ASPECTS OF SPECIES DIFFERENCES IN DRUG METABOLISM

by

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TO MY PARENTS

ABSTRACT

This thesis describes the investigations carried out on the metabolism of quinic acid and 3-indolylacetic acid in various species.

The fate of orally administered quinic acid has been examined in 22 species, including man. In man and the Old World monkeys, quinic acid is extensively aromatized (20-67%) and excreted in the urine as hippuric acid. In New World monkeys, lorisformes, the tree shrew, eleven species of lower mammals and one species of aves, the pigeon, the degree of aromatization is small (0-11%).

Treatment with neomycin sulphate before oral administration of quinic acid, or administration of the acid by intraperitoneal injection, considerably reduces the degree of aromatization in Rhesus monkeys.

Shikimic acid, given orally, is excreted as hippuric acid (26-56%) in Rhesus monkeys, but not in rats. If shikimic acid is given to Rhesus monkeys by intraperitoneal injection, no increase in urinary hippurate is observed.

The results indicate that quinic acid and shikimic acid are aromatized by the intestinal flora. The difference between those species which aromatize these polyhydroxycarboxylic acids

to benzoic acid and those that do not, may be related to differences in intestinal flora.

The fate of [^{14}C] quinic acid has been studied in the rat and Rhesus monkey. This has confirmed the low aromatization to benzoic acid in the rat and high aromatization in the Rhesus monkey. Further observations indicate that, on oral administration, quinic acid passes directly into the lower intestine, where it is metabolized.

The role of the intestinal flora in the aromatization reaction has been studied using [^{14}C] quinic acid. It would appear that specific strains of bacteria may be responsible for specific stages in the metabolism of quinic acid.

The metabolism of [^{14}C] indolylacetic acid has been investigated in 17 species of vertebrates, including 8 sub-primates and 9 primates. The amino-acid conjugation mechanism employed shows an interesting species distribution. The 8 sub-primates conjugate indolylacetic acid with glycine, as do the Prosimians. The New World monkeys use both glycine and glutamine conjugation, but in man and the Old World monkeys, only the glutamine conjugate is formed.

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CHAPTER 1

GENERAL INTRODUCTION

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INTRODUCTION

Over a hundred years ago, Claude Bernard stated that the solution of a physiological or pathological problem often depends solely on the appropriate choice of the animal for the experiment. Present day studies on species variations in the metabolism of foreign compounds would surely be an ideal example of his observation.

The number of foreign chemicals being introduced into our environment is continually increasing. These chemicals may be used as contraceptives, cosmetics, detergents, drugs or food additives, in which case they are deliberately brought into contact with the human body; or they may be in the form of pesticides, industrial waste products, automobile fumes etc. when they are contaminating our environment. The screening of drugs and many of these potentially toxic compounds is carried out in common laboratory animals such as rats, mice, cats, dogs and rabbits, and the decision to use these chemicals is often determined by results obtained from studies performed on these species. The problem immediately arises that species variations are found, not only in the metabolism of these chemicals, but also in the toxicological and pharmacological response.

Before considering some of the general trends that are beginning to emerge in the metabolism and in the pharmacological and toxicological responses observed in various species on administration of various foreign compounds, it is pertinent first to consider some of the basic principles of metabolism, and some specific examples where species variations have been observed.

GENERAL PRINCIPLES OF THE METABOLISM OF FOREIGN COMPOUNDS

In general, foreign compounds are metabolized in two phases. During the first phase the compound undergoes asynthetic reactions which may be classified as oxidations, reductions or hydrolyses. In the second phase, the products of the phase I reactions undergo synthetic or conjugation reactions (Williams, 1959). Not all drugs undergo both phases of metabolism. Some may be completely metabolized by a phase I reaction (e.g. ethanol is oxidized to carbon dioxide) whereas others need only conjugation in order that excretion occurs (e.g. benzoic acid is conjugated with glycine).

During each phase of metabolism, the polarity of the foreign compound tends to be increased. Phase II products are usually highly polar and frequently strongly acidic. The toxicity of the foreign compound is thus decreased; partly because

excretion is more rapid due to an increased polarity, and partly because its decreased lipid solubility reduces the likelihood of the compound passing cellular barriers and reaching a potential site of action (Brodie and Hogben, 1957).

Several drugs are known which do not undergo any metabolic changes in the body. Highly polar compounds, whether acidic or basic, are excreted unchanged, e.g. arsanilic acid, hexamethonium salts. However, examples are known of compounds which, although relatively non-polar, are not metabolized. These include such widely different chemicals as barbitone, halothane, dieldrin and cyclopropyl ethers (Williams, 1967a).

Phase I reactions

Many of the metabolic transformations in this category are catalysed by the enzymes of the endoplasmic reticulum (microsomal fraction) of the liver and other tissues. When the tissues are homogenized, the cells are disrupted and the endoplasmic reticulum is fragmented to form small vesicles known as "microsomes", which can be separated by centrifugation. These microsomal enzymes metabolize mainly foreign compounds, although certain "natural" compounds such as steroids and lipids also act as substrates for these enzymes (Mason, North and Vanneste, 1965).

Foreign compounds are also metabolized by non-microsomal enzyme systems. These may be present in the mitochondria (e.g. amine oxidase and enzymes which aromatize cyclohexane-carboxylic acids), in the soluble fraction (e.g. alcohol dehydrogenase, aldehyde oxidase and xanthine oxidase) or in the plasma (e.g. amine oxidases and esterases). These enzymes also take part in "normal" metabolism, hence they have both natural and foreign substrates.

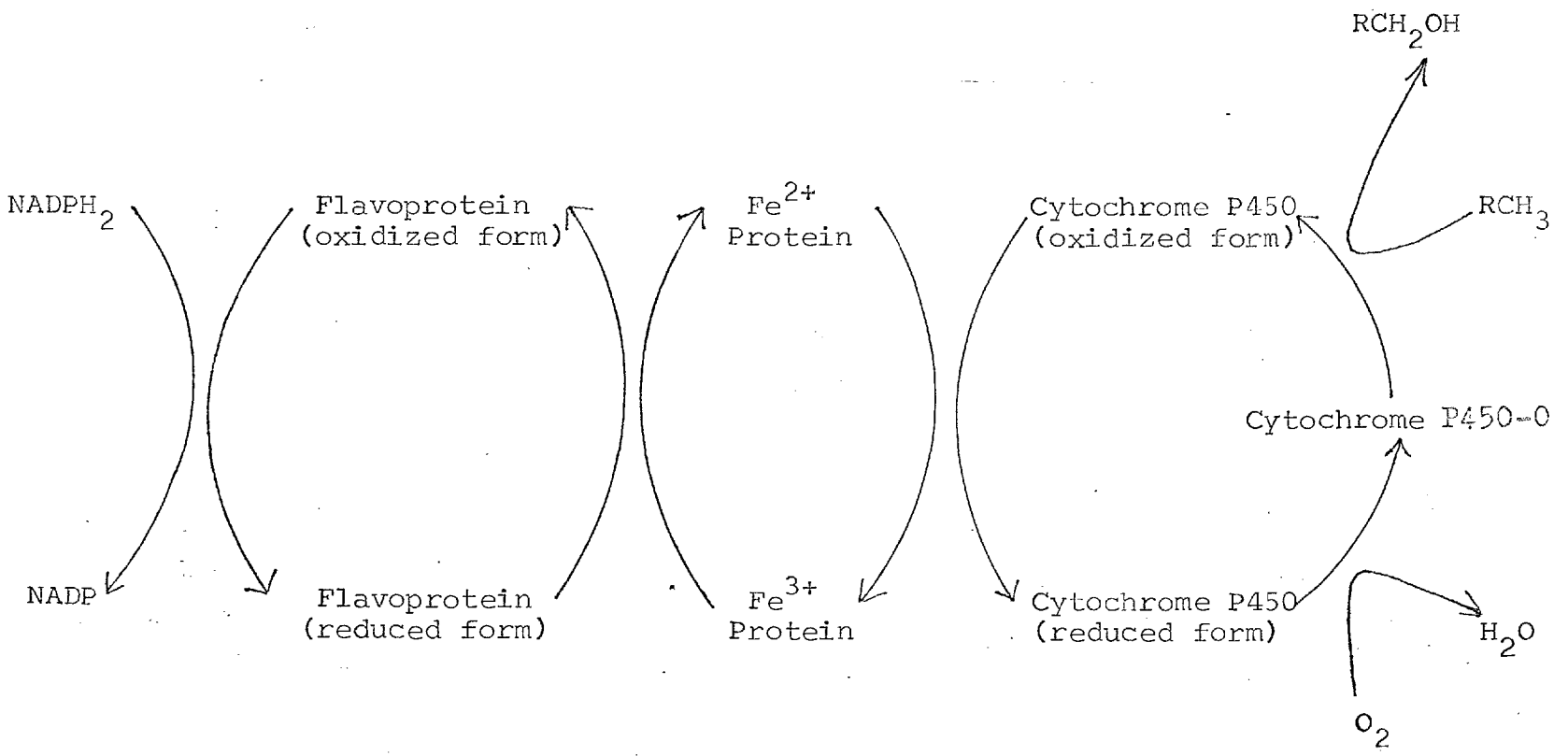
Phase I reactions can be further classified as:-

(a) Oxidation by microsomal enzymes

The microsomal oxidations include a wide variety of reactions all of which appear to follow the common mechanism of hydroxylation. The hepatic microsomal oxidizing system contains $NADPH_2$, a flavoprotein and a non-haeme iron protein, which together probably constitute $NADPH_2$-cytochrome C dehydrogenase, and a haemoprotein known as CO-binding pigment or cytochrome P450 (Omura, Sato, Cooper, Rosenthal and Estabrook, 1965). The electron transport system involved in microsomal hydroxylation has been formulated as shown in Fig. 1.1.

The P450 cytochrome at the end of the electron transport chain combines with oxygen to produce an "active oxygen"

Fig. 1.1. Electron transport system involved in hepatic microsomal hydroxylations*



* Data from Omura et al., 1965

complex. This then reacts with the substrate to form a hydroxylated product. The reactions that can be classified in this group include hydroxylation of aromatic and aliphatic groups, epoxidation, N-hydroxylation of amines, oxidation of nitrogen or sulphur, dealkylation, deamination and desulphuration.

(b) Non-microsomal oxidation

The mitochondria and soluble fractions of tissue homogenates contain many oxidases and dehydrogenases which catalyse oxidative deamination, oxidation of alcohols and aldehydes and the aromatization of alicyclic compounds.

(c) Reduction by microsomal enzymes

The endoplasmic reticulum contains enzymes capable of reducing aromatic nitro- and azo- compounds. Both reductase systems are flavoproteins with FAD as their prosthetic group.

(d) Non-microsomal reduction

Some of the enzyme systems associated with non-microsomal oxidations equally catalyse the reverse reaction. Hence foreign compounds may be reduced, for example, aldehydes and ketones are reduced to alcohols. Other non-microsomal

reductions include the reduction of double bonds, disulphides, sulphoxides and N-oxides, and the reductive dehydroxylation of hydroxamic acids, catechols and certain aliphatic hydroxyl derivatives.

(e) Hydrolysis

Foreign esters and amides are hydrolysed by hydrolytic enzymes occurring in the plasma and the liver. Some of these have been shown to be present in the liver microsomal fraction.

(f) Miscellaneous reactions

Many other transformations occur (dehydroxylation of phenolic acids, dehalogenation, ring scission, ring formation, reduction of unsaturated compounds etc.), but, at present, insufficient data is available to classify them in one of the above groups. However it should be pointed out that the intestinal microflora are capable of carrying out a wide variety of chemical conversions. These are considered in Chapter 4.

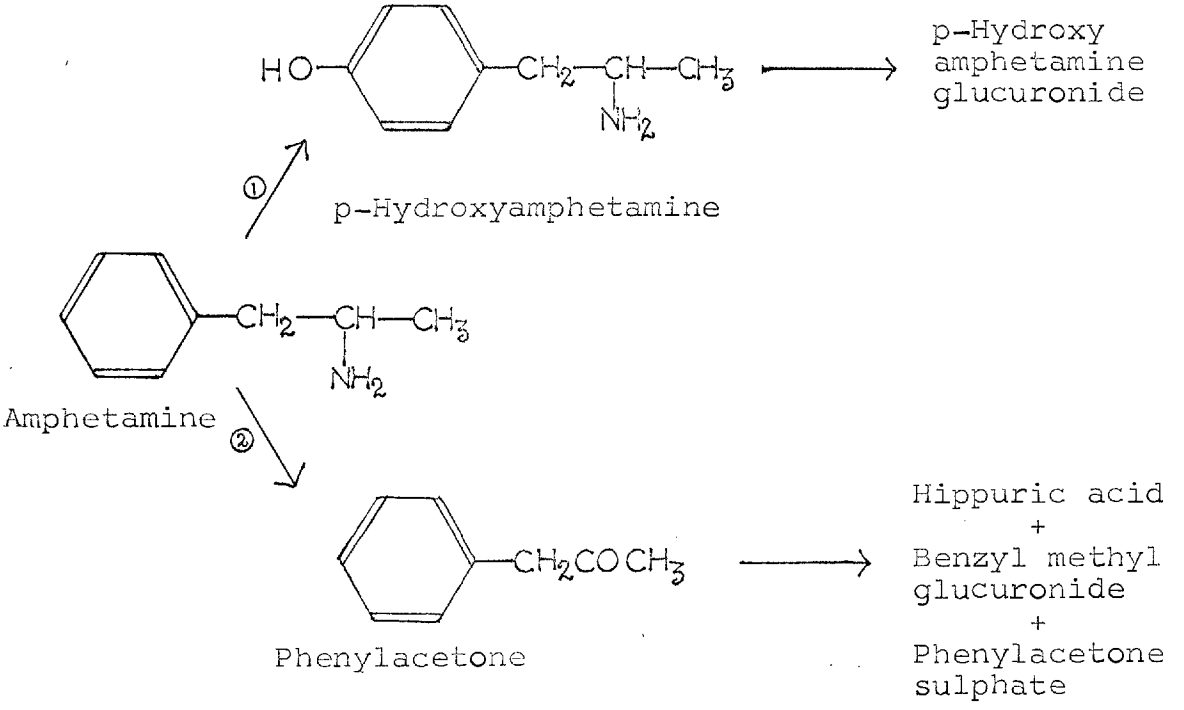
Examples will now be presented of some of these reactions where species variations have been observed.

