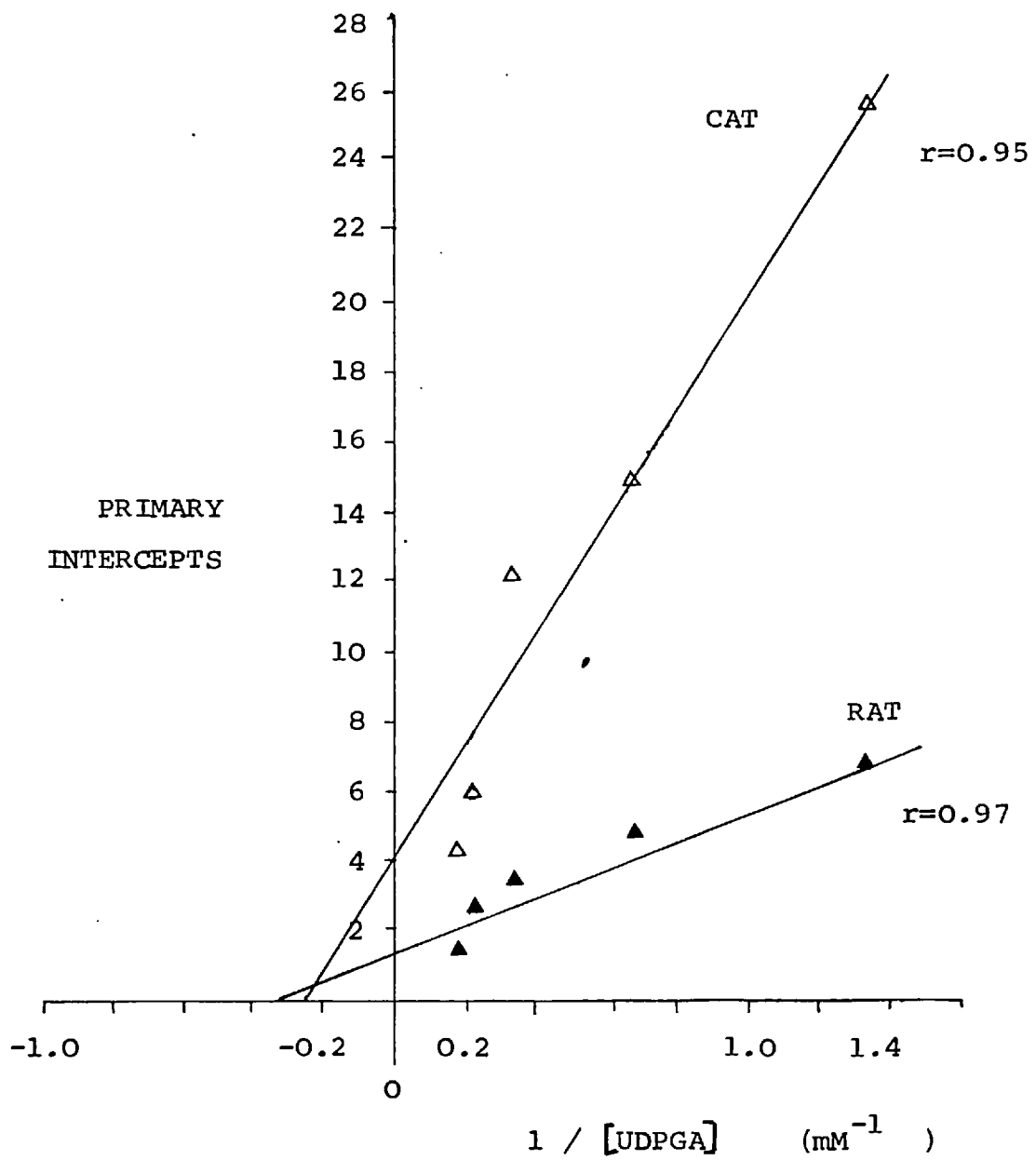


Figure 6.6

Initial rates of activity were determined as described in legends to Figures 6.1 and 6.3, and Materials and Methods (Chapter 2). The slopes of lines plotted in Figures 6.1 and 6.3 are plotted here as a function of the reciprocals of each fixed concentration of UDPGA. The intercepts on the abscissae are $1 / K_{UDPGA}$ for the enzymes in the two species. The intercepts on the ordinates are equal to $K_{1\text{-NAPHTHOL}} / V_{max}$, and substituting the respective V_{max} values obtained from Figure 6.5, the $K_{1\text{-NAPHTHOL}}$ values in μM, for the two species can be calculated.

Units of activity are nmoles of 1-naphthol converted per min. per mg. liver.
r is the correlation coefficient.

Figure 6.6: Determination of K_{UDPGA} and $K_{1\text{-NAPHTHOL}}$ for UDP-glucuronyltransferase in 600g. Supernatants from the Rat and Cat Livers.

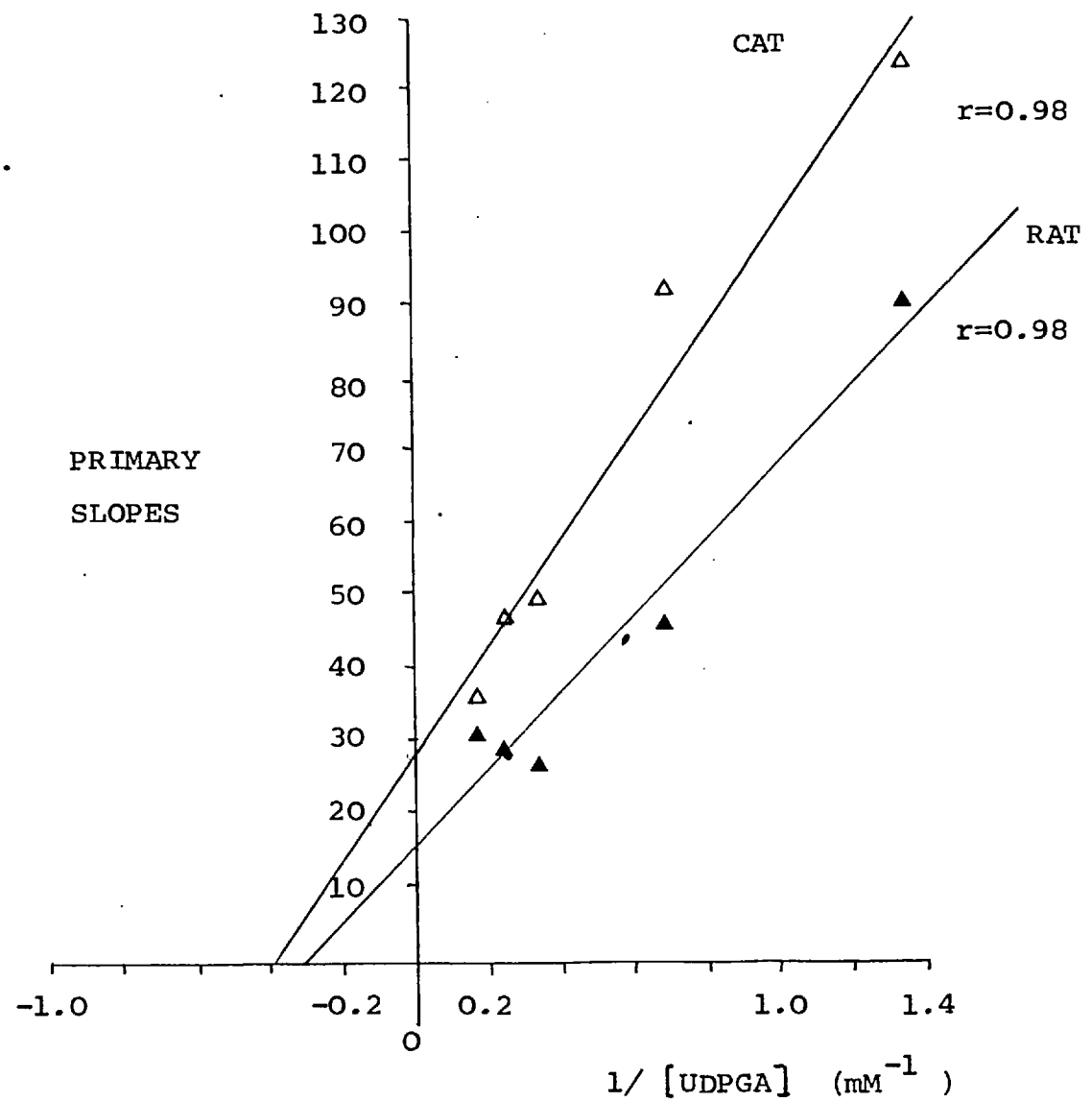


TABLE 6.1

* K_c refers to the K_M for the co-substrate, the co-substrates being UDPGA for UDP-glucuronyltransferase and PAPS for phenolsulphotransferase.

K_a refers to the K_M for the xenobiotic.

The units for the K_M terms are mM, and the V_{max} is in nmoles of the xenobiotic converted per min. per mg. liver.

The values of V_{max} and K_M were determined graphically after replots of the primary data as in Figures 6.5 and 6.6 respectively.