(a) Oxidation

(i) Aromatic hydroxylation

The drug amphetamine can undergo three possible metabolic

changes, viz. aromatic hydroxylation, oxidative deamination or β-hydroxylation in the side chain (Smith and Dring, 1970). Little is known about the β-hydroxylation, although this reaction may be of considerable pharmacological significance.



(1) is the major route in the rat

(2) is the major route in other species examined.

Amphetamine is incompletely metabolized by man, Rhesus monkey, greyhound and mouse, which excrete 29-42% of the dose

unchanged. In these species the main metabolic products are those resulting from oxidative deamination of the side chain. In the rabbit, guinea pig and rat, amphetamine is more completely metabolized. The main metabolites in the rabbit and guinea pig are again those resulting from oxidative deamination, whereas in the rat, the main metabolite is p-hydroxyamphetamine (Table 1.1).

The variation in the extent of hydroxylation by different species may be due to

(1) the amount of active enzyme (amphetamine p-hydroxylase) varying from one species to another

(2) the occurrence of a competitive deaminizing reaction (hydroxylation in the rat may be high because deamination in this species is low), or

(3) the presence of an inhibitor in certain species but not in others.

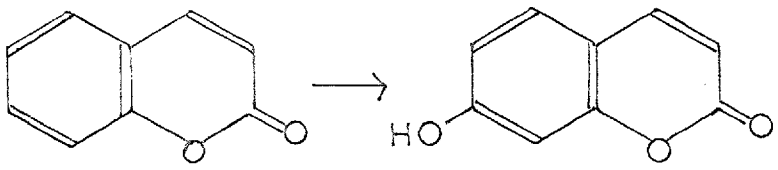
The liver microsomes of the dog and rat contain a heat-labile inhibitor of the deamination of amphetamine. When these microsomes are added to those of rabbit liver (which can deaminize amphetamine) then deamination is inhibited (Axelrod, 1955).

Table 1.1. Species variations in the metabolism of amphetamine*

Species	Percentage of dose excreted in the urine as:-		
	unchanged amphetamine	p-hydroxy amphetamine	products of oxidative deamination
Man	34	3	24
Rhesus monkey	29-42	2-6	34
Greyhound	30	2-6	31
Mouse	29-42	14	32
Rat	14	60	2
Rabbit	4	6	53
Guinea pig	18	0	62

* Data from Smith and Dring, 1970

Another interesting example of aromatic oxidation is the hydroxylation of coumarin to 7-hydroxycoumarin.



Coumarin

7-Hydroxycoumarin

Table 1.2 shows the species variations in the coumarin hydroxylating activity of the liver microsomal enzymes. The absence of coumarin 7-hydroxylating activity from rat liver is due to both lack of the enzyme, coumarin-7-hydroxylase, and to the presence of an inhibitor (Creaven, Parke and Williams, 1965a).

Incubations of coumarin with the liver microsomes of two strains of guinea pig and several strains of rabbit (both sexes) showed that this reaction, as well as exhibiting marked species variations, also shows strain and sex differences (See Table 1.3).

(ii) Aliphatic oxidation

Hexobarbital is a short-acting barbiturate which is inactivated by hydroxylation of the cyclohexenyl group, N-demethylation and scission of the barbiturate ring.

The hydroxylation of hexobarbital to 3'-hydroxyhexobarbital

Table 1.2. 7-Hydroxylation of coumarin by the liver microsomal enzyme, showing species variations in activity*

	Coumarin-7-hydroxylase activity (μM/g. liver/hr.)
Coypu	1.32
Cat	0.30
Hen	0.20
Pigeon	0.12
Frog	0.10
Rat	0
Mouse	0
Ferret	0

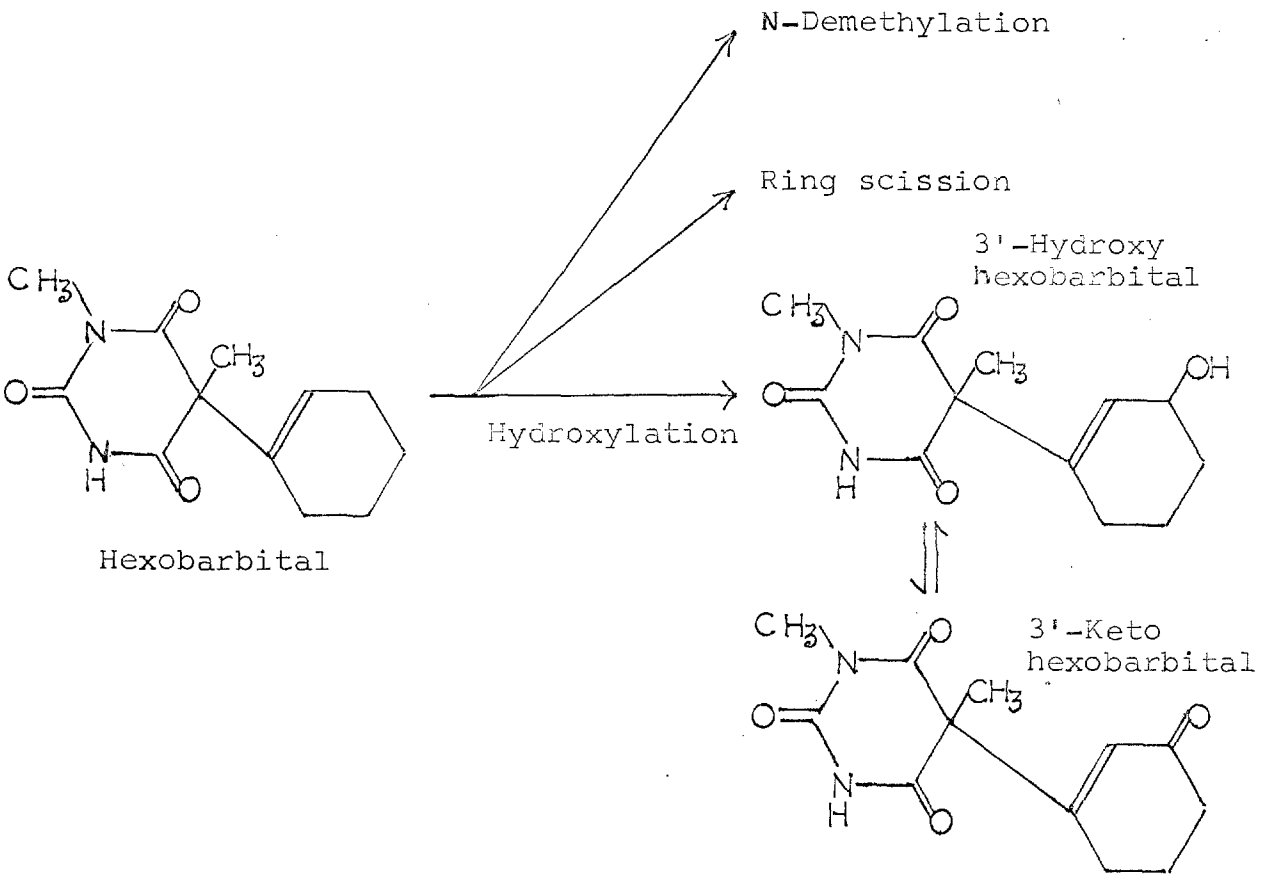
* Data from Williams (1963)

Table 1.3. 7-Hydroxylation of coumarin by the liver microsomal enzyme, showing strain and sex differences in activity*

Species	Strain	Sex	Coumarin-7-hydroxylase activity (μM/g. liver/hr.)
Guinea pig	Coloured	?	0.45
"	Albino	?	0.07
Rabbit	English	F	1.4
"	"	M	0.5
"	Chinchilla	F	0.9
"	"	M	0.5
"	Copenhagen white	F	0.15
"	Dutch	F	0.1
"	Wild	F	0.08
"	Chinchilla X Albino	F	0.54

* Data from Williams (1963)

is brought about by an $NADPH_2$-dependent, microsomal enzyme system of the liver; whereas the subsequent reversible oxidation to 3'-ketohexobarbital is carried out by a soluble liver enzyme.



The duration of action of hexobarbital varies from one species to another (Table 1.4).

Table 1.4. Species differences in the metabolism of hexobarbital

Species	Dose (mg/kg.)	Sleeping time (mins)	Serum level of hexobarbital on awakening (μg/ml.)
Man[1]			20
Dog[1]	50	315	19
Rat[1]	100	90	64
Mouse[1]	100	12	89
Rabbit[1]	100	49	57
Squirrel monkey[2]	50	136	15
Rhesus monkey[2]	50	63	27

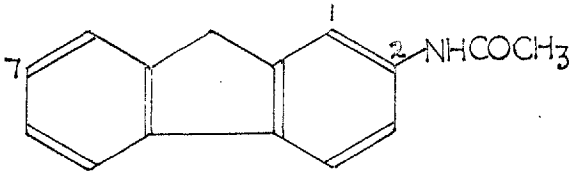
1 Data from Quinn, Axelrod and Brodie (1958)

2 Data from Mitoma and Scholler (1967)

Mice, in general, metabolize drugs more rapidly than do other animals (Quinn, Axelrod and Brodie, 1958). This is reflected in the much shorter sleeping-time observed for this species than for the other species examined. The sensitivity of the responding tissue may also be a factor in species differences. Thus the central nervous system of dog, squirrel monkey, Rhesus monkey and man is far more sensitive to the action of hexobarbital than that of the lower mammals studied, viz. rat, mouse and rabbit.

(iii) N-Hydroxylation of amines

Certain carcinogenic chemicals may not be effective by themselves, but are converted by metabolism into the biologically active material. The capacity to do this varies from one species to another. Thus 2-acetylaminofluorene (2AAF) is not carcinogenic to the guinea pig because it is hydroxylated at carbon 7 and not at the nitrogen atom.



2-Acetylaminofluorene

In the rat and other species where 2AAF is carcinogenic, metabolism involves hydroxylation on other carbons also, and

to a significant extent on the nitrogen atom. The latter reaction produces the carcinogen N-hydroxy-2-acetylaminofluorene (N-OH-AAF) (Weisburger, Grantham, Vanhorn, Steigbigel, Rall and Weisburger, 1964). Administration of N-hydroxy-2-acetylaminofluorene to the guinea pig causes adenocarcinomas of the small intestine if given orally, and sarcomas if injected (Miller, Miller and Enomoto, 1964).

The enzymic N-hydroxylation of 2-acetylaminofluorene has been shown to occur in the liver microsomes of rabbit, hamster, dog, cat, chicken, rat and mouse (Irving, 1964). Enzymic formation of N-hydroxy-2-acetylaminofluorene by guinea pig or human liver microsomes could not be shown, although patients with malignant disease do form the N-hydroxy metabolite (see Table 1.5).

(iv) Oxidative dealkylation

The enzyme system for the dealkylation of monomethyl-4-aminoantipyrine has been located in the liver microsomes of rabbit, rat and guinea pig (La Du, Gaudette, Trousof and Brodie, 1955).

Chickens, pigeons, alligators and tortoises were found to have a similar enzyme system for this conversion. But 4 species of fish, several frogs and salamanders were unable to

Table 1.5. Hydroxylation of 2-acetylaminofluorene in various species*

Species	Carcinogenicity	% of dose as:-	
		N-Hydroxy AAF	7-Hydroxy AAF
Man[1]	?	4-14	25-30
Monkey	?	1.8-2.7	9-18
Cat	+	1.5	11
Dog	+	5.2	0.9
Rabbit	+	13-30	15-29
Hamster	+	15-20	35-39
Mouse	+	1.8-2.3	16-20
Rat	+	0.3-15	19-27
Steppe lemming	-	trace	42
Guinea pig	-	0	72

1 Five patients with various malignant diseases.

* Data from Weisburger, Grantham, Vanhorn, Steigbigel, Rall and Weisburger (1964).

oxidise monomethyl-4-aminoantipyrine. The compound was excreted unchanged, presumably through the gills of the fish and the skin of the frogs and salamanders (Gaudette, Maickel and Brodie, 1958). Toads, unlike frogs, have skins which are not permeable to drugs, and these amphibia metabolize monomethyl-4-aminoantipyrine to 4-hydroxyantipyrine; the oxidative system for this conversion is located in the soluble fraction of liver homogenates (Brodie and Maickel, 1962). It has been suggested that fish and amphibia can dispose of lipid-soluble organic compounds by passive diffusion through the skin. Toads, a terrestrial off-shoot of aquatic amphibia, have lost this ability. Hence administered compounds must be converted into excretable products. However, it appears that the oxidative system in the toad is different from that found in reptiles, birds and mammals.

More recently several workers have shown that foreign compounds can be metabolized by the livers of both amphibia and fish, e.g.

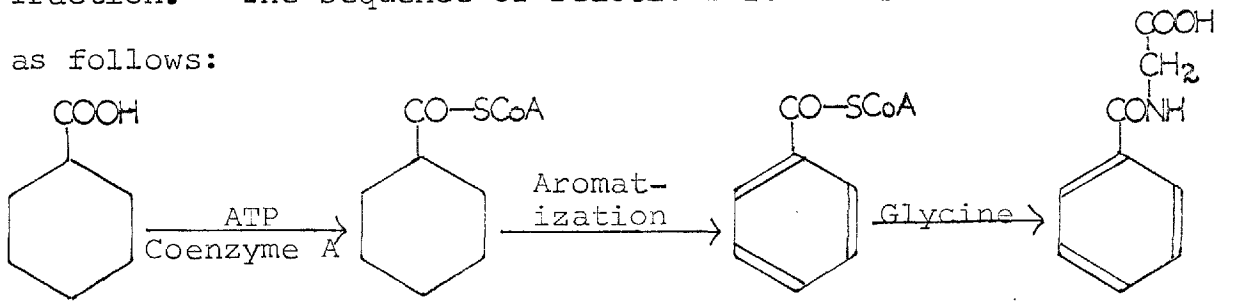
(1) 4-hydroxylation of biphenyl is brought about by liver preparations of 11 species including the frog and trout (Creaven, Parke and William, 1965b).

(2) Buhler (1966) has demonstrated hepatic hydroxylation of aniline, N-dealkylation of aminopyrine and reduction of nitrobenzoic acid in 9 strains of fish.

Optimal enzyme conditions in mammals (temperature, pH, substrate concentrations, activators, inhibitors) are probably not optimal in marine vertebrates (Adamson, 1967). Thus future studies of drug metabolism in fish, carried out under the correct conditions, may clarify firstly, whether foreign compounds are metabolized by all species of fish and secondly, whether gill clearance and skin diffusion of lipid-soluble foreign compounds play a significant role in excretion by these species.

(b) Non-microsomal oxidation

The aromatization of cyclohexanecarboxylic acid to hippuric acid is brought about by an enzyme system localised in the mitochondrial fraction of guinea pig liver homogenate (Mitoma, Posner and Leonard, 1958). The mitochondria alone, however, are inactive unless fortified by the boiled supernatant fraction. The sequence of reactions for this conversion is as follows:



Cyclohexane-carboxylic acid

Hexahydro-benzoyl-Coenzyme A

Benzoyl-Coenzyme A

Hippuric acid

Guinea pig and rabbit liver mitochondria show comparable aromatizing activity, a very weak activity is detected with rat liver mitochondria, and the mitochondria from cat, mouse, dog, monkey, toad, human, chicken and pigeon livers do not oxidize cyclohexanecarboxylic acid. Mitoma et al. suggest that the failure to demonstrate the oxidation of cyclohexanecarboxylic acid with human and dog liver preparations, in spite of positive in vivo findings by other workers, points to the lability of this enzyme system.

(c) Reduction

The ability of enzymes in the liver of fish, amphibia, reptiles, birds and mammals to reduce nitro- and azo-compounds has been compared by Adamson, Dixon, Francis and Rall (1965). The metabolic pathways studied were the reduction of the aromatic nitro-group of p-nitrobenzoic acid, and reductive cleavage of the azo-linkage of neoprontosil. Incubations were carried out at 37°C and/or the environmental temperature of the animal studied (Table 1.6).

The fish, amphibia, reptiles, birds and mammals all possessed hepatic azo-reductase activity, although the activity was much higher in mammals and the pigeon than in other species. Nitro-reductase could not be found in amphibia or the

Table 1.6. Azo- and nitro-reductase activity in the liver of various species*

Species	Temperature °C	Azo-reductase (μmoles of sulphanilamide formed/g. liver/hr.)	Nitro-reductase (μmoles of p-amino benzoic acid formed/g. liver/hr.)
FISH			
Lemon shark) Elasmobranch	26	0.61	0
Sting ray) Elasmobranch	26	0.76	0
Barracuda) Teleost	26	0.76	0.49
Yellow tailsnapper) Teleost	26	0.78	0.66
AMPHIBIA			
Frog (R. pipiens)	21	0	0
	37	1.34	0
Frog (R. catesbeiana)	21	0.58	0
	37	1.18	0
Toad	26	0.68	0
	37	1.60	0
REPTILE			
Turtle	21	0.50	2.52
	37	1.44	0.13
Horned Toad	37	4.48	0.44
BIRD			
Pigeon	37	7.05	1.08
MAMMAL			
Mouse	37	6.80	2.72
Rat	37	5.88	2.08
Guinea Pig	37	8.97	2.04

* Data from Adamson, Dixon, Francis and Rall (1965)

elasmobranch fishes. Buhler (1966), however, found nitro-reductase activity in the livers of 9 types of fish, as well as in the more primitive lamprey eel and sturgeon.

Studies on mammalian nitro-reductase and azo-reductase have shown that the enzyme systems involved are located predominantly in the soluble fraction of the cells, but also in the microsomes (Fouts and Brodie, 1957; Fouts, Kamm and Brodie, 1957). The livers from guinea pig and mouse show greater activity than those of rabbit and rat for both the reduction of p-nitrobenzoic acid to p-aminobenzoic acid and the reduction of prontosil to sulphanilamide (Table 1.7).

(d) Hydrolysis

Many esterases are found in all organisms, and hydrolysis of ester links in foreign compounds is a common detoxication mechanism. Prediction of the extent of hydrolysis of a particular foreign compound by a given species is difficult, but it has been found that introduction of an ester link into a toxicant often enables a useful selective action to be obtained, e.g. in the organophosphate insecticide malathion which is toxic to many insects, but virtually non-toxic to all mammals (O'Brien, 1965).

^{32}P-Malathion is rapidly metabolized in mammals, principally by hydrolysis of ester bonds, to give malathion monoester

Table 1.7. Enzyme activity of nitro-reductase and azo-reductase in various mammals*1

Species	Relative activity of nitro-reductase	Relative activity of azo-reductase
Guinea pig	100 *2	100 *3
Mouse	103	82
Rabbit	33	59
Rat	11	32

*1 Data from Fouts and Brodie (1957); Fouts, Kamm and Brodie (1957).

*2 Activity of 100 is equivalent to the formation of 1.53 µmoles of p-amino benzoic acid

*3 Activity of 100 is equivalent to the formation of 4.01 µmoles of sulphanilamide

and malathion diacid (Fig. 1.2). In insects, oxidation to malaoxon or cleavage of the phosphate thioester bond to give O,O-dimethylphosphorodithionate and O,O-dimethylphosphorothionate are the principal routes (Table 1.8). The toxicity of malathion in insects is probably due to its oxidation to malaoxon which is about 1000 times more active than malathion as an anticholinesterase.

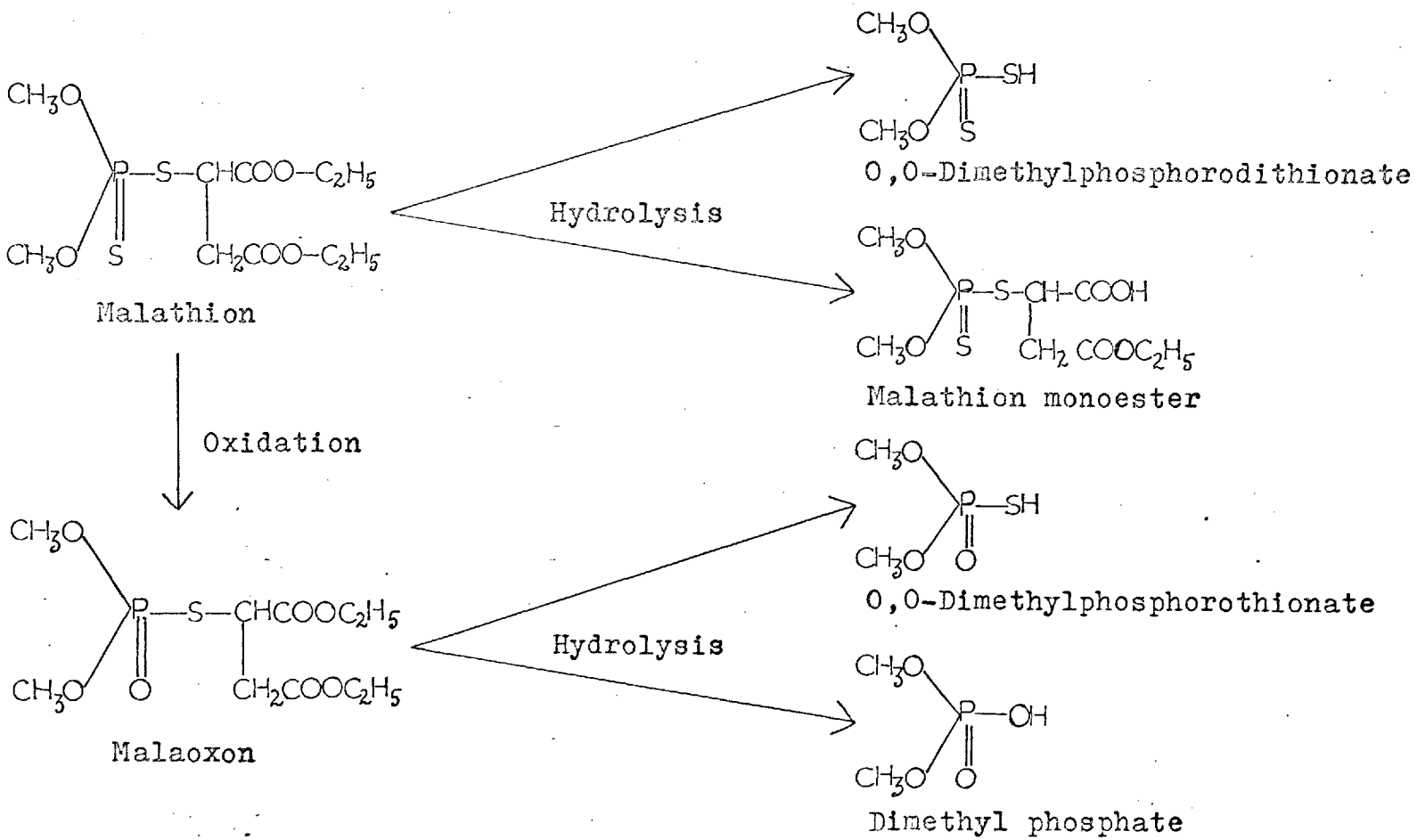
Table 1.8. Malathion hydrolysis in various species*

Species	% Hydrolysis via carboxyesterase	% Hydrolysis via phosphatase
Cow	80	20
Dog	67	30
Rat	59	24
Mouse	68	21
Housefly	12	67
American cockroach	44	50
German cockroach	36	47

* Data from O'Brien (1965)

Fig. 1.2.

Pathways of malathion metabolism



Phase II reactions

The biosynthetic reactions between a foreign compound (or its phase I metabolite) and a substance provided by the body (conjugating agent) are termed phase II reactions. These syntheses are catalysed by specific enzymes which require certain activated nucleotides for their activity. The intermediate nucleotide may contain either the conjugating agent or the foreign compound or its phase I metabolite. These two types of conjugation reactions may be represented as:-

(1) Conjugating agent $\xrightarrow{\text{energy source}}$ Activated conjugating agent $\xrightarrow[\text{transferase}]{\text{foreign compound} +}$ Conjugated product

(2) Foreign compound $\xrightarrow{\text{energy source}}$ Activated foreign compound $\xrightarrow[\text{transferase}]{\text{conjugating agent} +}$ Conjugated product

The first class of detoxication mechanisms includes the formation of glucuronides and ethereal sulphates, acetylation and methylation. The appropriate transferase enzymes for these reactions are located in the microsomes or supernatant fraction of liver homogenates.

Peptide conjugations with glycine, glutamine and ornithine come under the second class of detoxication mechanisms (Williams, 1969). In these cases, the appropriate transferase enzyme is associated with the mitochondria.

The most commonly occurring conjugation reactions are glucuronide formation, glycine conjugation, mercapturic acid synthesis, methylation, acetylation and ethereal sulphate synthesis. These will now be considered in some detail, with particular reference to any species variations from the general trend (Table 1.9).

Table 1.9. Common conjugation reactions and species where conjugation is defective or replaced

Foreign Compound	Conjugation commonly used	Exceptional species	
		a where conjugation is at a low level or absent	b where an alternative conjugation mechanism is used
Phenol	Glucuronide synthesis	Cat	Insects (glucose)
Benzoic acid	Glycine conjugation		Ticks, spiders, scorpions (arginine). Hen, turkey, goose, duck (ornithine).
3-Indolylacetic acid	Glycine conjugation		Man, Old World monkeys (glutamine)
Benzyl chloride	Mercapturic acid synthesis	Guinea pig, hen, duck and man	
p-Aminobenzoic acid	Acetylation	Dog	
Isobarbituric acid	Ethereal sulphate formation	Pig	

Glucuronide formation

Conjugation with glucuronic acid is widespread among species, occurring in mammals (Teague, 1954), marsupials (Hinks and Bolliger, 1957), birds (Baldwin, Robinson and Williams, 1960), reptiles and amphibia (Smith, 1964). In insects, the mechanism appears to be replaced by β-glucoside formation. This is a characteristic insect detoxication and probably occurs in other invertebrates as well (Smith, 1968). Glucuronide formation can occur with compounds possessing -OH, -COOH, $-NH_2$ and -SH groups. The mechanism of glucuronide synthesis can be described as:

1) α-Glucose-1-phosphate + UTP $\xrightarrow{\text{Uridyl transferase}}$ UDP glucose + pyrophosphate

2) UDP glucose $\xrightarrow[\text{DPN}]{\text{UDP glucose dehydrogenase}}$ UDP glucuronic acid

3) UDP glucuronic acid + Aglycone $\xrightarrow{\text{UDP transglucuronylase}}$ Aglycone + UDP glucuronide

Reaction 3 occurs whether the aglycones are endogenous (certain steroids, thyroxine and bilirubin) or foreign compounds (e.g. morphine and codeine). In all cases, the enzyme UDP transglucuronylase is in, or associated with the microsomes (Dutton, 1961). The domestic cat yields negligible amounts of

glucuronides when dosed with a variety of glucuronidogenic compounds (Robinson and Williams, 1958). This has been attributed to a deficiency of the enzyme glucuronyltransferase, and not to a lack of formation of the activated conjugating agent UDPGA. However, cats do form bilirubin glucuronide. Thus it seems possible that the glucuronyl transferase may differ from one substrate to another.

Glycine conjugation

Glycine conjugation has a wide zoological distribution and has been found in most species including mammals (Williams, 1967b), Columbiforme birds (Baldwin, Robinson and Williams, 1960), reptiles (Smith, 1958) and insects (Friedler and Smith, 1954).

Conjugation with glycine is potentially a reaction of all carboxylic acids. The following acids have been observed to undergo conjugation with glycine:

(a) aromatic acids (carboxylic and heterocyclic)
(b) substituted acetic acids (where one substituent is aromatic)
(c) 3-arylpropionic acid
(d) β-substituted acids
and (e) certain steroid acids.