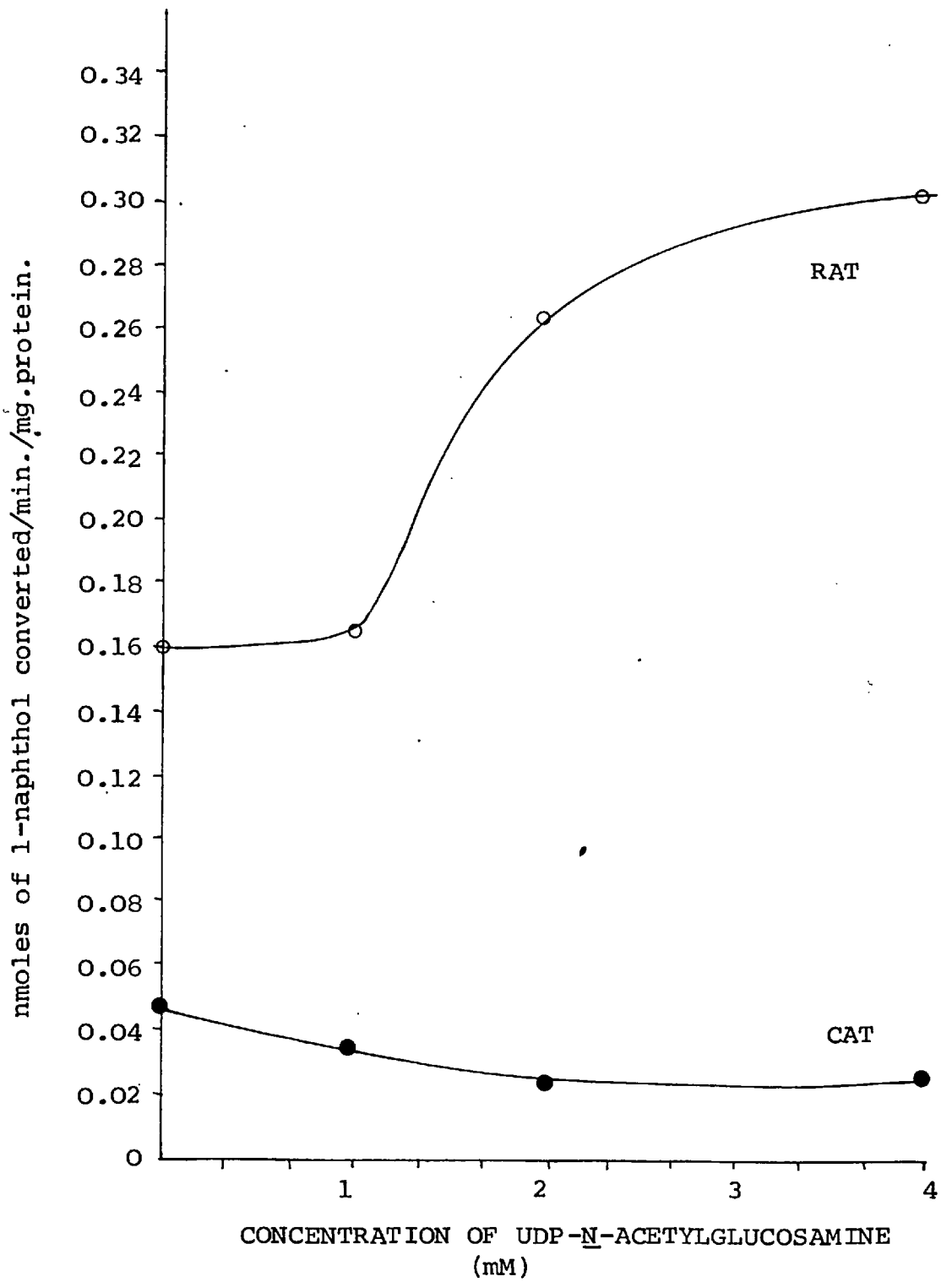
TABLE 6.1: Kinetic Parameters of UDP-glucuronyltransferase and Phenolsulphotransferase

Enzyme	Substrate	Species	Kinetic Parameters *		
			K_c	K_a	V_{max}
UDP-glucuronyltransferase	1-Naphthol	Rat	3.33	0.0116	0.750
		Cat	2.63	6.89	0.243
	Phenolphthalein	Cat	1.54	0.1692	0.385
Phenolsulphotransferase	1-Naphthol	Rat	0.017	0.0043	0.269

Figure 6.7

Rat and cat microsomes were assayed for UDP-glucuronyltransferase activity as described in Materials and Methods (Chapter 2).The concentration of UDPGA was 1.5mM in all assays, and the 1-naphthol concentration, 20μM in the rat, and 1000μM in the cat enzyme assays.UDP-N-acetylglucosamine at different concentrations was added to incubation media dissolved in 0.25M tris-HCl buffer, pH 7.4. Each point represents a mean of two determinations carried out on livers from three animals.

Figure 6.7: Effect of UDP-N-acetylglucosamine on the Rat and Cat Liver Microsomal UDP-glucuronyltransferase.



Discussion

The Possible Mechanisms for the Reactions Catalysed by UDP-glucuronyltransferase and Phenolsulphotransferase

The initial rates of UDP-glucuronyltransferase activity when plotted in a double reciprocal form (Figures 6.1-6.4) indicate that with the concentration of the fixed substrate increasing, the apparent V_{max} of the varied substrate increases, and further the intercept on the 1/[S] axis and the K_M value depend upon the concentration of the other substrate. Similar observations were also made with the glucuronidation of phenolphthalein in the cat and with sulphation of 1-naphthol in the rat. These results, therefore, show that studies of enzymic 1-naphthol and phenolphthalein glucuronidation, and 1-naphthol sulphation at a single concentration of either substrate may not provide the best K_M and V_{max} values. For all these reactions, the ratios of apparent K_M values to apparent V_{max} values were found not to be constant, i.e. the apparent K_M values for the enzymes did not differ to a very great extent when the concentrations of the fixed substrates were varied, whereas the apparent V_{max} values did. This suggests that the mechanisms for the UDP-glucuronyltransferases in the rat and cat livers with the two aglycones as well as for phenolsulphotransferase in the rat liver are sequential, i.e. both substrates (the co-substrate and the aglycone) must bind to the enzyme before any product is released (Cleland, 1963). Similar mechanisms have been proposed for the glucuronidation of oestrone by pig kidney (Rao et al., 1976), for p-nitrophenol by guinea pig liver (Vessey and Zakim, 1971, 1972b) and for morphine by rat liver (Sanchez and Tephly, 1975), and for the sulphation of p-nitrophenol by guinea pig liver (Bannerjee and Roy, 1968). It is not possible from the present data to propose the exact reaction mechanisms, since product inhibition and the kinetics of the reverse reactions need to be investigated before the mechanisms can be proposed.

Kinetic Parameters for phenolsulphotransferase in the Rat

Table 6.1 shows the kinetic parameters obtained for the rat liver phenolsulphotransferase with 1-naphthol as the aglycone. Thus, the V_{max} is much lower than that for rat liver UDP-glucuronyltransferase with the same substrate (Table 6.1). This is consistent with the differences in the rates of glucuronidation and sulphation for 1-naphthol observed in the rat *in vitro* as well as in *vivo* (see Chapter 5). The K_M for 1-naphthol for sulphation is about a third of that for glucuronidation indicating that the affinity of the phenolsulphotransferase for the aglycone in greater than that of UDP-glucuronyltransferase.

Bisubstrate kinetic analysis of the cat liver phenolsulphotransferase has not been carried out because the rates of conversion of 1-naphthol to its sulphate are very high at very low substrate concentrations, and due to limitations of the assay methods used, the kinetic parameters for this reaction can only be determined by saturation analysis (see Chapter 5). However, preliminary studies, in agreement with those reported in Chapter 5, did indicate that the values for $K_{1\text{-naphthol}}$ and K_{PAPS} would be very much lower than those for the rat enzyme.

UDP-glucuronyltransferase

1-Naphthol as the Aglycone in the Rat and the Cat

Comparing the kinetic data in Table 6.1 for the rat and cat liver UDP-glucuronyltransferases with 1-naphthol as the aglycone shows firstly that the V_{max} for the rat enzyme is very much greater (approximately 3-fold) than that for the cat enzyme. Secondly, the K_{UDPGA} values in the two species are similar, whereas in contrast, there is a very large difference (approximately 500-fold) between the $K_{1\text{-naphthol}}$ values. Thus, the rat enzyme has a considerably greater affinity

for 1-naphthol than the cat enzyme. Therefore, the low glucuronidating capacity for 1-naphthol in the cat appears to be due to a diminished affinity of the enzyme for the xenobiotic substrate rather than the co-substrate, UDPGA, in addition to a lower enzyme activity at V_{max}.

These findings for the cat liver UDP-glucuronyltransferase are in contrast to those obtained in the Gunn rat with p-nitrophenol as the aglycone where the enzyme has a reduced affinity for UDPGA compared with normal. (Nakata et al., 1976). Gunn rats have a genetically-determined deficiency in their capacity to synthesize glucuronides such that there is no detectable activity of UDP-glucuronyltransferase assayed with bilirubin as the substrate in liver microsomes, but the ratesof glucuronidation of o-aminophenol and o-aminobenzoic acid are about 20% of normal, and that for p-nitrophenol is decreased only slightly (Dutton, 1966; Schmid, 1972; Zakim et al., 1973d). Thus, to explain the slightly defective conjugation of p-nitrophenol in kinetic terms, Nakata et al. (1976) have shown that whereas the activity at V_{max} and values of the K_M for p-nitrophenol are identical in rats of Gunn and Wistar strains, the enzyme from the Gunn rat has a much lower affinity for UDPGA. This reduced affinity for the co-substrate appears to be due to abnormal interactions between the enzyme and the UDP-portion of UDPGA.

Phenolphthalein as the Aglycone in the Cat

When we consider the data obtained for the cat liver UDP-glucuronyltransferase with phenolphthalein as the substrate (Table 6.1), it can be seen that the activity at V_{max} is greater (approximately 1•5-fold) than that for 1-naphthol in the same species. The $K_{phenolphthalein}$ value, which is approximately 40-fold less than that for 1-naphthol in the cat, indicates that phenolphthalein is a much better substrate

for the cat hepatic UDP-glucuronyltransferase than 1-naphthol. The K_{UDPGA} value with phenolphthalein as the aglycone is slightly less (approximately 1.5-fold) than that for 1-naphthol in the same species. Therefore, the better capacity of the cat for the glucuronidation of phenolphthalein, compared with 1-naphthol, seems mainly due to an increased affinity of the enzyme for the aglycone, but may also be in part due to a slightly greater affinity for the co-substrate and a somewhat higher enzyme activity at V_{max}.

Possible Explanations for the Differences Observed in the UDP-glucuronyltransferase Activities

In trying to explain the differences observed in the relative extents of glucuronic acid conjugation of various phenols (phenol, 1-naphthol, 2-naphthol, morphine and phenacetin versus phenolphthalein) *in vivo* in the cat, it has been suggested by Capel *et al.* (1974a) that since there may exist several forms of UDP-glucuronyltransferases (see Table 1.10), the low glucuronidating capacity of the cat for a number of phenols may be due to a deficiency of one or more forms of glucuronyl-transferases. Thus, the form of the enzyme conjugating 1-naphthol in the cat may not be the same as that conjugating phenolphthalein, or that conjugating 1-naphthol in the rat. The kinetic data for 1-naphthol obtained in the present studies show that although the affinity of UDP-glucuronyltransferase in cat liver for the aglycone is very much lower than that in the rat, the cat is not *completely* lacking in the enzyme activity towards this phenol. However, *purely* on the basis of this kinetic data, it cannot be stated that the enzymes conjugating 1-naphthol, in the rat and cat, or the enzymes conjugating the two phenols in the cat are necessarily different. Several possible explanations exist for these observed differences.