In order that glycine conjugation occurs, the foreign compound (acid) must be activated; i.e. a class 2 mechanism is involved. For example, the synthesis of hippuric acid occurs in three steps:-

1) Benzoic acid + ATP $\longrightarrow$ Benzoyl-adenylate + pyrophosphate

2) Benzoyl-adenylate + CoA-SH $\longrightarrow$ Benzoyl-CoA + adenylic acid

3) Benzoyl-CoA + Glycine $\longrightarrow$ Hippuric acid + CoA-SH

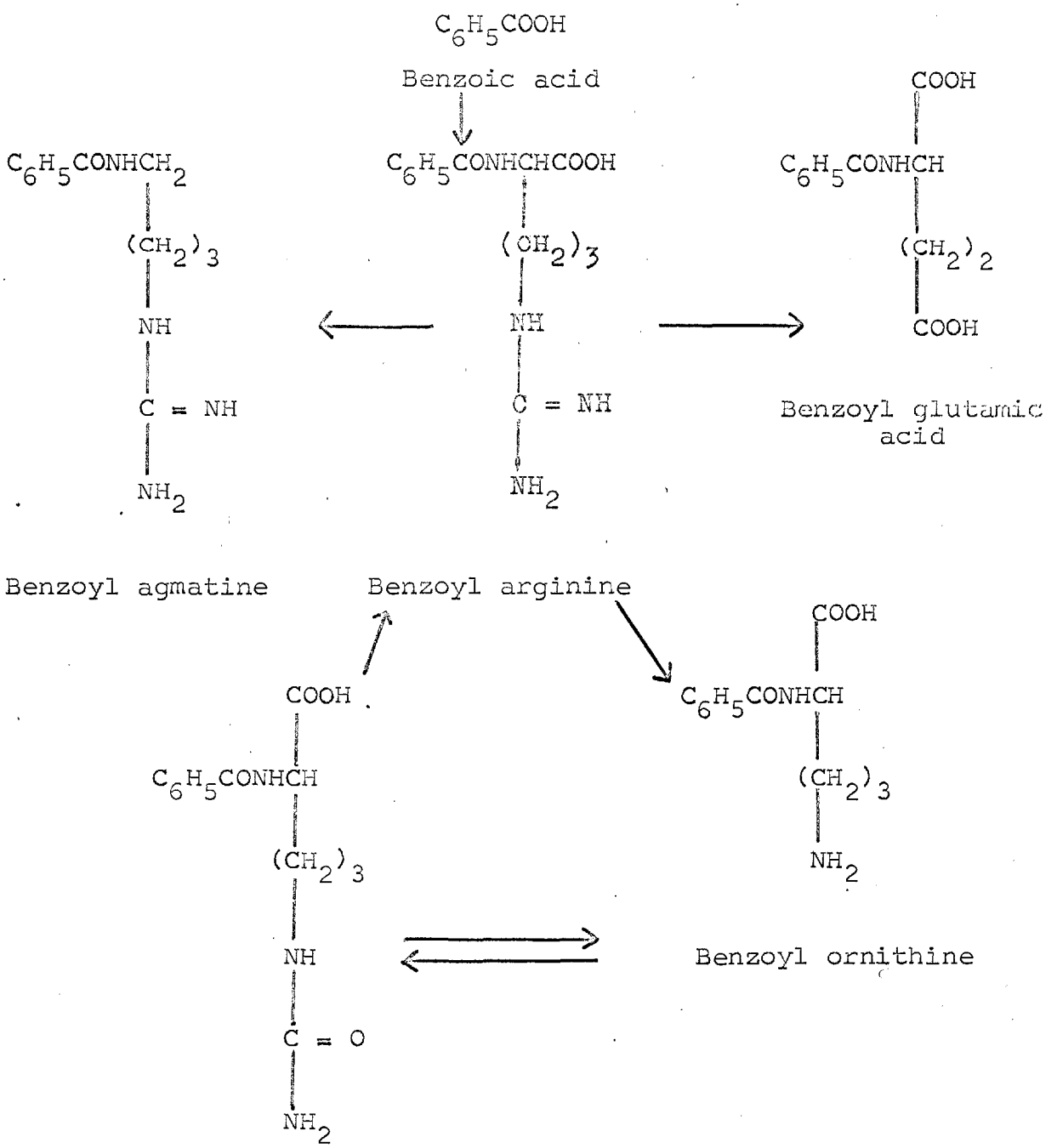
The first two steps result in the activation of benzoic acid. The active benzoyl-CoA is then conjugated with glycine, the reaction being catalysed by the enzyme glycine-N-acylase. This process takes place in the mitochondria (Schachter and Taggart, 1953).

In certain species, this glycine conjugation is replaced by an alternative pathway for specific compounds. Thus, the arylacetic acids (3-indolylacetic acid, phenylacetic acid and 3,4-dihydroxy-5-methoxyphenylacetic acid) are excreted as conjugates of glutamine in man and the Old World Monkeys (see Chapter 5).

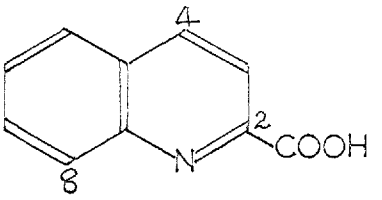
Birds classed as Galliformes (hens, turkeys) and

Anseriformes (ducks and geese) excrete aromatic acids and arylacetic acids as ornithine conjugates. Ornithine conjugation is not characteristic of all birds since the pigeon and dove (Columbiformes) excrete aromatic acids as glycine conjugates (Baldwin, Robinson and Williams, 1960). Reptiles, which have a close phylogenetic relationship to birds, are also capable of conjugation with ornithine, though, in the species examined, glycine conjugation is also found. Thus, urine from tortoises dosed with aromatic acids was found to contain both ornithine and glycine conjugates; similar results were found with a grass snake, two species of lizard and an alligator (Smith, 1964).

Extensive conjugation of aromatic acids is found in Arachnids. Although the excreta may contain conjugates with arginine, glutamine, glutamic acid, citrulline, ornithine and agmatine, it is thought that, with the exception of the glutamine conjugate, all these are derived from the original arginine conjugate (Hitchcock and Smith, 1966). (See below for metabolism of benzoic acid in Arachnids.)


C6H5COOH
Benzoic acid
C6H5CONHCH2
(CH2)3
NH
C = NH
NH2
Benzoyl agmatine
C6H5CONHCHCOOH
(CH2)3
NH
C = NH
NH2
Benzoyl arginine
COOH
C6H5CONHCH
(CH2)2
COOH
Benzoyl glutamic acid
COOH
C6H5CONHCH
(CH2)3
NH2
Benzoyl ornithine
COOH
C6H5CONHCH
(CH2)3
NH
C = O
NH2
Benzoyl citrulline

The aromatic acid quinoline-2-carboxylic acid, and certain hydroxylated derivatives of this acid, give rise to very rare conjugation reactions in certain species. In the rat, as might be expected, the acid is conjugated with glycine. In the cat, however, a major metabolite of this acid is quinaldylglycyltaurine, and a lesser metabolite is quinaldylglycylglycine (Kaihara and Price, 1965).

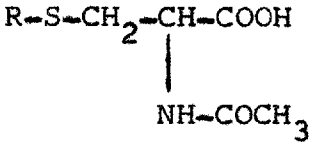


Quinoline-2-carboxylic acid

The cat is also known to form the glycyltaurine conjugate of 4-hydroxyquinoline-2-carboxylic acid. The dihydroxylated derivative, 4,8-dihydroxyquinoline-2-carboxylic acid, is excreted in the rat as two glucuronides (8-monoglucuronide and 4,8-diglucuronide) each with a serine residue attached to the 2-carboxyl group; whereas in the rabbit, the hydroxyl groups are sulphated, but again, a serine residue is attached to the 2-carboxyl group (Rothstein and Greenberg, 1957).

Mercapturic acid synthesis

Mercapturic acid synthesis occurs readily in the rat, rabbit, dog, cat and pig, but is at a very low level in man, guinea pig, duck and hen (Williams, 1964). The foreign compound RX (where R = aryl or alkyl group) will form a mercapturic acid with the general formula



Four kinds of group X are known to be replaced by the acetylcysteyl residue, namely, hydrogen, halogen, nitro and methane-sulphonoxy (CH_3-SO_3-).

From the point of view of mechanism, there appear to be two types of mercapturic acid formation. The first when the acetylcysteyl residue replaces a halogen or nitro group, e.g. in bromopropane, benzylchloride, nitrobutane. These mercapturic acids are synthesized in four steps, viz.

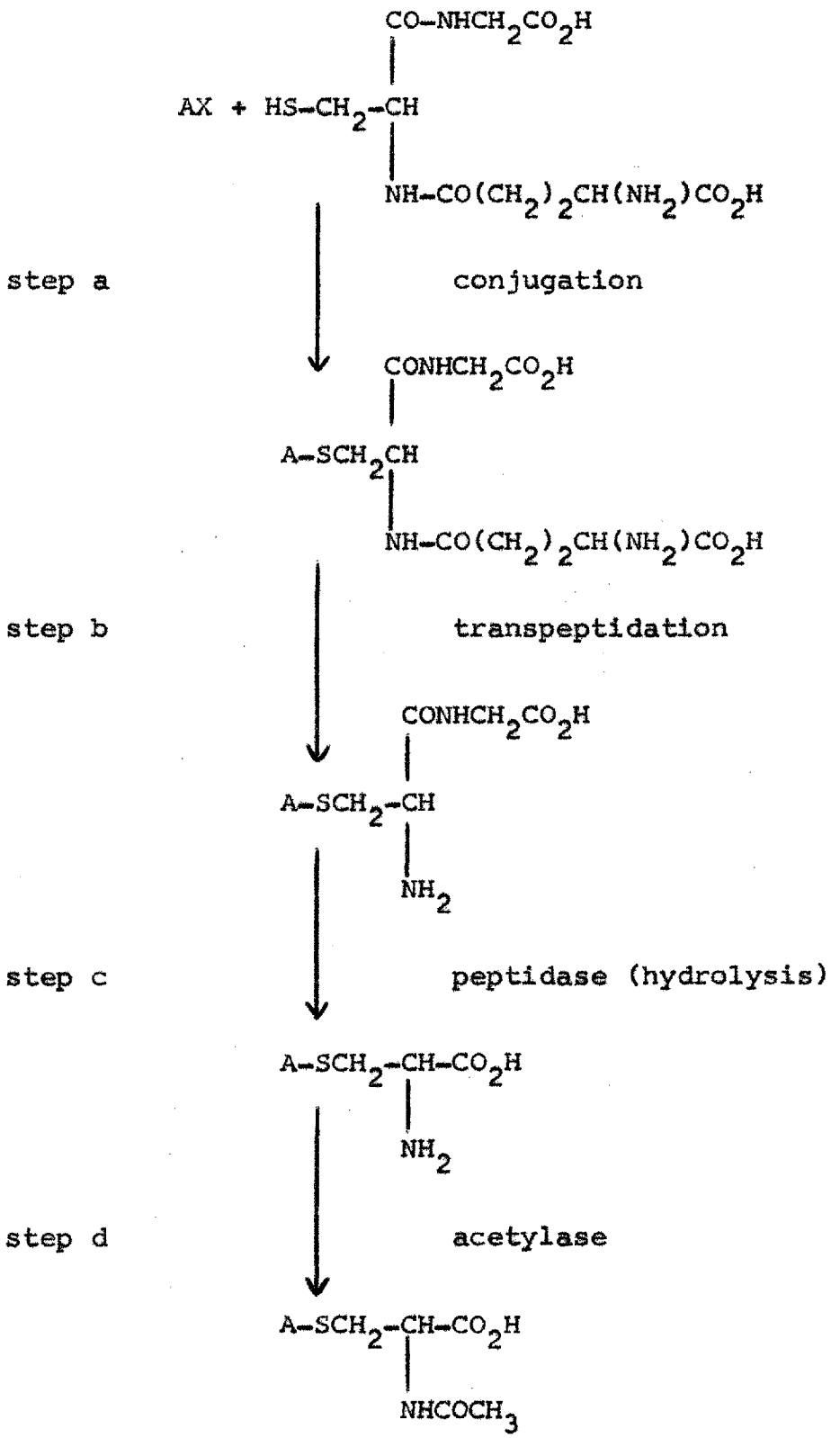
(a) conjugation of the foreign compound with glutathione;

(b) removal of the glutamyl residue from the conjugate, by transpeptidation, producing an S-cysteinyl glycine conjugate;

(c) hydrolysis by peptidases to give an -S-cysteine conjugate;

(d) N-acetylation of the S-substituted cysteine to a mercapturic acid.

Thus, if X is a halogen atom or a nitrogroup in the compound AX, then:

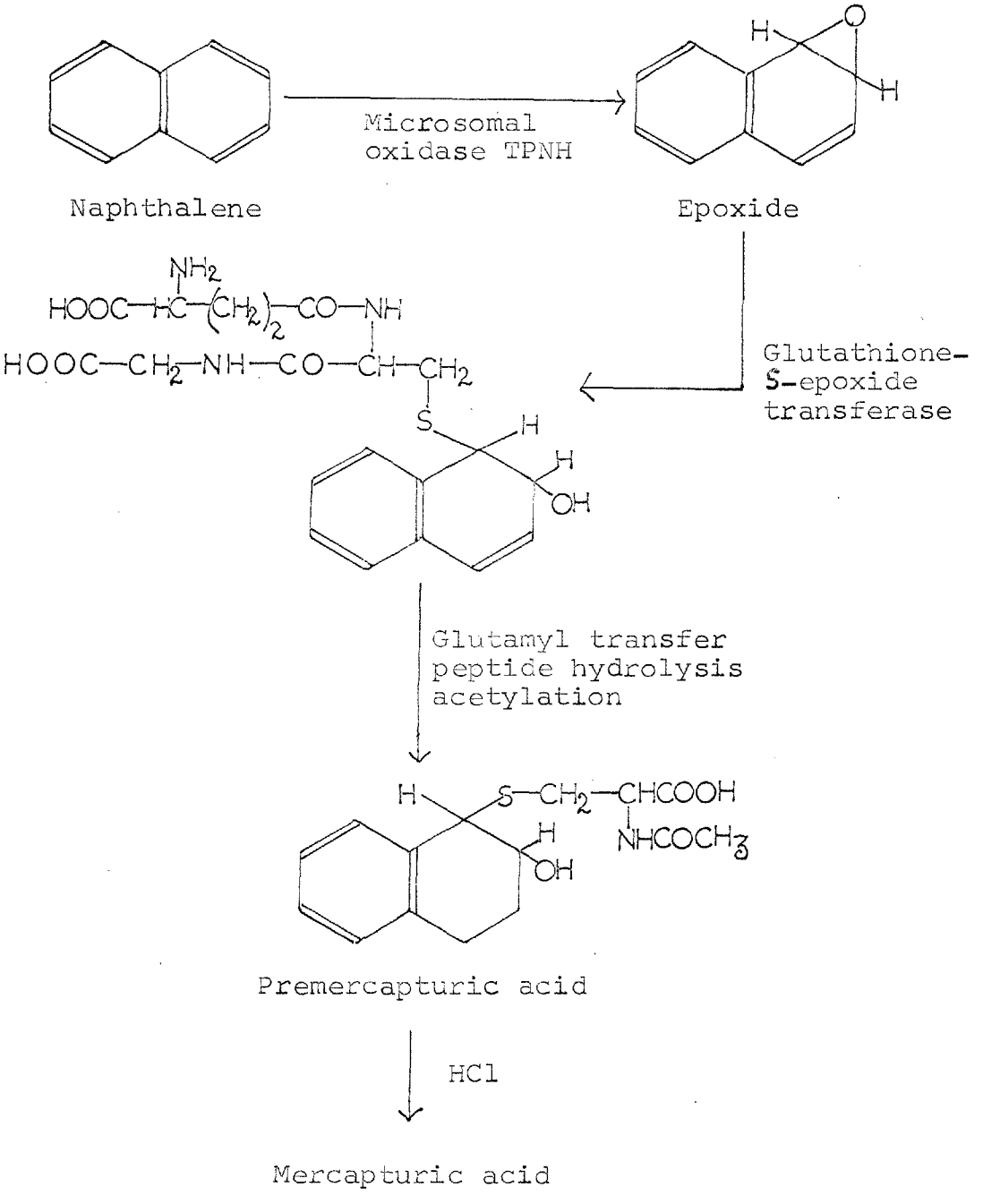

CO-NHCH2CO2H
AX + HS-CH2-CH
NH-CO(CH2)2CH(NH2)CO2H
step a
conjugation
CONHCH2CO2H
A-SCH2CH
NH-CO(CH2)2CH(NH2)CO2H
step b
transpeptidation
CONHCH2CO2H
A-SCH2-CH
NH2
step c
peptidase (hydrolysis)
A-SCH2-CH-CO2H
NH2
step d
acetylase
A-SCH2-CH-CO2H
NHCOCH3

The enzymes for the conjugation with glutathione (step a) differ according to whether the foreign compound is a substituted aliphatic compound or a substituted aromatic compound (Grover and Sims, 1964). Liver preparations from hens and ducks contain very low levels of glutathione-S-aryltransferase. Hence it is probably the first step in mercapturic acid synthesis which is defective in these birds. In the guinea pig however, it is the last step which is defective, since the guinea pig is unable to acetylate arylcysteines (Bray, Franklin and James, 1959).