Nakata *et al.* (1976) have put forward a hypothesis that

different UDP-glucuronyltransferases may contain a common UDPGA binding subunit. This hypothesis may help to explain the defective function of the enzyme in Gunn rats and the defective function of multiple enzymes. Thus, a single mutation in this common subunit could lead to defective function of several UDP-glucuronyltransferases. Interactions of this subunit with different aglycone binding subunits could modify the properties of the binding site for UDPGA, thereby leading to variability in the extent of the defect of glucuronidation of different aglycones.

If this hypothesis applies it could be suggested that the binding site for UDPGA in the cat is not defective on account of the similar K_{UDPGA} values obtained for 1-naphthol in both the rat and the cat. The large differences in $K_{1\text{-naphthol}}$ values in the two species and the difference between $K_{1\text{-naphthol}}$ and $K_{phenolphthalein}$ in the cat could then be attributed to the presence of a defective aglycone binding subunit for 1-naphthol in the cat. The slight differences in K_{UDPGA} values (2.63 mM for 1-naphthol and 1.54 mM for phenolphthalein) could be due to differences in interactions of the two aglycone binding subunits with the common UDPGA binding unit. However, it is also possible that the cat liver is completely deficient in the usual 1-naphthol or phenolphthalein glucuronidating forms of the enzymes, and that the conjugation of these substrates is catalysed by another form of enzyme (for example that normally conjugating bilirubin) with differing affinities for these two aglycones. If this is the case, then the slight differences in K_{UDPGA} values for 1-naphthol and phenolphthalein could perhaps be explained in terms of differences in interactions of these two substrates with the enzyme which may modify the UDPGA binding site, and hence, the affinity for UDPGA.

Another explanation for the observed differences in enzyme activity may lie in the fact that UDP-glucuronyltransferase is

a membrane-bound enzyme, there being a considerable amount of existing evidence that the enzyme activity is very much dependent on the phospholipid environment (for reviews see Zakim and Vessey, 1974; 1977). Therefore, it is possible that the differences in substrate affinity observed both between rat and the cat for 1-naphthol, and also between 1-naphthol and phenolphthalein in the cat are caused not by different enzymes but by the membrane environment in which the enzyme is located (Mulder, 1974). This environment could well influence the kinetic parameters for different substrates since their physico-chemical properties may determine the accessibility to the enzyme site, and hence their rate of conjugation. (Mulder and Van Doorn, 1975). Thus, variations in physico-chemical properties may be the cause of differences in properties of UDP-glucuronyltransferase observed with different substrates, and in that case may misleadingly suggest enzyme heterogeneity (Mulder, 1974; Mulder and Van Doorn, 1975; Dutton and Burchell, 1976). The possible influence of the membrane environment on the glucuronidation of 1-naphthol in cat liver is investigated and further discussed in the following chapter.

Thus, in terms of current ideas on the regulation of UDP-glucuronyltransferase, the differences observed in glucuronidation may be explained in terms of:

(1) different enzymes or aglycone binding subunits sharing a common UDPGA binding unit;

(2) a single enzyme or aglycone binding unit with differing affinities for different aglycones; and

(3) the possible influences of the membrane environment on enzyme activity.

Availability of Co-substrates for the Conjugation Reactions

The K_M for phenolsulphotransferase in the rat for the co-substrate, PAPS, is very similar to that for the aglycone

substrate, in comparison with the differences between the K_M's for UDPGA and the aglycones for UDP-glucuronyltransferases in both the rat and cat. Similar observations have been made by Bannerjee and Roy (1968) where the respective K_M values for PAPS and the aglycone (p-nitrophenol) were 0.036 mM and 0.070 mM, by McEvoy and Carroll (1971) where they were 0.014 mM and 0.051 mM respectively, and by Ryan and Carroll (1976) where they were 0.013 mM and 0.006 mM (aglycone, in this case, dehydroepiandrosterone) respectively.

A possible reason for this could be that since PAPS formation occurs in the same part of the cell (the cytosol) as the transfer of sulphate to a phenolic aglycone (Roy & Trudinger, 1970; De Meio, 1975), the cosubstrate availability is not likely to be hampered by transport processes.

This is in contrast to the reaction catalysed by UDP-glucuronyltransferase, where the affinity for the co-substrate, UDPGA, *in vitro* is rather poor compared with the aglycone substrate; thus, K_{UDPGA} in the rat liver is 3.33 mM compared with a $K_{1\text{-naphthol}}$ of 0.0116 mM (Table 6.1; see also data for other aglycones: Potrepka and Spratt, 1971; Vessey and Zakim, 1971, 1972b; Zakim *et al.*, 1973b; Sanchez and Tephly, 1974; Del Villar *et al.*, 1974). This is probably due to the low permeability of the microsomal membrane to UDPGA. Recent studies indicate that charged substrates of M.W. above 90 do not penetrate microsomes (Nilsson *et al.*, 1971; Depierre and Dallner, 1975). UDPGA which is synthesized in the cytosol (Strominger *et al.*, 1957), would thus need to be actively transported to the active site of UDP-glucuronyltransferase. Some support for the low permeability of the microsomal membrane is also provided by the 'coupled transglucuronidation' studies of Berry and Hallinan (1974) which have indicated that o-aminophenol is glucuronidated at a greater rate in the presence of UDP and p-nitrophenyl glucuronide than with UDPGA, suggesting that the microsomal vesicles are more permeable to UDP and

p-nitrophenyl glucuronide than to UDPGA, and that since UDPGA is excluded from the inside of the vesicle, then the UDP-dependent hydrolysis of p-nitrophenyl glucuronide generates a greater amount of UDPGA on the inside of the vesicle than can be achieved by diffusion of this substrate from the outside.

Since the physiological concentration of UDPGA is small compared with other UDP-sugars (0.12-0.41 mole/g. wet wt. of rat and guinea pig livers; Zhivkov, 1970; Keppler et al., 1970). and since the concentration of UDPGA reaching the enzyme site may also be affected by the permeability barrier, for efficient function in vivo, UDP-glucuronyltransferase must have a high selectivity for the binding of UDPGA. This has been demonstrated in untreated microsomal preparations where the transferase is specific for UDPGA, but is extensively inhibited by the end-product of the reaction, UDP, at the overall concentration found in liver (Winsnes, 1969, 1972b; Zakim and Vessey, 1974, 1977). As the concentration of UDP in vivo is nearly equal to that of UDPGA (Keppler et al., 1970), inhibition by UDP (or UTP) could become important and limit severely the rate of glucuronidation reactions in intact animals. Therefore, in vivo the most useful manner for modulating the interactions between UDPGA and UDP-glucuronyltransferase would be to maximize affinity for the glucuronic acid portion and to minimize that for UDP.

The Effect of UDP-N-Acetylglucosamine on the Rat and Cat Microsomal UDP-glucuronyltransferases

UDP-N-acetylglucosamine, a physiological metabolite, has an activating effect on UDP-glucuronyltransferase in vitro in a number of species, namely, the rat, guinea pig and mouse (Winsnes, 1972a; Marniemi and Laitinen, 1975; Otani et al., 1976; Zakim and Vessey, 1977a). It does this by enhancing the affinity of the enzyme for UDPGA and decreasing that for

the UDP-moiety, and furthermore, this activation does not alter enzyme-glucuronic acid interactions sufficiently to reduce specificity of the binding of UDP-sugars. (Zakim et al., 1973c; Zakim and Vessey, 1977a). The activation by UDP-N-acetylglucosamine therefore seems to be important for the function of UDP-glucuronyltransferase under conditions likely to exist in vivo.

The K_{UDPGA} values, obtained in the rat and cat in the present investigations with 1-naphthol as the aglycone, are very similar, suggesting that the permeability characteristics of the microsomal membrane in the two species towards UDPGA are also similar. To determine whether increasing the affinity of the cat liver UDP-glucuronyltransferase for UDPGA and relieving any possible inhibition of the enzyme by UDP or UTP by means of UDP-N-acetylglucosamine activation increases the rate of glucuronidation of 1-naphthol in the cat liver microsomes, studies have been carried out in the cat liver microsomes, and compared with those in the rat (Figure 6.7). As the results show, increase of rat liver enzyme activity towards 1-naphthol is obtained at 1,2 and 4 mM concentrations of UDP-N-acetylglucosamine, but not at all in the cat at any of the concentrations studied. It is possible that UDP-N-acetylglucosamine exerts its effect on UDPGA binding in the cat liver enzyme in a similar fashion to that in the rat, though it does not increase the rate of 1-naphthol glucuronidation; a bi-substrate kinetic analysis of the cat liver enzyme in the presence of UDP-N-acetylglucosamine would be needed to investigate this possibility.

These experiments therefore support the suggestions made earlier that the low glucuronidating activity towards 1-naphthol in the cat is not due to a reduced affinity of the enzyme for UDPGA nor is it due to inhibition of the UDP-glucuronyltransferase by atypically high levels of UDP or UTP. The

stimulation of enzyme activity by UDP-N-acetylglucosamine has also been attributed to inhibition of UDPGA breakdown by the microsomal UDPGA - pyrophosphatase (Pogell and Leloir, 1961), as well as increasing the membrane permeability of UDPGA by assisting a proposed permease (Winsnes, 1972a). By whichever mechanism UDP-N-acetylglucosamine may stimulate UDP-glucuronyltransferase, the suggestion that the cat liver UDP-glucuronyltransferase is not hampered by co-substrate availability still stands.

Thus, to summarize, the data obtained from the present investigations indicate that the low glucuronidating capacity for 1-naphthol in the cat compared with the rat is due to poor affinity of the enzyme for the aglycone and not the co-substrate, UDPGA. Conversely, phenolphthalein is a much better substrate for the cat liver UDP-glucuronyltransferase, this being due to the higher affinity of the enzyme for the aglycone, and also perhaps a slightly greater affinity of the enzyme for the co-substrate. Studies with UDP-N-acetylglucosamine support the suggestions that the 1-naphthol glucuronidating form of cat liver enzyme is not hampered in any way by UDPGA availability. The bisubstrate kinetic data for the rat phenolsulphotransferase and UDP-glucuronyltransferase shows that the affinity of the latter for 1-naphthol is slightly less than that of the former; however, the activity at V_{max} for the glucuronyltransferase is very much greater than that for the phenolsulphotransferase, thus, agreeing with the studies reported in previous chapters.