The second type of mercapturic acid formation occurs when the acetylcysteyl residue replaces a hydrogen atom, e.g. in benzene, chlorobenzene or aniline. When these compounds are administered to animals, they are converted into substances which are excreted in the urine. The latter are converted into mercapturic acids on acidification of the urine, and are termed premercapturic acids (Knight and Young, 1957).

It has been suggested (Boyland, 1962) that the first step in premercapturic acid formation is epoxidation. The epoxide then reacts with glutathione in a reaction catalysed by glutathione-S-epoxidetransferase, to form the glutathione precursor of the premercapturic acid. (This enzyme, again, is different from glutathione-S-aryl transferase.) Subsequent glutamyl transfer, hydrolysis by peptidases and acetylation

produce the premarcapturic acid. The latter conjugate can be converted into a mercapturic acid by the action of dilute acid (Boyland, Sims and Solomon, 1957), e.g. consider the metabolism of naphthalene



Methylation

Compounds containing amino or substituted amino groups, hydroxyl or sulphydryl groups, can be methylated. Methylation of the phenolic hydroxyl group is a major route of detoxication for adrenaline in mammals, and a variety of substituted catechols are also methylated by the same enzyme, catechol-O-methyl transferase (Iverson, 1967). The reaction occurs in man, rat, cow, pig, mouse, guinea pig, cat and rabbit (Smith, 1968). The N-methylation of pyridine derivatives has been observed in man, dog, pig, goat, rabbit, frog, rat, mouse, hamster, turtle and hen. However, no data is available on the relative rates of methylation in different species.

Methylation requires an active form of methionine, namely S-adenosylmethionine. Under the influence of a methyl transferase, S-adenosylmethionine transfers its methyl group to an appropriate acceptor and is itself converted into S-adenosylhomocysteine.

Acetylation

Studies on the acetylation of the aromatic amine, sulphanilamide, have shown that man, rabbit, chicken, mouse, rat, guinea pig, cat, pig, cow, horse, monkey and fish can carry out this conversion (Marshall, 1954). The dog and fox (both members of the Canidae family) are the only species of those so

far investigated, that are unable to acetylate aromatic amines (Bridges and Williams, 1963). The dog is also unable to acetylate hydrazine groups, although this reaction does occur in man, Rhesus monkey, rabbit and rat.

The acetyl group used in the acetylation of amines must be in the activated form of acetyl-coenzyme A. The transfer of the acetyl moiety to the amino group is catalysed by the enzyme transacetylase.

The inability of dogs to acetylate sulphanilamide is probably due to the presence of an inhibitor of the transacetylase enzyme (Leibman and Anaclerio, 1962), although at present the nature of this inhibitor is unknown.

Sulphate conjugation

Conjugation of phenols with sulphate to form ethereal sulphates occurs in most mammalian species, in birds, reptiles, amphibia, anthropods and insects, but not in fish. Sulphate conjugation is so widely distributed, in fact, that it has been suggested that it may be the most primitive of the detoxication mechanisms (Williams, 1967b).

Sulphate conjugation is usually associated with phenols, but certain aliphatic alcohols and aromatic amines are also known to be conjugated with sulphate.

The enzymic synthesis of ethereal sulphates involves

primarily the formation of an active sulphate. This is the compound 3'-phosphoadenosine-5'-phosphosulphate (PAPS). The type of acceptor to which sulphate can be transferred is determined by the nature of the transferring enzyme. PAPS, in the presence of the appropriate enzyme, can transfer sulphate to phenols, phenolic steroids, 3-β-hydroxysteroids, aromatic amines, choline and mucopolysaccharides.

The pig appears to be the only mammal, so far investigated, in which sulphate conjugation is impaired. Pigs do not excrete ethereal sulphates of phenol, iodophenol or chlorophenol; and the amount of isobarbituric acid conjugated with sulphate is much lower than that found in man, dog and rabbit (Stekol, 1936).

CONSEQUENCES OF THE METABOLISM OF FOREIGN COMPOUNDS

During the first phase of metabolism, a foreign compound may be oxidized, reduced or hydrolysed. The process is aimed at making the compound more polar, thus facilitating its excretion. As a result of these reactions there are three possibilities:

(a) the compound becomes less active, e.g. phenobarbitone, the hypnotic is inactivated by hydroxylation to hydroxyphenobarbitone (Butler, 1954).

(b) the compound is activated - thus prontosil becomes active as an antibacterial agent, when reduction of the azo-linkage releases sulphanilamide (Fuller, 1937),

and (c) the activity of the compound is changed. The narcotic, codeine, undergoes O-demethylation to produce the strongly narcotic morphine, which also has analgesic properties (Mannering, Dixon, Baker and Asami, 1954).

In the second phase of metabolism, the large majority of foreign compounds (or their phase I metabolites) are converted into water-soluble, non-toxic conjugates. These are frequently strong organic acids. Occasionally a phase II reaction will result in an "intoxication". Thus fluoroacetate is metabolized to fluorocitrate, the latter compound exerting its lethal effect by jamming the tricarboxylic acid cycle (Peters, 1963).

FACTORS AFFECTING THE METABOLISM OF FOREIGN COMPOUNDS

Many factors are now known to affect the enzymes involved in the metabolism of drugs. These factors may be "intrinsic" in that they belong naturally to the enzyme system under consideration; on the other hand the factors may be forced

upon the enzyme system by environmental conditions, in which case they might be considered as "extrinsic".

Bearing in mind the content of the first part of this "Introduction" perhaps the most salient intrinsic factor is that of the species. Both qualitatively and quantitatively, species variations in metabolism are mainly due to variations in the enzymes catalysing the different metabolic reactions. These differences in enzymic activity may be attributed to

(1) the absolute amount of enzyme varying from one species to another, e.g. coumarin-7-hydroxylase;

(2) the amount of inhibitor varying. Thus it is thought that the inability of the dog to acetylate aromatic amines is due to the presence of an inhibitor;

(3) the presence of a second enzyme which catalyses the reverse reaction. In chicken and pigeon it has been found that on feeding sulphanilamide, both N^4-acetylase and deacetylase are highly active (Bridges, 1963);

(4) the presence of a second enzyme which competes for the same substrate, e.g. competitive deamination and hydroxylation of amphetamine.

Within one particular species, different strains of that species may possess differing enzymic activity. The case of

coumarin-7-hydroxylase activity varying amongst various strains of the rabbit species has been dealt with, in the preceding text.

Furthermore, even on consideration of a particular strain of a species, the pattern of metabolism of foreign compounds may vary according to the sex of the animal under consideration. It has been shown that male rats oxidize drugs more quickly than do female rats (Davies, Gigon and Gillette, 1968).

Another "intrinsic" factor known to influence the metabolism of foreign compounds, is that of age. Newborn rabbits are unable to metabolize many drugs. At two weeks of age, the activities of various enzymes vary between 5 and 37% of the adult; but by four weeks, rabbits possess the full enzymic activities of an adult (Fouts and Adamson, 1959).

In an environment where the number of synthetic chemicals is continually on the increase, the effects of one foreign compound on the metabolism of another may have serious consequences. These effects, whether stimulatory or inhibitory, may change the toxicity and pharmacological activity of a particular drug. This "synergism" of drug action is particularly important in the case of patients needing more than one drug. Thus, if patients being treated with the anticoagulant bishydroxycoumarin are given phenobarbital, the plasma level of bishydroxycoumarin is reduced and consequently the anticoagulant action is decreased (Cucinell, Conney, Sansur

and Burns, 1965).

Another example of an extrinsic factor which influences the metabolism of drugs, is that of diet. When rats are fed low calcium diets, the metabolism of hexobarbital by microsomal enzymes is impaired (Dingell, Joiner and Hurwitz, 1966). Vitamin C appears to be another important constituent of the diet, since vitamin C deficient guinea pigs show an increased sensitivity to the action of various drugs. This has been attributed to a decrease in the activity of the microsomal enzymes (Conney, Bray, Evans and Burns, 1961). An indirect effect of diet would be that of a change of diet causing a change in the composition of the intestinal flora. In this context, any factor changing the nature of the intestinal flora, e.g. disease of the alimentary tract or treatment with antibiotics, could change the pattern of metabolism of a compound which is metabolised by the flora. The role of gut bacteria in the metabolism of foreign compounds is discussed in Chapter 4.

CONSEQUENCES OF SPECIES VARIATIONS IN THE METABOLISM OF FOREIGN COMPOUNDS

Classification is one of the fundamental concerns of science. Facts and objects must be arranged in an orderly

fashion before their unifying principles can be discovered and used as the basis for prediction. Perhaps the most valuable prediction in the field of metabolism of foreign compounds is that of the toxicological and pharmacological response in man to a particular therapeutic agent, this prediction being based on data obtained from the study of other species.

The relatively few examples of species variations previously cited may give some idea of the quandary faced by the biochemist, pharmacologist and toxicologist at the present time. Not only does the pattern of drug metabolism vary from one species to another, but also the rate of metabolism varies, and too, in some cases, the sensitivity of the target organ.

Finally, even in considering the one species, man, we find that responses to certain drugs may vary from one individual to another.

As more data on comparative studies becomes available it appears that the general pattern of metabolism is much more similar between man and monkey than between man and lower mammals, such as the dog (see Table 1.10). This information has led to the establishment of regional primate centres and a number of smaller primate colonies, thus enabling scientists to carry out comparative studies on a large number of primates. It now appears that the macaque monkeys are the most promising

Table 1.10. Presence or absence of certain metabolic pathways in various species*

Metabolic pathway	Man	Rhesus Monkey	Dog
Glutamine conjugation	+	+	-
Acetylation of aromatic amines and hydrazines	+	+	-
Mercapturic acid formation	slow	- or slow	rapid
N-Methylation of amantadine	-	-	+
Phosphate conjugation of 2-naphthylamine	-	-	+
Aromatization of quinic acid	+	+	-
Sulphadimethoxine glucuronide formation	+	+	-

* Data from Smith (1967)

species, as regards similarity to man. It has also been suggested (Smith, 1967) that the New World "owl" monkeys and the Prosimian "bush babies" deserve more attention.

However, it has already been shown that the Rhesus monkey is not the ideal species from which to project observations to man. The drug ICI-33828 is metabolized 20 times more rapidly, and antipyrine, phenylbutazone, amidopyrine and oxphenbutazone 6-10 times more rapidly in Rhesus monkeys than in man (Brodie and Reid, 1967). ICI-33828 inhibits pituitary gonadotropic function at vastly different doses in various species. But, despite a 200-fold variation in daily dosage, each species, including man, shows an inhibitory response at the same plasma concentration of 3 μg/ml (Duncan, 1963). Several other examples are now known of cases where the drug response is much more closely related to the plasma level than to the dosage.

For example:

(1) the duration of action of the mild tranquilizer caprisoprodol varies in 4 species from 0.1 hr. in the mouse to 10 hr. in the cat, but the plasma levels are almost identical on recovery of righting reflex (Gillette, 1965).

(2) phenylbutazone exerts antirheumatic effects in man at a daily dose of about 5 mg/kg., compared with

300 mg/kg. required to protect against glycerol-induced inflammation in the rabbit's eye. In each case, plasma levels are 100-150 µg/ml. (Brodie and Reid, 1967).

Another possible source of species variations is the sensitivity of the target organ. Thus in the example of hexobarbital previously quoted, the serum level of hexobarbital on awakening varies from 15 to 89 µg/ml. However, it should be pointed out that this 6-fold variation is much smaller than the 30-fold variation in sleeping time associated with the administration of the drug. It has been suggested that a more enlightened approach to drug therapy in man and to screening tests in animals, would place greater reliance on plasma levels rather than on pharmacological response.

On consideration of the whole problem of "prediction" at the present time, it would appear that metabolic studies on animals may be an important guide as to what will happen in man although, as yet, insufficient comparative data are available. Future research into the comparative aspects of drug metabolism may show that one particular species (or even a strain of that species) responds to one particular chemical grouping in a similar way to man.

The problem of individual variation within the one species

of man still remains. The drug isoniazid is inactivated by acetylation, rapidly by 50% of the population and slowly by the other 50%. That is, there is a bimodality in the distribution of 'fast' and 'slow' acetylators of isoniazid. This bimodality is also found in the acetylation of sulphadimidine. Rapid acetylators of sulphadimidine are also rapid inactivators of isoniazid (Clarke, 1962). Another example where individual patterns in the rate of metabolism are beginning to emerge, occurs in work done by Hammer and co-workers (1969). A good correlation was found between the rates of metabolism of oxyphenylbutazone, desmethylimipramine and nortriptyline in a group of 5 patients. Thus, when drugs have been classified into chemical groups, and animals into species appropriate to a particular chemical group, perhaps the next stage will be to classify our own species according to their reactions to certain well-chosen "typing" drugs.