CHAPTER SEVEN

The Nature of the Micro-environment of UDP-glucuronyl-transferase from Rat and Cat Livers

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Introduction

An abundance of evidence indicates that the properties of UDP-glucuronyltransferase, one of the many membrane-bound enzymes, are intimately related to interactions with the microsomal phospholipids (Zakim and Vessey, 1974, 1977a; Graham et al., 1974a). Thus, high degrees of activation of the enzyme activity can be observed in vitro when native microsomal preparations are treated with, for example, detergents, phospholipases, sonication, bilirubin, urea, chaotropic agents, and lysophosphatides (Leuders and Kuff, 1967; Van Roy and Heirwegh, 1968; Winsnes, 1969; Vessey and Zakim, 1971; Mulder, 1971; Hanninen and Puukka, 1971; Vainio, 1973; Henderson, 1970; Sanchez and Tephly, 1973b). It is uncertain at present whether UDP-glucuronyltransferase is sensitive to specific modifications within its environment or whether activation reflects a non-specific response to a variety of different changes within the membrane.

Due to the firm association of the enzyme with the endoplasmic reticulum attempts to purify active forms of the enzyme have not been very successful (see Chapter 1). As a result, studies on understanding the regulatory properties of UDP-glucuronyltransferase have so far relied on the use of untreated and detergent and phospholipase-treated microsomal preparations (Zakim and Vessey, 1977). Furthermore, investigations involving membrane perturbants have also contributed somewhat towards an understanding of the defect in the function of UDP-glucuronyltransferase in homozygous Gunn rats (Zakim et al., 1973d; Nakata et al., 1975), as well as in comparing the microenvironments of the enzyme(s) in different species (Vessey and Zakim, 1972a; Graham and Wood, 1972).

It has been shown by the bisubstrate kinetic analyses of the rat and cat UDP-glucuronyltransferase activities towards

1-naphthol that the low glucuronidating capacity in the cat is due to the reduced affinity of the enzyme for the aglycone and not for the co-substrate, UDPGA. However, phenolphthalein is not as poor a substrate for the cat liver enzyme as 1-naphthol (see Chapter 6). There is now a considerable amount of indirect evidence suggesting that there are several substrate-specific forms of UDP-glucuronyltransferases (see Chapter 1). Taking this into consideration, Capel et al. (1974a), from their in vivo studies in the cat with phenolphthalein and various other phenols, suggested that the form of enzyme conjugating phenolphthalein in the cat is not the same as that required to conjugate phenol or 1- and 2-naphthols with glucuronic acid.

Since the membrane environment is an integral part of UDP-glucuronyltransferase, the heterogeneity of the enzyme may arise due to differences in its micro-environment (Mulder, 1974; Dutton et al., 1977). The activities of membrane-bound enzymes may be affected in various ways by the lipid environment such as the physical state of the lipid phase (Byrne and Chapman, 1964; Chapman and Collin, 1965) and phospholipid fluidity (Kimelberg and Papahadjopoulos, 1972) which depends on the cholesterol content and the chain length and degree of saturation of fatty acids (Van Der Neut-Kok et al., 1974). Furthermore, different xenobiotics have different physico-chemical properties and therefore, each of these may have different influences on the micro-environment of the enzyme and hence, the expression of enzyme activity (Mulder, 1974; Mulder and Van Doorn, 1975).

It is possible that the differences observed for UDP-glucuronyltransferase activity towards 1-naphthol in the rat and the cat are due to differences in the micro-environments of the enzyme in the two species, in a similar fashion to what has already been observed for the Gunn rat (Zakim et al., 1973d),

which also has a low glucuronidating capacity for certain xenobiotics (e.g. o-aminophenol, o-aminobenzoate, umbelliferone, menthol; Schmid, 1972). If such a situation exists in the cat liver microsomes, then the perturbation of protein-phospholipid interactions in the microsomal membrane, which in vitro appear to be a regulator of enzyme activity (Zakim and Vessey, 1977), may somehow modify the low glucuronidating capacity for 1-naphthol in the cat.

Therefore, to investigate into these possibilities, the effects of various membrane perturbants, namely, phospholipases A and C, Triton X-100 and bilirubin have been compared towards rat and cat microsomal UDP-glucuronyltransferases with 1-naphthol as substrate, and furthermore, the phospholipid and cholesterol contents and fatty acid composition of the microsomal membrane in the two species have been examined.

Results

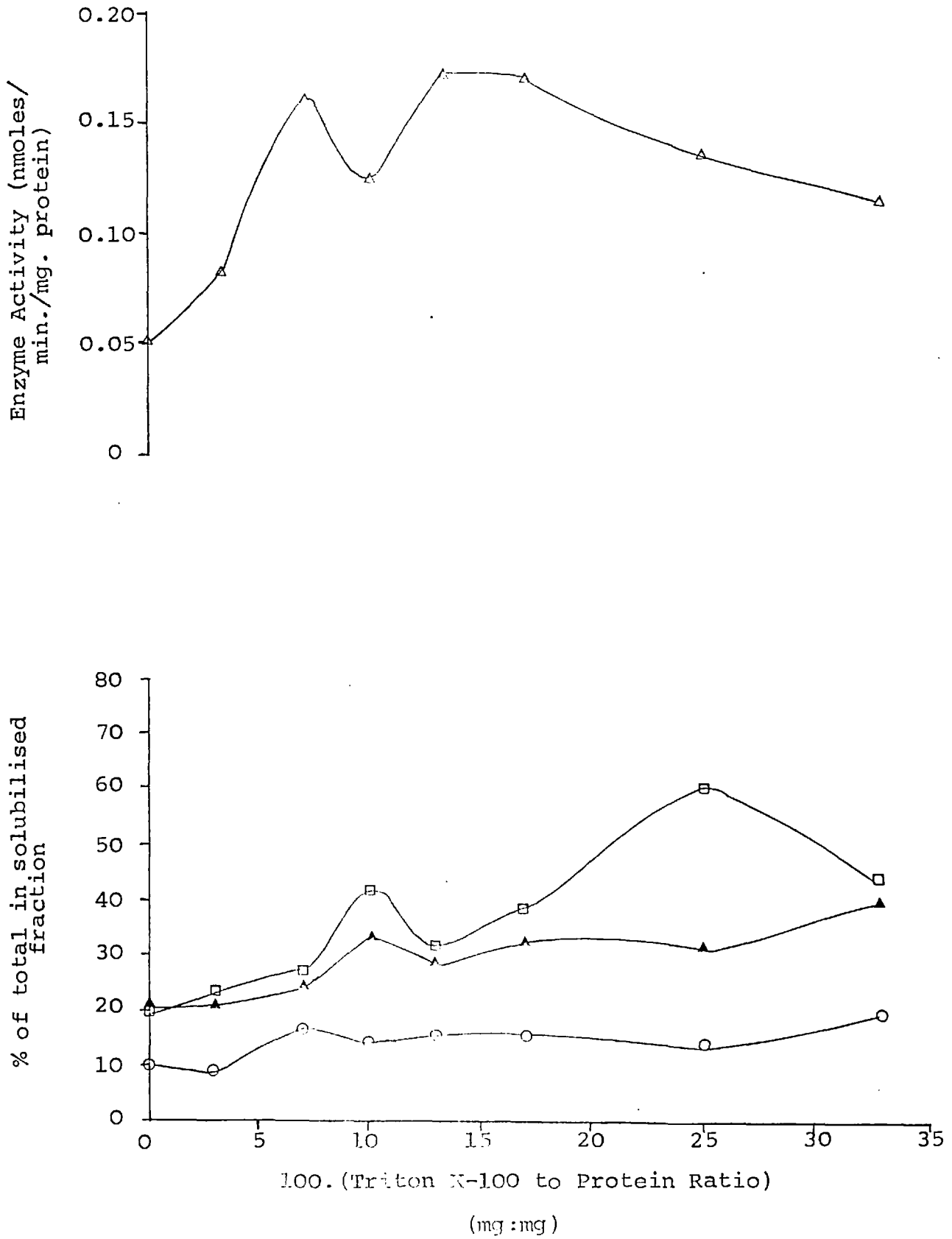
Figures 7.1 and 7.2 illustrate the solubilisation of protein and phospholipid of the microsomes from rat and cat livers as well as the effect on UDP-glucuronyltransferase activity (1-naphthol as the substrate) with increasing Triton X-100 to microsomal protein ratios. The effect of increasing bilirubin concentration on rat and cat liver UDP-glucuronyltransferase is shown in Figure 7.3. The effects of phospholipases A and C (Table 7.1) on enzyme activity in the two species were determined after preincubation with the two phospholipases over 60 minute periods though here only the maximal activation obtained after the appropriate preincubation time period are presented. In each case, appropriate controls were included (see Materials and Methods, Chapter 2). Details of the various treatments on the microsomal enzymes in the two species are given in legends to the respective figures or table. The phospholipid to protein and the phospholipid to cholesterol ratios, and the fatty acid compositions of mixed phospholipids

Figure 7.1

Microsomes from rat liver were treated with Triton X-100 at the indicated Triton X-100 to microsomal protein ratios and assayed for UDP-glucuronyltransferase activity as described in Materials and Methods. The concentration of UDPGA was 1.5 mM in all assays and the 1-naphthol concentration, 20μM. The enzyme activity is presented as nmoles of 1-naphthol converted/min./mg.microsomal protein. Similarly treated microsomal preparations, and containing [1-^{14}C]-1-naphthol and cold 1-naphthol (final concentration being 20μM), were centrifuged at 80,000g for 30 minutes to separate the solubilised fraction from the unsolubilised , and the phospholipid, protein and ^{14}C contents of the two fractions determined; the amounts of these various constituents found in the solubilised fraction are presented as percentages of the total found in the solubilised and unsolubilised fractions. Each point represents the mean value of four determinations carried out on livers from nine rats.

- □ Protein
- ▲ Phospholipid
- ○ 1-naphthol
- △ UDP-glucuronyltransferase activity.

Figure 7.1: Effect of Triton X-100 on Rat Liver Microsomal protein and phospholipid, and on UDP-glucuronyltransferase Activity.



Legend to Figure 7.2:

Microsomes from cat livers were treated with Triton X-100, and determined for UDP-glucuronyltransferase activity, phospholipid, protein and ^{14}C contents as described in the legend to Figure 7.1. The concentration of 1-naphthol in the incubation media and for determining the distribution between the solubilised and unsolubilised fractions was 1000μM. Each point represents the mean value of two determinations carried out on livers from three cats.

□ Protein

▲ Phospholipid

o 1-naphthol

△ UDP-glucuronyltransferase activity.

Figure 7.2: Effect of Triton X-100 on Cat Liver Microsomal protein and phospholipid, and on UDP-glucuronyltransferase Activity.

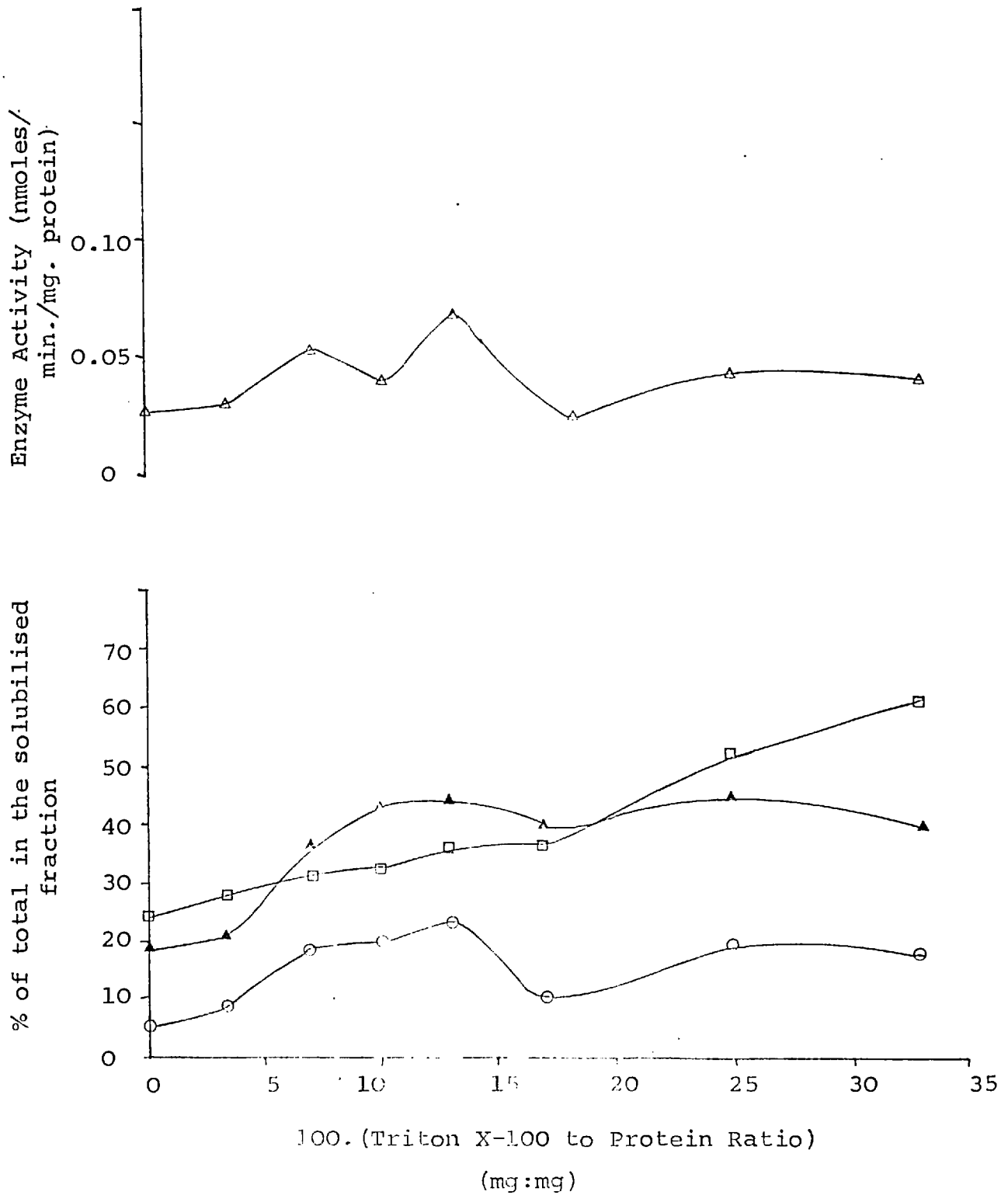


Figure 7.3

Rat and cat liver microsomes were assayed for UDP-glucuronyltransferase activity as described in Materials and Methods (Chapter 2).The concentration of UDPGA was 1.5mM in all assays, and the 1-naphthol concentration , 20μM in the rat and 1000μM in the cat. Bilirubin at different concentrations was added to incubation media dissolved in 0.07N NaOH. Each point represents a mean of two determinations carried out on livers from three animals.

Figure 7.3: Effect of Bilirubin on the Rat and Cat Liver Microsomal UDP-glucuronyltransferase.

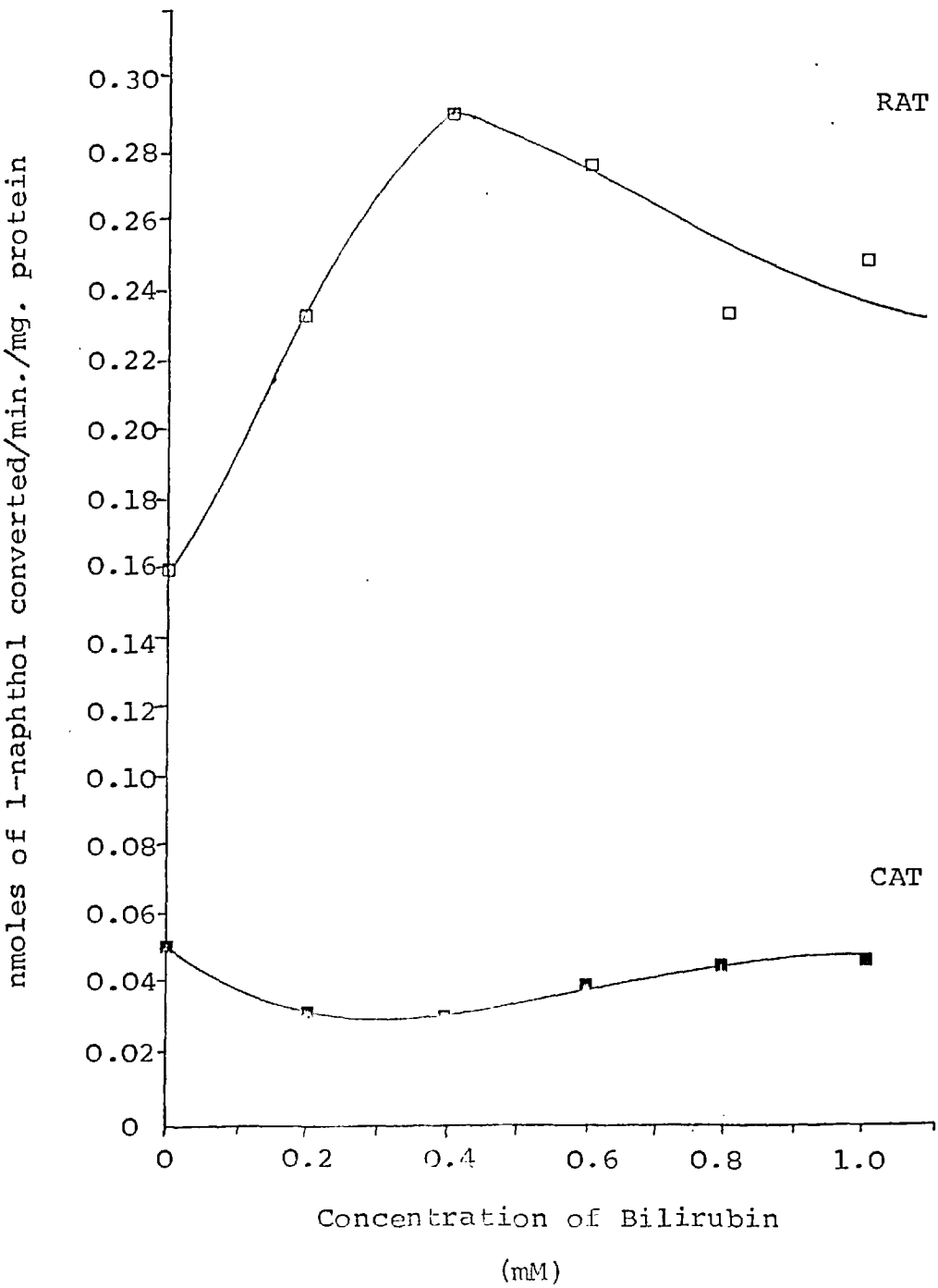


TABLE 7.1

Microsomes from rat and cat livers were treated with either phospholipase A from Naja naja venom (microsomal to phospholipase A protein ratio of 100:1) or phospholipase C from C. welchii (microsomal to phospholipase C protein ratio of 16:1) in 12.5 mM tris-HCl buffer, pH 8.0, and 2.5 mM calcium Chloride, at 23°C. Aliquots were removed at various times and assayed at 37°C for UDP-glucuronyltransferase activity as described in Materials and Methods (Chapter 2). The concentration of UDPGA was 1.5 mM in all assays, and the 1-naphthol concentration, 20μM in the rat, and 1000 μM in the cat assays. The incubation media contained 3mM EDTA to inhibit the further action of the phospholipases. The controls were treated as for test incubations except that the phospholipases had been excluded.

* Presented as means ± S.E.M.
Determined on livers pooled from 3 animals, each value being a mean of 2 determinations.

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TABLE 7.1: Effect of Phospholipases A and C on UDP-glucuronyltransferase activity in the rat and cat liver microsomes

Species	Treatment	Time of preincubation (min)	nmoles of 1-naphthol converted/min/mg.protein.*
Rat	Control	-	0.058 ± 0.007
	Phospholipase A	5	0.115 ± 0.014
	Phospholipase C	5	0.208 ± 0.003
Cat	Control	-	0.022 ± 0.002
	Phospholipase A	10	0.039 ± 0.016
	Phospholipase C	40	0.042 ± 0.028

of microsomes from the rat and cat livers are given in Tables 7.2 and 7.3 respectively.