Although species differences in drug response may pose many problems to the pharmacologist, there are many useful applications. Thus all herbicides, insecticides, rodenticides, anthelmintics and bactericides can only be used because species differences in toxicity occur. Decisions on the acceptable daily intake of pesticides in man, again rest upon tests carried out in other species of animals. In these cases, man must be assumed to behave like the most

sensitive species tested (Stoner, 1964). The maximal acceptable daily intake for a sensitive species of animal (in terms of mg/kg. body weight/day) can be extrapolated to man in two ways:

(i) dividing the dose by an arbitrary factor, usually 100, or

(ii) using the safety factor proposed by Gaddum (1957) where the acceptable dose is the threshold daily dose less 6 standard deviations.

A more academic application of species variations in the metabolism of foreign compounds would be that of confirming, or in some cases clarifying, the existing taxonomical classification of animal species. This would result in a classification of species based on their biochemical characteristics, as compared with the current classifications in which species are identified by their physiological and anatomical properties. From the data available at the present time it would appear that the pattern of the phase II conjugation reactions may be of great use in such a biochemical classification. Thus glucose conjugation of phenols is a reaction characteristic of insects; arginine conjugation of aromatic acids, characteristic of Arachnids; ornithine conjugation characteristic of reptiles and certain

birds; and glutamine conjugation of substituted acetic acids, a reaction common to man and Old World monkeys.

One case where a metabolic study may have helped to clarify a doubtful classification is that of the fate of sulphadimethoxine in the tree shrew. The latter are small animals which look, act and eat like other Insectivores, and for a long time were classed with the Insectivora order. Closer inspection revealed that, although these animals are small, the brains and eyes are slightly enlarged, the part of the cortex serving sight is a little increased and that serving smell, reduced. All these latter characteristics suggest that the tree shrew may be a primate. Thus, on an anatomical basis, classification of the tree shrew is precarious. A study of the fate of sulphadimethoxine in primates compared with other species has shown that the main metabolite of the drug in the urine of man, Rhesus monkey, baboon, squirrel monkey, capuchin, bush baby, slow loris and the tree shrew is sulphadimethoxine-N^1-glucuronide. In other animals such as the dog, rat, mouse and guinea pig, the main metabolite is N^4-acetylsulphadimethoxine (Adamson, Bridges, Kibby, Walker and Williams, 1970). It would appear that N^1-glucuronide formation is a major reaction of sulphadimethoxine only in primates; furthermore, in this respect, the tree shrew behaves as a primate.

AIMS OF THE PRESENT INVESTIGATION

This thesis describes investigations into the species variations in the metabolism of two anutrients.

The first is that of the conversion of quinic acid into benzoic acid. This conversion entails the loss of six hydrogen and 4 oxygen atoms - thus it is a phase I reaction. When benzoic acid is formed it is subsequently conjugated with glycine and excreted as hippuric acid - a phase II reaction. Species variations in this stage of the metabolism of quinic acid have not been investigated.

The second is that of the pattern of conjugation of 3-indolylacetic acid amongst various species, with particular emphasis on the formation of the glutamine conjugate in primates. This conversion has been chosen as an example of a phase II reaction which exhibits species differences.

CHAPTER 2

SPECIES VARIATIONS IN THE AROMATIZATION OF QUINIC ACID

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INTRODUCTION

Physical properties of quinic acid

l-Quinic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid) is a naturally occurring acid with the following physical properties:-

Melting point	162°C
Solubility in water at 9°C	40%
Solubility in 95% aqueous alcohol at 14°C	2%
$[\alpha_D^{20}]$ (c. = 19.74 water)	-44.03
pK at 14.1°C	3.56

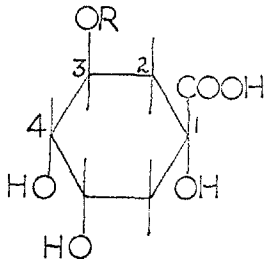
l-Quinic acid has the configuration shown below [I] (Fischer and Dangschat, 1932). The enantiomorph, d-quinic acid has been isolated by resolution of the racemate, the latter being produced via the racemization of the lactone of l-quinic acid by heat (Lohmar, 1957). Throughout the text, the term quinic acid will refer to the laevorotatory form of the acid.

Occurrence of quinic acid in nature

Quinic acid was first isolated from cinchona bark (whence its name) by Hoffmann in 1790. It is widely found as the free acid in plant materials including coffee beans, blueberry

and bearberry leaves, cedar and larch needles, many species of gymnosperm and in a number of fruits (Posternak, 1965).

Quinic acid occurs frequently as depsides (Gr. depsein to tan) with caffeic acid or p-coumaric acid. The most widely occurring ester is chlorogenic acid (II) quinic acid-3-caffeate, which was first isolated from coffee (Coffee Arabica) over a hundred years ago.



I, R = H

II, R = Caffeyl

Chlorogenic acid occurs in a wide variety of fruit including apple, bilberry, grape and pear, in solanaceous plants, tobacco leaves, grass and several other sources. A second isomer of chlorogenic acid, quinic acid-5-caffeate, has been isolated from green coffee and more recently other depsides, whose structures are at present unknown, have been isolated from sweet potato, peaches and several different fruits. The 1,4-dicaffeyl ester of quinic acid, cynarine was isolated from artichokes by Pannizi and co-workers (Swain, 1962). Quinic acid has also been shown to be present in many fruits

and vegetables as a depside with p-coumaric acid; and recently a 1,4-di-p-coumarate of quinic acid has been found in pineapple (Posternak, 1965).

It is apparent that quinic acid is widely distributed in nature, in the plant world, and is therefore a normal constituent of the diet.

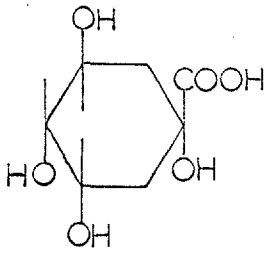
It has been suggested that quinic acid may be a major non-aromatic precursor of urinary hippuric acid (Blatherwick and Long, 1923) (Fig. 2.1). Humans and rats receiving a purified diet (lactalbumin-dextrin diet) excrete only small amounts of hippuric acid, i.e. benzoylglycine (1.5-4 mg./kg./day) (Armstrong, Chao, Parker and Wall, 1955). This amount appears to be formed endogenously. The normal output of hippuric acid is 22.5 to 26.5 mg./kg./day, hence the major proportion of hippuric acid in rat and human urine is presumably derived from dietary precursors. The amount of hippuric acid found in urine cannot be accounted for solely by the benzoic acid content of ordinary diets (Cotran, Kendrick and Kass, 1960).

Aromatization of quinic acid in various mammalian species

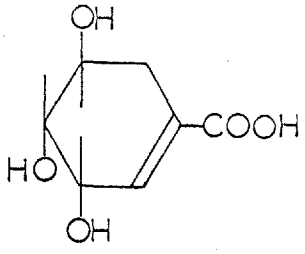
The aromatization of quinic acid was first shown to occur in man in 1863. It was found that orally administered calcium quinate was converted to hippuric acid, which was isolated from urine (Lautemann, 1863). This reaction was

Fig. 2.1.

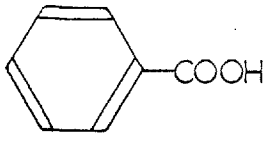
Quinic acid and related compounds



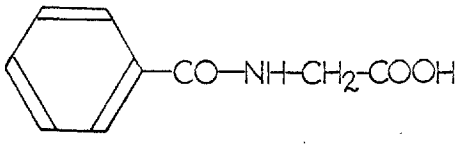
Quinic acid



Shikimic acid



Benzoic acid



Hippuric acid

studied in man, rat, rabbit, guinea pig and cat by Beer, Dickens and Pearson (1951) and the results are tabulated below (Table 2.1).

It can be seen that man is the only species in which quinic acid is converted to hippuric acid in good yield. It should be pointed out that in this work some species were given quinic acid orally whereas others were given the acid by subcutaneous injection. Bernhard, Vuilleumier and Brubacher (1955) showed that quinic acid leads to an increase in urinary hippurate not only in man but also in guinea pig. In man, 65% and 82% of orally administered quinic acid (3 g., 6 g.) was converted to hippuric acid, and in guinea pig, oral doses of 930, 680 and 1000 mg./kg. were aromatized to the extent of 63%, 72% and 47% respectively. The difference between the work of Beer et al. (1951) who found no increase in urinary hippurate after giving quinic acid to guinea pigs, and Bernhard et al. (1955), who detected an increase of about 61%, could be due to the fact that in Beer's work, the guinea pigs were injected subcutaneously, whereas in Bernhard's work, the animals were given the acid orally. Adamson, Bridges and Williams (1966) found that aromatization of an orally administered dose of quinic acid (300 mg./kg.) occurred to the extent of 70% of the dose in man, 40-70% in Old World monkeys, but in the New World monkeys, lorisform lemurs and the tree

Table 2.1. Urinary excretion of benzoic acid in various species following the administration of quinic acid*

Species	Dose (g.)	Route of administration	Control benzoic acid level (mg/day)	Benzoic acid (mg/day) after giving quinic acid	% aromatization of quinic acid
[1]Man	6	Oral	620	3,210	68
[1]Man	6	Oral	620	2,270	43
[2]Rabbit	1.9	Oral	15.8	45.7	2.5
[2]Guinea pig	0.31	Subcutaneous	12.4	18.6	3.2
[2]Rat	0.33	Oral	7.6	13.9	3
[2]Cat	2	Subcutaneous	31.9	9	0
[2]Cat	2	Subcutaneous	No urine	8.1	0

1 Hippuric acid estimated by isolation procedure

2 Hippuric acid estimated colourimetrically

* Data from Beer, Dickens and Pearson (1951)

shrew, little or no aromatization was detected.

Effects of neomycin pretreatment and routes of administration on the extent of aromatization of quinic acid

Although several workers had investigated the occurrence of this aromatization reaction, the site of the reaction was unknown. Beer et al. (1951) suggested that the intestinal flora may be responsible for the conversion. Cotran, Kendrick and Kass (1960) showed that oral neomycin, in doses sufficient to inhibit bacterial multiplication in the intestinal tract, prevented the conversion of orally administered quinic acid (6 g.) to hippuric acid in man. When quinic acid (300 mg.) was given parenterally to guinea pigs it was not converted to hippuric acid, although when given orally, it led to the prompt excretion of amounts of hippuric acid sufficient to account for 30% of the ingested quinic acid. Thus, it appears that in agreement with the suggestion of Beer, aromatization of quinic acid in man and guinea pigs is achieved by intestinal bacteria.

These conclusions were supported by the work of Asatoor (1965) (see Table 2.2). Pretreatment of rats with neomycin sulphate (200 mg./day) not only reduced urinary hippuric acid output to below the normal level (0.26 mg./mg. creatinine compared with 0.65 mg./mg. creatinine) but also prevented any

Table 2.2. Urinary excretion of hippuric acid following oral administration of quinic acid or shikimic acid in normal and neomycin pretreated rats*

Acid administered orally	Normal rats		Rats pretreated with neomycin sulphate	
	Control level of hippuric acid (mg./mg. creatinine)	Hippuric acid (mg./mg. creatinine) after administration of acid	Control level of hippuric acid (mg./mg. creatinine)	Hippuric acid (mg./mg. creatinine) after administration of acid
Quinic acid (500 mg.)	0.65	1.95	0.26	0.15
Shikimic acid (200 mg.)	0.5	2.7	< 0.2	$\ll 0.2$

Values represent mean of 6 animals

* Data from Asatoor (1965)

increase in hippuric acid after oral administration of quinic acid (500 mg.). Neomycin pretreatment also prevented the increase in urinary hippuric acid found to be associated with the oral administration of shikimic acid to rats. Shikimic acid (see Fig. 2.1) often accompanies quinic acid in nature and it is known that quinic acid is converted into shikimic acid in fruit during ripening (Posternak, 1965).

Thus it can be concluded that the intestinal flora plays a predominant role in the production of hippuric acid from aromatic precursors such as quinic acid and shikimic acid.

Mitoma, Posner and Leonard (1958) were unable to show oxidation of quinic acid with human liver homogenate, although they demonstrated the oxidation of hexahydrobenzoic acid to hippuric acid by guinea pig liver mitochondria fortified with the boiled supernatant fraction.

This chapter deals with the investigation of the species occurrence of the aromatization of quinic acid in a number of primate and sub-primate species. An attempt has been made to demonstrate the conversion of quinic acid to benzoic acid or hippuric acid by Rhesus monkey liver homogenate. A comparison is made between the increase in urinary hippuric acid following oral administration and intraperitoneal injection of quinic acid to Rhesus monkeys. The effect of pretreatment with neomycin sulphate on the aromatization of quinic acid has been studied in both rats and monkeys. Finally, the possibility of shikimic acid acting as an intermediate in the conversion of quinic acid to benzoic acid has been investigated by measuring the degree of aromatization of orally administered shikimic acid in rats and Rhesus monkeys.

MATERIALS AND METHODS

Chemicals

(1)-Quinic acid (m.p. 164°C), α_D^{20} -42.2 (2% in water) and benzoic acid (m.p. 122°C), (Hopkin & Williams Ltd., Chadwell Heath, Essex); hippuric acid (m.p. 187°C) (Harrington Bros. Ltd., London); shikimic acid (m.p. 190°C), phenol (b.p. 182°C), catechol (m.p. 105°C), o-hydroxybenzoic acid (m.p. 159°C), m-hydroxybenzoic acid (m.p. 201°C), p-hydroxybenzoic acid (m.p. 214°C), 2,3-dihydroxybenzoic acid (m.p. 204°C), 2,4-dihydroxybenzoic acid (m.p. 218°C), 3,5-dihydroxybenzoic acid (m.p. 233°C), 3,4-dihydroxybenzoic acid (m.p. 199°C), 3,4,5-trihydroxybenzoic acid (m.p. 253°C), benzene (b.p. 80°C), phenylacetic acid (m.p. 76°C) and methyl benzoate (b.p. 195°C) (Koch-Light Labs. Ltd., Colnbrook, Bucks.) were all purchased. Benzoyl glucuronide (m.p. 180°C) was a sample prepared by Baldwin, Robinson and Williams (1960) by feeding turkeys with benzoic acid.