Discussion

The effect of Triton X-100

Many studies in the past have shown that Triton X-100 enhances the rat liver UDP-glucuronyltransferase activity towards various different substrates (Lueders and Kuff, 1967; Winsnes, 1969; Mulder 1970, 1971; Graham and Wood, 1972; Zakim et al., 1973d). The present studies indicate that with 1-naphthol as substrate as well, the enzyme activity is increased with increasing concentrations of Triton X-100. At the point of maximal activation (Triton X-100 to protein ratio of 13.3), the increase in enzyme activity compared with the control, in both the rat and cat, is similar. Thus, in the cat, enzyme activity increases from 0.025 nmoles/min/mg. microsomal protein for the control to 0.07 nmoles/min./mg. protein on maximal activation (an increase of 2.8-fold), while in the rat, the increase is from 0.052 nmoles/min/mg. protein for the control to 0.171 nmoles/min./mg. protein on maximal activation (an increase of 3.3-fold), However, even at the point of maximal activation, the activity in the cat microsomes is still very much lower than that in the rat. Figures 7.1 and 7.2 also illustrate the patterns of solubilisation of microsomal protein and phospholipid with increasing Triton X -100 to protein ratios in the two species, and it can be seen that the detergent has similar effects on microsomal membrane solubilisation in the rat and the cat.

The mechanism of solubilisation of a biological membrane by a non-ionic detergent such as Triton X-100 is thought to be as follows. Triton X-100 is believed to bind primarily to hydrophobic regions on the proteins occupied by lipid in the native membrane. Lipid removal by detergent treatment would

TABLE 7.2: The Phospholipid to protein and Phospholipid to cholesterol ratios in microsomes from the rat and the cat livers

Species	Protein (mg/ml of microsomal fraction)	Phospholipid (mg/ml of microsomal fraction)	Phospholipid/Protein Ratio	Phospholipid/Cholesterol Ratio
Rat	13.25 ± 1.25*	38.6 ± 3.9*	3.02 ± 0.46*	24.9 ± 1.91†
Cat	13.5 ± 1.5**	26.3 ± 4.8**	1.93 ± 0.14**	36.5 ± 1.20†

The protein, phospholipid and cholesterol contents of microsomes from rat and cat livers were determined as described in Materials and Methods (Chapter 2). The data are presented as means±S.E.M.

* Number of animals = 12; number of determinations = 4.

** Number of animals = 3; number of determinations = 4.

† Number of animals = 3; number of determinations = 2.

TABLE 7.3: Fatty acid composition of mixed microsomal phospholipids in the rat and cat

Fatty acid	Fatty acid content in: (% of Total Fatty Acids)	
	Rat	Cat
Palmitic ($C_{16} = 0$)	16.5	10.0
Stearic ($C_{18} = 0$)	51.1	44.8
Oleic ($C_{18} = 1$)	5.0	10.1
Linoleic ($C_{18} = 2$)	13.2	14.7
Arachidonic ($C_{20} = 4$)	14.2	20.4

Legend: See Materials and Methods (Chapter 2) for details.

No. of determinations = 2

No. of animals = 3 of each species.

thus be an exchange of bound lipid for bound detergent which may form micelle-like regions on the surface of the protein (Pitt-Rivers and Impiomabato, 1968; Spatz and Strittmatter, 1971). According to Simons et al. (1973) depending on the ratio of detergent to membrane lipids, different steps of solubilisation may be achieved. Thus, in the first instance, when a small amount of detergent is present, the molecules of the detergent are incorporated into the membrane without breaking it. With increasing concentrations of detergent, the membrane is solubilised into a micellar solution containing mixed protein-lipid-detergent micelles in equilibrium with detergent micelles and free detergent molecules. Finally, when enough detergent is added, pure protein-detergent micelles may be obtained in equilibrium with detergent-lipid and detergent micelles (see also Gulik-Krzywicki, 1975; Helenius and Simons, 1975).

In the present studies, there appears to be a general trend towards an increase in protein and phospholipid contents of the solubilised fractions (Figures 7.1 and 7.2) with increasing concentrations of Triton X-100; however, a relationship between these events occuring with membrane solubilisation and activation of UDP-glucuronyltransferase cannot be recognised. The studies by Jansen and Arias (1975) on the solubilisation of UDP-glucuronyltransferase with deoxycholate, a bile-salt anion with similar detergent-like properties as Triton X-100 (Makino et al., 1973; Helenius and Simons, 1975) have also indicated that the enzyme activity was solubilised with the bulk of microsomal proteins, selective solubilisation having not been achieved. This is perhaps to be expected since for membrane-bound enzymes, the mechanism of regulation of enzyme activity are complex. Thus, for UDP-glucuronyltransferase, the membrane environment may either act as a permeability barrier for the substrates (Berry and Hallinan, 1974; Berry et al., 1975), and/or may influence the conformation and kinetic properties of the

enzyme (Zakim and Vessey, 1977); therefore, enzyme activation by Triton X-100, for instance, may mean either removal of membrane lipid thus improving the accessibility of the substrates to the enzyme site, and/or altering the conformation of the enzyme and hence, improving its ability to conjugate a substrate.

In the present investigations, when the hepatic microsomal fractions from the two species were treated with Triton X-100, 1-naphthol was also included in order to ascertain whether the permeability of the microsomal membrane in the two species towards the substrate is similar, since the accessibility of the substrate to the enzyme site is an important factor in determining the rate of glucuronidation (Mulder, 1974; Mulder and Van Doorn, 1975; Dutton et al., 1977; Depierre and Dallner, 1975). The results obtained show that in both species, in the controls, where the enzyme activity is most likely to be confined to the unsolubilised fraction, there is about 20% of the added 1-naphthol in the solubilised fraction suggesting that in the cat at the concentration of the phenol (1000 μM) when there is some measurable UDP-glucuronyltransferase activity, though considerably less than in the rat at 20 μM 1-naphthol, the microsomal membrane permeability for the substrate is similar to that in the rat. Thus, there is likely to be about 80% of added substrate within microsomes, in the vicinity of the enzyme, in both species. Similarly, at the point of maximal activation by Triton X-100 (detergent to protein ratio of 13.3), though the distribution of enzyme activity in the solubilised and unsolubilised fractions is not known since the effect of the detergent on enzyme activity was determined separately, the quantities of 1-naphthol in the solubilised fractions from the two species are not very different, that in the cat being 36% and in the rat, 32%. Therefore, it would appear that the detergent, Triton X-100 has similar effects on the microsomal

membrane solubilisation in the rat as well as the cat, and that the accessibility of the substrate to the enzyme sites in the two species are also similar; however, the expression of enzyme activity in the control and the maximally activated microsomes is still very much lower in the cat compared with the rat.

The Effects of Phospholipases A and C

In the rat, the enzyme could be activated maximally after preincubation of the microsomes with either phospholipases A or C for 5 minutes, the enzyme activity having increased approximately 3- and 4-fold respectively (Table 7.1). In the cat, on the other hand, maximal activation after treatment with phospholipases A and C could be observed after preincubation periods of 10 and 40 minutes respectively. Even then, in the cat microsomes, the enzyme activities on maximal activation by the two phospholipases are much lower than in the rat.

A number of investigations have been carried out on the effects of phospholipases A and C on UDP-glucuronyltransferase activity with variable results. Thus, Graham and Wood (1969) and Attwood *et al.* (1971) found that both phospholipases caused a decline in enzyme activity from guinea pig liver microsomes, while other workers (Isselbacher *et al.* 1962; Tomlinson and Yaffe , 1966; Hanninen and Puukka, 1971; Vessey and Zakim, 1971, 1972a; Winsnes, 1972a) have shown that in different species (rat, mouse, guinea pig) both phospholipases caused activation of the enzyme. It has as yet not been possible to explain these discrepances between the effects of the phospholipases though it has been suggested that the effect of phospholipase A could depend upon the species of animal being studied, the source of the phospholipase or the method of preparation of the microsomes. All these effects have now been studied with results that phospholipase A from two sources (from *C. adamenteus* and *Naja naja* venoms) increase activity in microsomes from guinea pig livers, and phospholipase A from *Naja naja* venom could

activate the enzyme from all the species studied (i.e. rat, mouse, rabbit, guinea pig and human; Vessey and Zakim, 1972a). Similarly the method of preparation of microsomes had no significant effect on the response of UDP-glucuronyltransferase to treatment with phospholipase A (Graham and Wood, 1973). In the present studies, since the effects of the two phospholipases on the rat and cat enzyme activities were studied under the same conditions, the differences observed can only be attributed to species differences.

The activation of phospholipase A does not appear to be due directly to degradation of membrane phospholipids, but due to the surface-active products (especially lysophosphatides) of the catalytic reaction of the phospholipase (Graham and Wood, 1974; Hanninen and Puukka, 1971). Thus, activation by phospholipase A resembles that by detergents and lysophosphatides (Graham et al., 1974b). Graham and Wood (1974) have further suggested that the enzyme activation by phospholipase C may also be due to surface-active compounds, even though the immediate products of this phospholipase action (diglycerides and phosphomonoesters) are not surface-active; rat liver microsomal fractions contain an acylhydrolase which liberates fatty acids from the diglycerides produced by phospholipase C (Cater and Hallinan, 1971; Michell et al., 1973). The effects of phospholipases A and C on various kinetic aspects of enzyme activation have also been studied (for review see Zakim and Vessey, 1977).

The effect of Bilirubin

With increasing concentrations of bilirubin in the incubation media, an increase in rat UDP-glucuronyltransferase activity was obtained, the maximal activation having been achieved at 0.4 mM bilirubin (Figure 7.3). In contrast, in the cat, stimulation of enzyme activity was not obtained

at any of the bilirubin concentrations examined. Sanchez and Tephly (1973b) reported that bilirubin enhanced the glucuronidation of morphine and p-nitrophenol in the rat by about 900 and 1,100 % of the control values, the maximal activation having been obtained at 1.0 mM concentration. Commercially obtained bilirubin may be contaminated with bile acids, and the latter are known to be potent activators of UDP-glucuronyltransferase (Leuders and Kuff, 1967; Van Roy and Heirweigh, 1968). The activation by bilirubin due to possible contamination by bile acids has been ruled out in the present studies by thin layer chromatography of bilirubin on silica gel plates (E. Merck, Darmstadt, Germany; Kieselgel 60F 254) in toluene: chloroform: glacial acetic acid (70:20:10) and by detection of the samples by a vanillin-sulphuric acid spray (Sanchez and Tephly, 1973b; Stahl, 1965). This detection method would have determined 1-5µg of conjugated or free bile acids.

The mechanism of activation by bilirubin is as yet not completely understood though Sanchez and Tephly (1973b, 1975) have suggested that bilirubin may be exerting its activating effect by altering somehow the enzyme in the phospholipid environment in the microsomes. Bilirubin, however, appears to differ from the effects of a detergent because it has no capacity for solubilising the enzyme. Therefore, bilirubin has certain characteristics which may be useful in situations when activation is desired without the release of the enzyme from the membrane. This might be the case in vivo where bilirubin has been found to promote the over-all rate of glucuronide excretion in rats treated with morphine and p-nitrophenol. (Sanchez and Tephly, 1973b).

Thus, the cat liver UDP-glucuronyltransferase with 1-naphthol as the substrate is somewhat influenced by Triton X-100 and Phospholipases A and C, though on maximal stimulation by these perturbants, the enzyme activities are still much lower than in the rat. In the Gunn rat various membrane perturbants were found not to stimulate UDP-glucuronyltransferase activity towards p-nitrophenol (Zakim et al., 1973d). Therefore,it was suggested that the Gunn rat, compared with the Wistar rat, had an alteration in interaction between UDP-glucuronyltransferase and its lipid environment. It would appear from the present studies that because similar patterns of microsomal membrane solubilisation with Triton X-100 were obtained in both the rat and the cat, and 1-naphthol does seem to be as much able to penetrate the cat microsomal membrane as in the rat, the low glucuronidating capacity for 1-naphthol in the cat is not due to an abnormal lipid environment of the enzyme but due to a low activity of the enzyme itself.

The Phospholipid and Cholesterol contents and the Fatty Acid Compositions of the Rat and Cat Liver Microsomal Membranes

To further study the nature of the microsomal membrane environments in the rat and the cat, various biochemical analyses have also been carried out. As Table 7.2 shows, the phospholipid to protein ratio is smaller and the phospholipid to cholesterol ratio greater in the cat, indicating that the cat microsomes contain less phospholipid and cholesterol than the rat microsomes. The fatty acids of the microsomal phospholipids from the two species have also been examined (Table 7.3) and the compositions of these are not very different, there being somewhat greater amounts of the unsaturated fatty acids (oleic, linoleic and arachidonic) in the cat. Thus, the ratio of saturated to unsaturated fatty acids in the rat is 2.1, while that in the cat is 1.2.

Phospholipids, besides providing a specific function to membrane bound enzymes such as, for example, providing an ion-specific site or as a cation-specific 'carrier' or 'pore' for the ($Na^+ + K^+$)- ATPase (Kimelberg and Papahadjopoulos, 1972), may have a non-specific function as providing the correct milieu for the enzyme. At physiological temperatures there is considerable motion within the membrane lipid bilayer (Hubbell and McConnell, 1969, 1971). In addition, there is lateral diffusion of lipid molecules (Trauble and Sackmann, 1972; Kornberg and McConnell, 1971) and proteins (Singer and Nicolson, 1972) within the plane of the membrane. The lipid portion of the membrane thus has the properties of a two-dimensional fluid. The fluidity of the membrane lipids is a function of both the fatty acid composition of the lipid chains and the temperature and depends also on the presence or absence of cholesterol. In general, most mammalian membranes are in a fairly fluid state at physiological temperatures as a result of the relatively large proportion of fatty acids containing unsaturated cis-double bonds (e.g. oleic, linoleic and arachidonic acids; Marsh, 1975). The fluidity of the chains would presumably increase the degree of hydrophobic association between the phospholipid and protein and allow freedom for conformational and topographical changes (Kimelberg and Papahadjopoulos, 1972).

The effect of cholesterol on phospholipid monolayers and vesicles is rather complex but in summary it appears to induce an intermediate fluid state (Ladbroke et al., 1968). This means that in bilayers below their transition temperatures, the presence of cholesterol causes them to be more fluid while at temperatures above their transition points, when they are in a crystalline state, the presence of cholesterol makes them less fluid.

In the case of UDP-glucuronyltransferase, the fluidity of the membrane lipids might be one of the properties of the

lipid phase which regulates the conformation of the enzyme. (Zakim and Vessey, 1977). Thus, Zakim and Vessey (1975b) concluded from their phase-transition studies on microsomal UDP-glucuronyltransferase that the specificity of substrate binding at the UDPGA site and the activation by UDP-N-acetyl-glucosamine, which are important physiological functions of the enzyme, are dependent upon the fluidity of the lipid portion of the microsomal membrane. Thus, the transition of lipids from a fluid to a rigid state is associated with a loss of substrate specificity and response to allosteric activation of microsomal UDP-glucuronyltransferase. In view of the present findings in the microsomal phospholipid, cholesterol and fatty acid contents in the rat and the cat, it would appear that the differences observed in their quantities in the two species are not markedly different so as to cause differences in the membrane fluidity and therefore, in the function of UDP-glucuronyltransferase towards 1-naphthol in the cat. Furthermore, such small quantitative differences in phospholipid, cholesterol and fatty acids are likely to be found between two such different species as the rat (a herbivore) and the cat (a carnivore), since they have different dietary habits, and the phospholipid and fatty acid compositions are known to differ, at least in part, according to diet (Dallner et al., 1966; Graham et al., 1974b; Shorland, 1962).

Thus, to summarise, the effects of Triton X-100 and phospholipases A and C were to enhance UDP-glucuronyltransferase activities in the rat as well as the cat to similar extents compared with the respective controls, but since on maximal activation the enzyme activity in the cat was much lower than in the rat, it would appear that the cat liver microsomal preparations are lacking in 1-naphthol glucuronidating activity. Bilirubin, in contrast to its effects in the rat microsomes, did not stimulate enzyme activity in the cat microsomes at any

of the concentrations studied. The native microsomal preparations from the two species seem to be permeable to 1-naphthol to a similar degree, and further as shown by the phospholipid, cholesterol and fatty acid analyses of the microsomes from both species and by the solubilisation of the various microsomal components by Triton X-100, the micro-environments of UDP-glucuronyltransferase do not appear to differ to any marked extent so as to have caused the lower ability of the cat to conjugate 1-naphthol with glucuronic acid.

CHAPTER EIGHT

Summary and Concluding Remarks

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Summary and Concluding Remarks

Considerable species differences in the metabolism of xenobiotics have been found (Williams, 1974). The underlying causes for interspecies variations are complex and may arise from differences in a number of factors (see Chapter 1; Gillette, 1977). However, the differences in pathways or rates of biotransformations are the most important factors, and the reason for these differences is generally likely to be a difference in the relative activities of the enzymes involved in the various pathways of xenobiotic metabolism, though the co-substrate supply may also become rate-limiting. Thus, glutathione conjugation can become impaired when the hepatic concentration of glutathione is reduced (Priestly and Plaa, 1970).

Of the two conjugation mechanisms studied here glucuronidation and sulphation, both of which can often accept the same susbtrate, sulphation is a readily saturable mechanism, being a zero-order reaction very much dependent upon co-substrate supply (Bray et al., 1952c,d; Levy and Matzuzawa, 1967; Bennett et al., 1975). Where two or more pathways of metabolism may be competing for the same substrate, saturation of a particular pathway may lead to a greater use of an alternative one, if this is available. This concept has been illustrated in the present studies by the in vivo conjugation of 1-naphthol at different dose levels in the rat, cat and guinea pig, and that of phenol and 1-naphthol in a few species of primates.

Glucuronidation and Sulphation as Competing Pathways of Conjugation in vivo

Non-primates

In the rat and guinea pig, which utilize both glucuronic acid and sulphate for the conjugation of phenols though in

different proportions, when the dose of 1-naphthol is increased, there appears to be only a slight, if any, increase in the proportion of the xenobiotic conjugated with glucuronic acid (Chapter 3). In the cat, which is known to have a low glucuronidating capacity for a number of phenols (Table 1.17), the sulphate conjugate predominates at all doses of 1-naphthol studied. However, at the highest dose level (25mg/Kg) investigated, a small amount of glucuronide is formed indicating that even in the cat the sulphation mechanism, which appears to have a high activity, is nevertheless saturable.

Primates

The studies in several primate species emphasize the importance of considering both substrate and dose level when making inter-species comparisons in metabolic patterns. There is a clear species difference in the conjugation pattern of 1-naphthol. Thus, with this substrate, the Old World monkeys (rhesus, cynomolgus and patas) and the Prosimians (bushbaby, treeshrew) all utilize sulphation as the major conjugation pathway at the dose levels studied. In the New World species (tamarin, capuchin), however, 1-naphthol is poorly sulphated, glucuronic acid conjugation being the major mechanism even at the lowest dose investigated (Figures 4.2 and 4.4).

In contrast, with phenol, dose is important in considerations of variation between species. Thus, at 10mg/Kg dose, a similar pattern to that of 1-naphthol is obtained (Figure 4.2); sulphate conjugation predominates in the Old World species and Prosimians, and glucuronidation in the New World species. In the capuchin monkey (a New World species), however, the pattern of conjugation of phenol is very much dependent on the dose (Figure 4.3) since sulphation, and not glucuronidation, is the major mechanism at the lowest dose studied (0.01mg/Kg), the reverse being true at 25mg/Kg dose

of phenol. With phenol, therefore, differences in conjugation patterns between the Old and New World species are much less marked at 0.01mg/Kg than at higher dose levels.

Correlation Between in vivo and in vitro studies.