Animals

The animals used are listed in Table 2.3, young adult females being used when possible. All animals were kept on a constant diet for several days before the administration of quinic acid or shikimic acid, and during an experiment (for

Table 2.3. Diets and methods of feeding quinic acid or shikimic acid to various species

Species*1	Diet	Method of feeding quinic acid
Man	Constant fruit, tea and coffee intake	Orally, in water
Rhesus monkey	2 bananas, 1 apple + 41B rat food ad. lib.	Concealed in bananas
Baboon	2 bananas, 2 apples + 41B rat food ad. lib.	Concealed in bananas
Green monkey	2 bananas, 1 apple + 41B rat food ad. lib.	Concealed in bananas
Spider monkey	"	Mixed in honey
Squirrel monkey	"	Mixed in honey
Capuchin	"	Mixed in honey
Bush baby	1 banana, 1 apple + 41B rat food ad. lib.	Mixed in honey
Slow loris	"	Mixed in honey
Tree shrew	"	Mixed in honey
Dog	"Lassie" dog food	Mixed in meat
Cat	"Kit-e-cat" + milk	Mixed in meat
Ferret	"	Orally, by needle
Rabbit	*2S.G.1 rabbit food ad. lib.	Orally, by stomach tube
Rat	*241B rat food ad. lib.	Orally, by needle
Mouse	"	Orally, by needle
Guinea pig	41B rat food + cabbage	Orally, by needle
Hamster	41B rat food	Orally, by needle
Lemming	41B rat food	Orally, by needle
Pigeon	Paul's layers mash No. 1	Orally, by stomach tube
Fruit bat	Fruit	Orally, by needle
Hedgehog	"Kit-e-cat" + milk	Orally, by needle

*1 All species were given water ad. lib.

*2 41B and S.G.1. supplied by E. Dixon and Sons.

details see Table 2.3). Quinic acid or shikimic acid was dissolved in water as the sodium salt for administration to man, rats, mice, guinea pigs, hamsters, lemmings, rabbits, ferrets, pigeons, fruit bats and hedgehogs; the powdered, solid form of the acid was mixed in the diet of the other species when studying aromatization of the orally administered acids,

(a) in meat for cats and dogs,

(b) in honey for squirrel monkey, spider monkey, capuchin, bush baby, slow loris and the tree shrew, and

(c) concealed in bananas for Rhesus monkey, green monkey and baboon.

The acids were dissolved in a minimal amount of sterilised water, as the sodium salt, for intraperitoneal injection (for dose levels, see appropriate Table).

To suppress the gut flora in rats, neomycin sulphate (100 mg. in water) was administered orally twice daily for 6 days. Control urine was collected over the 5th day, quinic acid (600 mg./kg.) was administered orally on the 6th day and the subsequent 24 hr. urine collected. Rhesus monkeys received neomycin sulphate (2 g.) concealed in bananas, daily for 4 days; control urine was collected over the 5th day, quinic acid

(350 mg./kg) given on the 6th day and the subsequent 24 hr. urine collected. Further doses of quinic acid were given periodically up to 38 days after neomycin treatment had been completed, in order to ascertain when the ability of the monkeys to aromatize quinic acid had returned to normal.

A side effect of oral neomycin is that it reversibly damages the small intestinal mucosa, producing a temporary malabsorption syndrome (Asatoor, Chamberlain, Emmerson, Johnson, Levi and Milne, 1967). Madribon (sulphadimethoxine, 50 mg./kg.) was therefore administered to the Rhesus monkeys on the 12th day of the experiment. The subsequent 24 hr. urine was collected and analysed for madribon metabolites (Bridges, Kibby and Williams, 1964, 1965).

Mercuric chloride (20 mg.) was added to all urine samples to prevent any possible bacterial breakdown of benzoic acid conjugates.

Fluorimetric determination of benzoic acid conjugates in urine

Hippuric acid in 70% sulphuric acid fluoresces strongly when irradiated with ultraviolet light, and this can be used for its fluorimetric estimation (Ellman, Burkhalter and La Dou, 1961). This method has several advantages over previously published methods:

1) much greater sensitivity (minimal significant

concentration in 70% sulphuric acid is approximately 0.2 μg/ml),

2) there is no loss of hippuric acid during extraction procedures,
3) there is no necessity for preliminary purification of the urine,
4) much smaller samples can be utilized.

Fresh 24 hr. urine was centrifuged at 2000 g. for 10 mins. Aliquots (8 μl) of the urine were withdrawn from the supernatant using a Hamilton microsyringe, mixed with 70% Analar sulphuric acid (3 ml.) and the fluorescence measured in an Aminco-Bowman spectrophotofluorimeter at the appropriate wavelengths for hippuric acid (λ_A = 259 nm.; λ_F = 366 nm. - see Table 2.4). The concentration of hippuric acid in urine could then be readily calculated from a standard curve of fluorescence intensity versus hippuric acid concentration.

Recent studies have shown that hippuric acid is the main urinary metabolite of benzoic acid in all the species studied here except the ferret and fruit bat (Bridges, French, Smith and Williams, 1970). In the two latter species benzoyl glucuronide is the main metabolite. The fluorescence of the urine of these two species was measured at the optimum conditions for benzoylglucuronide fluorescence (λ_A = 258 nm.; λ_F = 388 nm.).

The following compounds were examined for fluorescence to see if they would interfere with the fluorimetric estimation of hippuric acid:-

quinic acid, shikimic acid, benzoic acid, benzoyl glucuronide, phenol, catechol, o-hydroxybenzoic acid m-hydroxybenzoic acid, p-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, benzene, phenylacetic acid and methyl benzoate.

The fluorescence of each compound was examined at a concentration of 5 µg/ml. in 70% sulphuric acid (see Table 2.4).

Investigation of the aromatization of quinic acid by Rhesus monkey liver homogenate

A Rhesus monkey was killed by i.v. injection of pentobarbital (150 mg./kg.), the liver rapidly removed and homogenised in three times its own weight of 0.15 M.KCl and 0.001M. EDTA, in a Waring Blendor. A portion (2 ml.) of the homogenate was added to test tubes containing 0.1M phosphate buffer pH 7.4 (2 ml.) and quinic acid (5, 10 or 20 mg. in 1 ml. of water). Controls contained 1 ml. of water or benzoic acid (5 mg. in 1 ml. of water) instead of quinic acid. Incubations were carried out at 37° for 1 hour in a shaking water bath.

Trichloroacetic acid (2 ml. of 25% solution) was added to each tube, followed by ethanol (5 ml.). Tubes were shaken for 15 min. in a mechanical shaker then centrifuged at 2000 xg. for 10 mins. Samples of the supernatant were then estimated for benzoic acid or hippuric acid fluorimetrically as described previously. The recovery of benzoic acid was 99 $\pm$ 1%. The hydroxylation of biphenyl (Creaven, Parke and Williams, 1965b) and the acetylation of sulphadimethoxine (Bridges, Kibby, Walker and Williams, 1969) were also studied using other samples of the liver homogenate, to ensure that it was active enzymically.

RESULTS AND DISCUSSION

Suitability of the fluorimetric method

The fluorescence of 18 benzene derivatives in 70% sulphuric acid has been investigated (Table 2.4). Only four of these compounds showed an appreciable fluorescence, namely, hippuric acid, benzoic acid, benzoyl glucuronide and methyl benzoate. Quinic acid and shikimic acid showed no fluorescence under these conditions at concentrations of up to 500 µg/ml. Benzoic acid and its three derivatives showed a constant fluorescence intensity, stable for up to 4 hours.

Species variations in the aromatization of orally administered quinic acid

The excretion of hippuric acid following oral doses of quinic acid in 10 species of primates is shown in Table 2.5. The percentage of the administered dose which is aromatized to hippuric acid is assessed by subtracting the hippuric acid found in the urine for 24 hours before feeding from that excreted in urine during 24 hours after feeding. Since the normal output of hippuric acid is variable, small conversions of quinic acid to hippuric acid cannot be detected accurately by the fluorimetric technique. However, this can be done by feeding [^{14}C] quinic acid and then using the technique of

Table 2.4. Fluorescence of various benzene derivatives in 70% sulphuric acid

Compound	λ activation (nm)	λ fluorescence (nm)	Relative Intensity
Benzoic acid	258	388	100
Hippuric acid	259	366	54
Benzoyl glucuronide	258	388	38
Methyl benzoate	280	396	45
Catechol	251, 286	322	0.14
Phenol	288	322	0.14
o-Hydroxybenzoic acid	316	434	0.32
m-Hydroxybenzoic acid	-	-	0
p-Hydroxybenzoic acid	-	-	0
2,3-Dihydroxybenzoic acid	309	359	0.4
2,4-Dihydroxybenzoic acid	310	390	11
3,4-Dihydroxybenzoic acid	310	360	4.0
3,5-Dihydroxybenzoic acid	-	-	
3,4,5-Trihydroxy-benzoic acid	372	445	5.2
Phenylacetic acid	-	-	0
Benzene	-	-	0
*Quinic acid	-	-	0
*Shikimic acid	-	-	0

Concentration: 5 µg./ml. in 70% Sulphuric acid.
All readings taken immediately after the solutions were prepared.

* Non-fluorescent from 5-500 µg./ml.

Table 2.5. Conversion of quinic acid to hippuric acid in some primate species

Sub-order	Family	Species	Approx. body wt. (kg.)	Approx. normal hippuric acid output (mg./kg.)	Sex	% aromatization of dose
Anthropoidea	Hominidae	Man	66-80[3]	9-26	M F	64, 67 62
"	Cercopithecidae	Rhesus monkey	3-6[10]	32-57	M F	60, 23 45, 43, 43, 40 29*, 28*, 21*, 21*
"	"	Baboon	13-16[3]	19-53	M	51, 46, 20
"	"	Green monkey	1.4[2]	48, 66	M	47, 42
"	Cebidae	Spider monkey	5-6[3]	7-27	M	0+, 10+, 11, 2
"	"	Squirrel monkey	0.5[3]	0.6-2.1	F	0, 0, 0
"	"	Capuchin	1.7[2]	4.4, 10	M F	0 0
Prosimii	Lorisidae	Bush baby	0.6[2]	7, 9	M F	3 0
"	"	Slow loris	0.7[2]	3, 12	M F	9 3
"	Tupaiidae	Tree shrew	0.4[3]	0.1-4.4	F	0, 0, 0

Quinic acid was administered orally at a dose level of 350 mg./kg. except in man, where the dose was 6g. Average values for body wt. and normal hippuric acid levels are given, the number of animals being indicated by a superscript.

Individual values for aromatization are given.

* These monkeys had been kept on low levels of tetracycline by the animal dealer. This occurred just before feeding quinic acid.

+ Values obtained on separate occasions on the same animal.

isotope dilution to estimate [^{14}C]hippuric acid quantitatively. A detailed description of this method is found in the next chapter. Large conversions are readily shown by the fluorimetric method, and, as can be seen in Table 2.5, man and the Old World monkeys studied (Rhesus monkey, green monkey and baboon) convert large amounts of quinic acid to hippuric acid (21-67%). On the other hand, in the New World monkeys (spider monkey, squirrel monkey and capuchin) and Prosimii (bush baby, slow loris and tree shrew) small but variable amounts of hippuric acid are formed from quinic acid (0-11%).

The aromatization of quinic acid thus appears to follow the taxonomic division of the primates between the higher apes and Old World monkeys on the one hand, and the New World monkeys and Prosimii on the other (see Appendix).

The ability of 11 lower mammals and 1 species of bird, the pigeon, to aromatize quinic acid has been investigated, and the results are shown in Table 2.6. In hedgehog, fruit bat, lemming, hamster, guinea pig, mouse and ferret, no increase in urinary benzoate (free or conjugated) was detectable after feeding with quinic acid. In other lower mammals such as dog, cat, rabbit, rat and in the pigeon, slight increases in hippuric acid were detected (0.2-8%).

In these studies, aromatization of orally administered quinic acid could not be shown in 3 English guinea pigs, although

Table 2.6. Conversion of quinic acid to hippuric acid in some sub-primate species

Class	Order	Family	Species	Approx. body wt. (kg.)	Approx. normal hippuric acid (mg./kg.)	% aromatization of dose
Mammalia	Carnivora	Canidae	Dog	10^{2}	20, 75	0.2, 2
"	"	Felidae	Cat	2.4^{2}	56, 62	0, 0.4
"	"	Mustelidae	Ferret^{o}	0.5^{2}	165, 173	0, 0
"	Lagomorpha	Leporidae	Rabbit	2.5^{3}	47-132	3, 5, 5
"	Rodentia	Muridae	Rat	0.25^{3}	12-18	4, 6, 8
"	"	Muridae	Mouse*	0.02^{12}	37	0
"	"	Caviidae	Guinea pig	0.4^{2}	66, 74	0, 0
"	"	Cricetidae	Hamster	0.1^{3}	250-382	0, 0, 0
"	"	Cricetidae	Lemming^{+}	0.02^{2}	49, 49	0, 0
"	Insectivora	Erinaceidae	Hedgehog	0.9^{2}	21, 39	0, 0
"	Chiroptera	Pteropidae	Fruit bat^{o}	0.3^{1}	33	0
Aves		Columbidae	Pigeon*	0.5^{2}	82	2

Quinic acid was administered orally, 300 mg./kg. to rabbits, hamsters, guinea pigs, cats, ferrets, dogs, lemmings and pigeons, 350 mg./kg. to hedgehogs and fruit bats, and 600 mg./kg. to rats and mice. Average values for body wt. and normal urinary hippurate are given, the number of animals being indicated by superscripts. Individual values for aromatization are given. The animals used were females.

* - pooled urine, or in the case of pigeon, pooled excreta.

+ - one male, one female.

o - increases in hippuric acid and benzoyl glucuronide investigated.

previous workers (Bernhard et al., 1955; Cotran et al., 1960) had obtained values of 30-61% aromatization of the dose. As the conversion of quinic acid to benzoic acid shows great species variations, it is possible that 'strain' variations may also occur. Neither of the above mentioned workers indicated which strain of guinea pigs was used, but it is possible that the difference between their results and those obtained from the study of English guinea pigs is due to 'strain' variations in this conversion. Another possibility is that the previous workers may not have taken the precaution of maintaining their animals on an absolutely constant diet. In the study described here, great care was taken to ensure that all the species under investigation were kept on a strict diet.

On consideration of the animals studied here, it would appear that extensive aromatization of orally administered quinic acid to benzoic acid is peculiar to man and the Old World monkeys.