The UDP-glucuronyltransferase and phenolsulphotransferase activities towards 1-naphthol measured in vitro in the rat, cat and guinea pig (Chapter 5) are in agreement with the in vivo patterns of conjugation (Chapter 3). Thus, in the rat liver, both enzymes have similar affinities for 1-naphthol and in the guinea pig, the rate of conversion to 1-naphthylglucuronide is slightly greater than that for sulphation. Conversely, in the cat, the phenolsulphotransferase has a very high affinity for 1-naphthol, this being even greater than in the rat, while the affinity of UDP-glucuronyltransferase for 1-naphthol is very low, approximately 400-fold lower than in the rat (Chapters 5 and 6). In contrast, phenolphthalein is a much better glucuronogenic substrate in the cat, the affinity of the enzyme being only slightly less compared with the corresponding enzyme in the rat. This result is, therefore, in agreement with the findings reported by Capel et al. (1974a) that substantial amounts (60% of 25mg/Kg dose) of phenolphthalein glucuronide are formed in the cat. Hence, the patterns of conjugation observed in vivo in the cat, rat and the guinea pig can be correlated with the associated enzyme activities measured in vitro.

The Glucuronidating Capacity of the Cat and the Heterogeneity of UDP-glucuronyltransferase

Studies have been carried out to gain a greater insight into the low capacity of the cat to conjugate 1-naphthol with glucuronic acid. The bisubstrate kinetic data obtained for the cat liver UDP-glucuronyltransferase with 1-naphthol as the aglycone indicate that the low glucuronidating capacity for

1-naphthol compared with that in the rat is due to a poor affinity of the enzyme for the aglycone and not the co-substrate, UDPGA. In contrast, phenolphthalein is a much better substrate for the cat liver enzyme, this being due to a higher affinity of the enzyme for the aglycone and a slightly greater affinity for UDPGA. Furthermore, studies with UDP-N-acetylglucosamine, an endogenous metabolite which may enhance the affinity of UDP-glucuronyltransferase by a number of mechanisms, support the suggestion that the form of enzyme conjugating 1-naphthol in cat liver is not hampered in any way by co-substrate availability. (Chapter 6).

The activation of UDP-glucuronyltransferase in the rat and cat livers by Triton X-100 and phospholipases A and C suggests that the cat liver enzyme activity towards 1-naphthol is at a very low level. Moreover, as indicated by the solubilisation of the various microsomal membrane components (i.e. protein and phospholipid) by Triton X-100, and by the analyses of phospholipid and cholesterol contents, and the fatty acid compositions of the liver microsomal membranes, the micro-environments of the UDP-glucuronyltransferases in the two species do not differ so markedly so as to have caused the low affinity of the cat enzyme for 1-naphthol.

Therefore, these observations that the 1-naphthol glucuronidating form of the enzyme in the cat liver is not different from that in the rat with respect to its affinity for the co-substrate, UDPGA, or its micro-environment, suggest that the enzyme in the cat has a defective function in binding the aglycone susbtrate. That the cat can conjugate phenolphthalein at a much higher level than 1-naphthol, though at a slightly lower level than phenolphthalein in the rat, suggests that the cat liver UDP-glucuronyltransferase activity towards this substrate is not lacking. Whether the forms of enzymes catalysing 1-naphthol and phenolphthalein in the cat are

different cannot be resolved purely on the basis of kinetic data obtained in the present studies. The differences in the kinetic parameters for these two substrates in the cat could be due to different effects of these two phenols on the membrane environment as well as differences in rates of penetration of these aglycones through the microsomal membrane to the active site of the enzyme. Thus, for example, certain drugs are known to increase the microsomal membrane fluidity and/or alter the membrane conformation and thereby cause differences in rates of metabolism (Mulder, 1974a; Cater et al., 1974). That different aglycones and their conjugation products may have different influences on the membrane environment also makes it difficult to investigate the heterogeneity of the enzyme by means of, for example, competitive inhibition (Mulder and Van Doorn, 1975; Vessey and Zakim, 1974). Therefore, unless different UDP-glucuronyltransferase activities towards these two substrates can be isolated in purified forms, it cannot be ruled out that the change of substrate specificity is caused by alterations of the membrane environment influencing one single enzyme protein.

It is also possible that the form of UDP-glucuronyltransferase which normally conjugates 1-naphthol and phenolphthalein in other species is completely absent in the cat and that these substrates are glucuronidated by other form(s) of UDP-glucuronyltransferase(s) that are present in this species (e.g. the activities towards bilirubin or oestrone; see Lathe and Walker, 1958; Potrepka and Spratt, 1971; Heath and Dingell, 1974). The fact that the chemical structures of these phenols are different from those of the endogenous substrates does not necessarily argue for enzyme polymorphism. As noted by Axelrod et al. (1958), all of these substrates possess a nucleophilic functional group and that the formation of glucuronides is a nucleophilic substitution reaction in which the electron donating aglycone displaces the UDP moiety from the opposite side of the electrophilic anomeric carbon atom of

UDPGA (Figure 1.1). This formulation rationalises the inversion of the α-glycosidic bond of the nucleotide to the β-configuration of the glucuronide; furthermore, one enzyme could catalyse such a reaction. The sequential kinetics determined for bilirubin and p-nitrophenol conjugation (Vessey and Zakim, 1972b; Potrepka and Spratt, 1972) are consistent with such a mechanism. If 1-naphthol and phenolphthalein are glucuronidated by an enzyme normally conjugating an endogenous substrate, then the differences in the relative enzyme activities towards these substrates could perhaps be explained in terms of their different affinities for the active site on the enzyme as well as the variations in their physico-chemical properties and their influences on the membrane-environment of the enzyme.

In comparison with 1-naphthol, phenolphthalein may have more analogous properties to bilirubin. Thus, both phenolphthalein and bilirubin have very high affinities for protein (e.g. albumin), or more generally, for hydrophobic phases, and may, therefore, bind to microsomal membranes (Mulder, 1971; Tomlinson and Yaffe, 1966). One of the properties which may determine the membrane affinity and the glucuronidation rate of phenols is their lipid solubility (Mulder and Van Doorn, 1975). However, when we compare the lipid solubilities of phenolphthalein (log P = +0.56), bilirubin (log P = +0.45), and 1-naphthol (+1.62), as determined by the extraction of these compounds from an aqueous solution into an equal volume of n-octanol at 20^{o}C (see Parker, 1977), the lipid solubility of 1-naphthol is greater than that for phenolphthalein, while those for bilirubin and phenolphthalein are similar. There is evidence that certain drugs increase the fluidity of a lipid region in a membrane (Cater et al., 1974; Piper and Hargreaves, 1975; Mulder, 1974a) and hence, the rate of metabolism of a drug could be determined not only by its lipophilic nature but by its

ability to fluidise the membrane. As we have seen in the present studies (Chapter 7), bilirubin has an activating effect on the rat liver UDP-glucuronyltransferase, and as suggested by Sanchez and Tephly (1973b), this may be an important characteristic of this endogenous substrate that regulates UDP-glucuronyltransferase activity *in vivo*. It is possible that bilirubin, bearing a bulky structure, exerts its effect by increasing the membrane fluidity; phenolphthalein may also have an analogous effect to that of bilirubin. Perhaps the ability of phenolphthalein to alter membrane structure and enzyme conformation may help to explain the greater capacity of the cat to glucuronidate this phenol, or even the observations that this phenol is preferentially glucuronidated in most species examined so far (i.e. rat, dog, rabbit, guinea pig, monkey, sheep; Abou-El-Makarem, 1967). Although 1-naphthol has a greater lipophilicity, its smaller molecular size compared with phenolphthalein may allow the sequestration of this phenol into the microsomal membrane without extensively altering the membrane structure and fluidity.

Consequences of a Readily Saturable Conjugation Mechanism

Unconjugated phenols or quinols might be expected to be excreted by species unable to conjugate effectively with glucuronic acid, where reliance is placed on the more readily saturable sulphation mechanism. This has been shown to occur in the cat with doses of 40mg/Kg of phenol or 2,6-dimethoxyphenol, though not at a lower dose (20 mg/Kg; Miller *et al.*, 1973). The extent of formation of quinols may be important since the production of quinones, which inhibit mitochondrial respiration, from quinols, has been claimed to be responsible for the toxicity of phenols to cats (Miller *et al.*, 1973; see also Table 1.22).

1- and 2-naphthols could be further oxidized to various

isomers of dihydroxynaphthalene. Thus, the cat has been reported as being able to hydroxylate 2-naphthol to a dihydroxynaphthlene, this being excreted as a sulphate conjugate (Capel et al., 1974a). However, in the present studies as with those of Capel et al. (1974a), no such metabolites of 1-naphthol in the cat have been detected, though there is present some unchanged 1-naphthol at the highest dose level investigated (25mg/Kg), suggesting that the sulphation mechanism has become saturated, and the low availability of the glucuronidation pathway cannot cope with this level of the xenobiotic.

All the primate species examined here are able to use the glucuronidation pathway which is a first-order process not readily saturable (Bray et al., 1952a). However, there is a slight suggestion that the New World monkeys may not be able to conjugate phenols as efficiently as the Old World species since the highest levels of quinol metabolites were found in the capuchin and tamarin monkeys (Chapter 4). Oxidation of phenol to quinol may occur to a marked extent when the conjugation of phenol itself is sufficiently slow. Thus, Capel et al. (1972) and Miller et al. (1973) reported appreciable amounts of quinol sulphate as a metabolite of phenols in cats.

Therefore, studies in a species such as the cat indicate lower thresholds for toxicity as a possible consequence of dependence on a readily saturable pathway for detoxication. Such comparative studies further suggest that where two or more reactions may be competing for the same xenobiotics, it is important to know the extent to which one may compensate for deficiency in the other, especially in vivo. This may be important clinically where two drugs which compete for the same reactions are administered simultaneously. For example, interactions between the sympathomimetic, isoprenaline, which

is inactivated by conjugation with sulphate and other drugs (e.g. paracetamol), also similarly metabolised, could occur and be potentially dangerous (Bennett et. al., 1975).

Furthermore, the realisation that certain metabolites can react with endogenous substrates that can readily be depleted, also has important implications in toxicity. For example, depletion of sulphate by acetaminophen decreases the carcinogencity of N-hydroxy-2-acetylaminofluorene because the formation of the chemically reactive N-hydroxysulphate conjugate of the carcinogen is decreased (Miller, 1970 ; Weisburger and Weisburger, 1973). On the other hand, depletion of hepatic glutathione by diethyl maleate increases the covalent binding and toxicity of carbontetrachloride even though the latter does not by itself decrease glutathione levels in liver (Gillette et al., 1974). It is evident from these considerations that nontoxic substances can affect the severity of the injury caused by toxicants even though the nontoxic substance is neither an inducer nor an inhibitor in the usual sense of the word.

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