Aromatization of quinic acid by Rhesus monkey liver homogenate

Incubations of Rhesus monkey liver homogenate with quinic acid yielded no increase in benzoic acid or hippuric acid, although the homogenate was active in the 4-hydroxylation of biphenyl (1.3 μmole/g. of liver/hour) and the N^4-acetylation of sulphadimethoxine (0.16 μmole/g. of liver/hour).

As aromatization of quinic acid by liver homogenate could not be demonstrated, it was decided to investigate the possibility that the conversion of quinic acid to benzoic acid is brought about by gut bacteria.

Aromatization of quinic acid by gut bacteria

The possible participation of gut bacteria in the aromatization of quinic acid has been studied by two methods.

Firstly, a comparison is made between the degree of aromatization of orally administered quinic acid, and that of intraperitoneally injected quinic acid, in the Rhesus monkey (Table 2.7).

When quinic acid was given orally to the Rhesus monkeys in these experiments, the percentage conversion of the dose to hippuric acid was 21%. This is lower than might be expected, owing to the fact that the monkeys had been kept on low levels of tetracyclin by the animal dealer, prior to this experiment. After intraperitoneal injection of quinic acid there was no increase in urinary hippuric acid. In fact, in each case, there was a slight fall in the level of hippuric acid. Hence it appears that quinic acid must pass through the intestinal tract of Rhesus monkeys, if aromatization is to occur.

Secondly, the alteration in the degree of aromatization of orally fed quinic acid, due to neomycin pretreatment, is

Table 2.7. Effect of route of administration on the aromatization of quinic acid in the Rhesus monkey

Rhesus Monkey No.	Route of administration	Normal hippuric acid level (mg./day)	Hippuric acid level (mg./day) after feeding quinic acid	% of dose aromatized
*4	Oral	114.5	502.7	20.6
*5	Oral	90	488.8	21.3
4	Intraperitoneal	157.3	98.5	0
5	Intraperitoneal	122	98.5	0

* - Monkeys had been kept on low levels of tetracyclin by animal dealers. This occurred just before the experiment.

Dose of quinic acid = 350 mg./kg.

described in the rat and Rhesus monkey. Oral neomycin is widely used in medicine to reduce the number of colonic bacteria (Asatoor, Chamberlain, Emmerson, Johnson, Levi and Milne, 1967). It has a wide spectrum of activity and can eliminate both Aerobes (coliforms and streptococci) and Anaerobes (bifids and clostridia) (Finegold, Posnick and Miller, 1965).

In normal rats there was a small, but consistent increase in urinary hippurate on feeding orally with quinic acid. In neomycin pretreated rats, however, there was no increase in hippuric acid associated with the oral administration of quinic acid. Hence it appears that the small amount of aromatization found in normal rats is probably brought about by gut bacteria (Table 2.8).

The effect of neomycin sulphate pretreatment on the aromatization of quinic acid is more marked in the Rhesus monkey, since this species converts quinic acid to hippuric acid to a much greater extent than does the rat. The decrease in the ability to aromatize oral doses of quinic acid (350 mg./kg.) by 3 Rhesus monkeys pretreated with neomycin sulphate (2 g./day for 4 days) is shown in Fig. 2.2. The values used in plotting the histogram represent the average of the values obtained from the 3 animals.

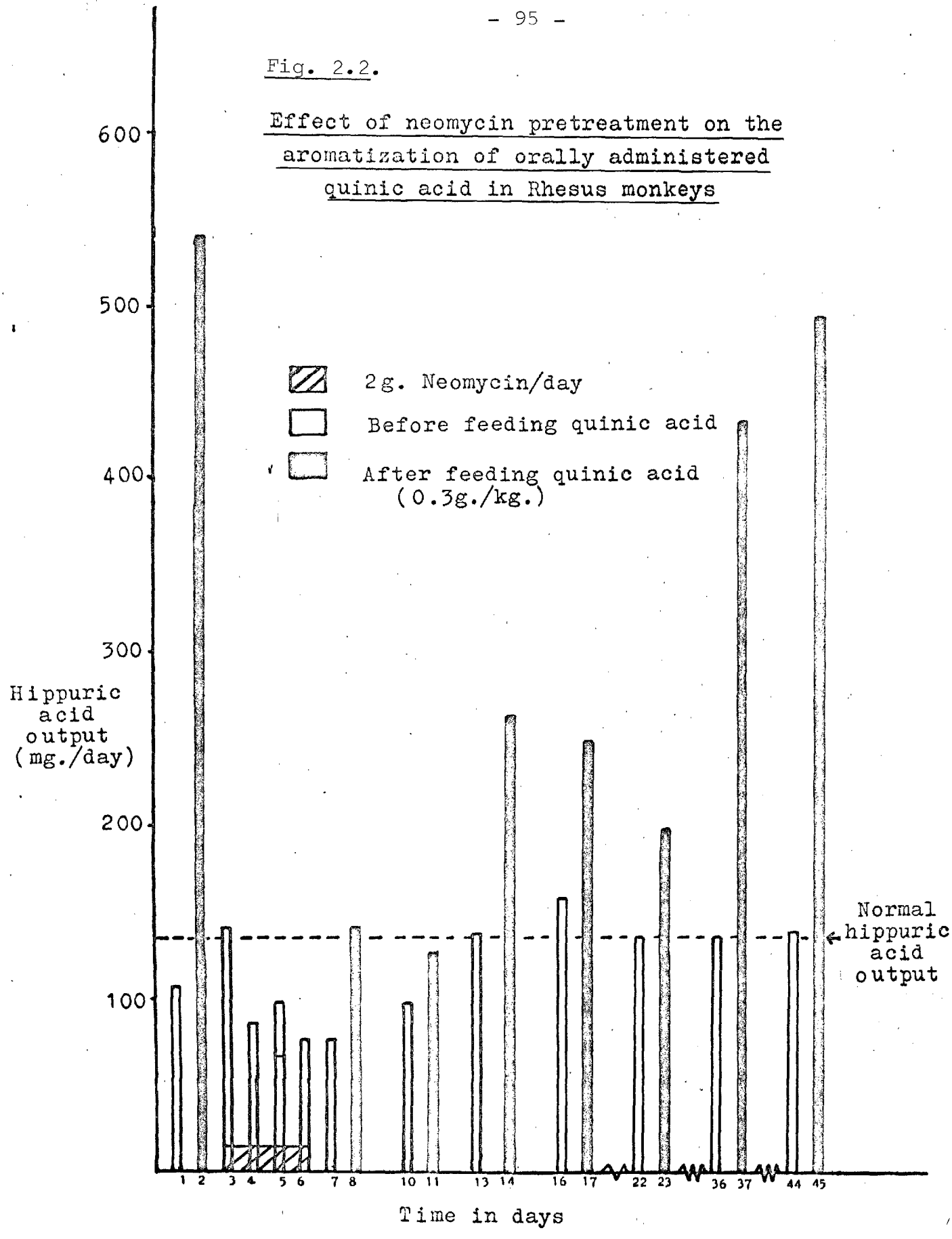
Before treatment with neomycin, the increase in hippuric on feeding quinic acid (350 mg./kg.) represents 24% of the dose.

Table 2.8. Effect of neomycin sulphate pretreatment on the aromatization of quinic acid in the rat

Treatment		Control level of hippuric acid (mg./day)	Hippuric acid level (mg./day) after feeding quinic acid (600 mg./kg.)	% Aromatization
None	1.	9.0	12.3	3.6
	2.	2.6	6.6	4.0
	3.	3.7	7.8	4.2
Neomycin	4.	7.9	7.9	0
	5.	7.9	6.8	0

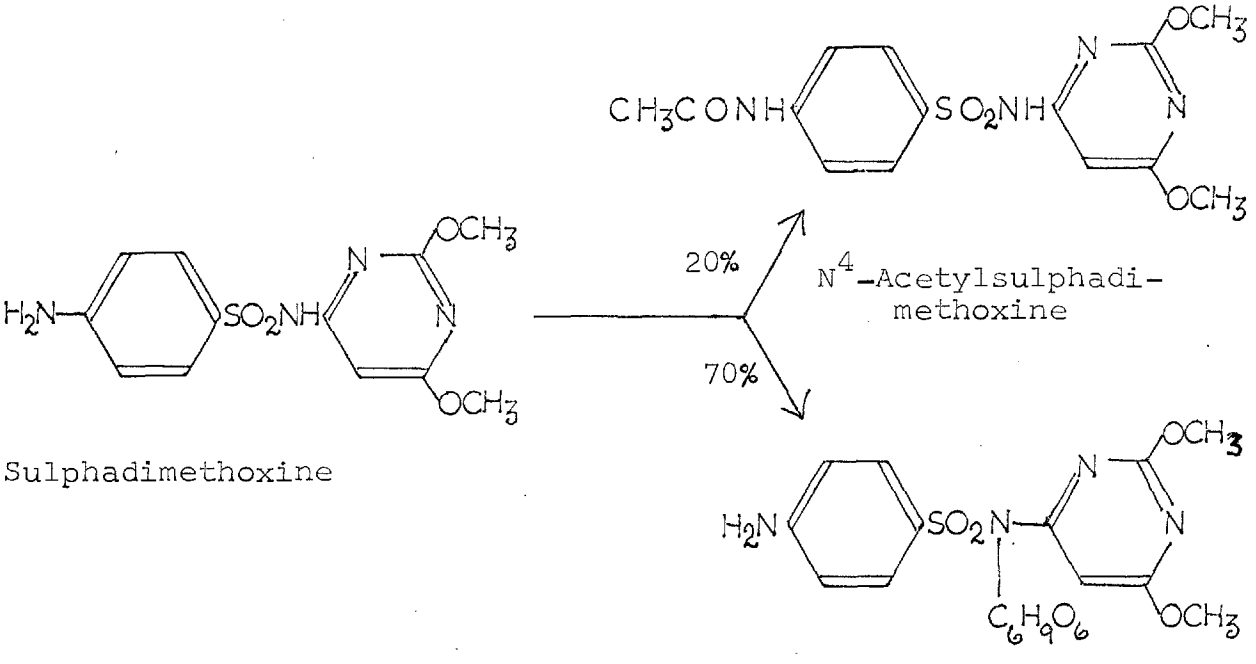
Fig. 2.2.

Effect of neomycin pretreatment on the aromatization of orally administered quinic acid in Rhesus monkeys



After 4 days of neomycin treatment, however, only 3.5% of the dose was aromatized. Over a period of time (≏ 30 days) the monkeys gradually recovered the ability to convert quinic acid to hippuric acid; on day 37 of the experiment 16% of a dose of quinic acid was aromatized and on day 44, 20% was aromatized.

Madribon (sulphadimethoxine, 50 mg./kg.) was administered to the monkeys on the 12th day of the experiment to see to what extent neomycin was interfering with absorption. It was found that madribon was excreted in urine 70% as the N^1-glucuronide and 20% as the N^4-acetyl derivative, as has been previously reported (Adamson, Bridges and Williams, 1966).



Sulphadimethoxine-N^1-glucuronide

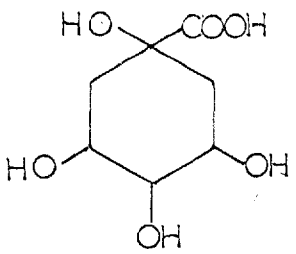
Thus it appears that, at the dose levels of neomycin used here, absorption is probably not significantly affected.

The suppression of certain gut bacteria (coliforms, streptococci, bifids, clostridia) in Rhesus monkeys greatly reduces their ability to aromatize quinic acid. Hence, it seems that in normal Rhesus monkeys it is the gut bacteria that brings about this conversion.

Another interesting observation from Fig. 2.2. is that the level of urinary hippuric acid is considerably lowered in these monkeys after treatment with neomycin (76 mg./day compared with a normal level of 135 mg./day). This observation is in agreement with the work of Asatoor et al. (1967) who showed that some bacterial metabolites reappeared rapidly after discontinuance of neomycin intake, in humans, whilst others remained abnormally low for many days, e.g. hippuric acid and faecal amines.

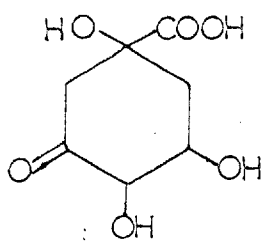
Aromatization of shikimic acid by the rat and Rhesus monkey

In the course of studying aromatic biosynthesis in a group of aromatic polyauxotrophs which require part or all of a group of 5 aromatic metabolites (tyrosine, phenylalanine, tryptophan, p-aminobenzoic acid and p-hydroxybenzoic acid), Davis and Weiss (1953) have proposed the following scheme:-

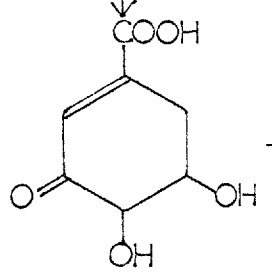


Quinic acid

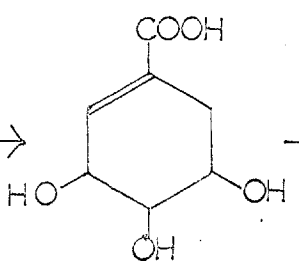
Aerobacter
side reaction



5-Dehydroquinic acid



5-Dehydroshikimic acid



Shikimic acid

Tyrosine
Phenylalanine
Tryptophan
p-Aminobenzoic acid
p-Hydroxybenzoic acid

It is suggested that quinic acid is not a normal intermediate in aromatic biosynthesis, but a non-essential compound which Aerobacter (both wild-type and mutants) can convert into dehydroshikimic acid and thence via shikimic acid

to various aromatic metabolites.

Thus it seemed possible that shikimic acid would be an intermediate in the metabolism of quinic acid by mammals. Rats and Rhesus monkeys were given shikimic acid orally and the degree of aromatization of the dose to benzoic acid estimated fluorimetrically (Table 2.9).

In Rhesus monkeys, 41% of the dose of shikimic acid (300 mg./kg.) is converted to hippuric acid. This value is comparable with that obtained on feeding quinic acid. Hence it seems possible that shikimic acid may be an intermediate in the conversion of quinic acid to benzoic acid.

If the sequence of reactions leading from quinic acid to benzoic acid is as follows:-

$$\text{QUINIC ACID} \xrightarrow{1} \text{SHIKIMIC ACID} \xrightarrow{2} \text{BENZOIC ACID}$$

(Stages 1 and 2 consist of several steps)

then the difference between animals that convert quinic acid to hippuric acid to an appreciable extent and those that do not, could be due to the fact that the latter are unable to carry out stage 1. If this were the case, oral administration of shikimic acid (600 mg./kg.) to the rat (a species which does not aromatize quinic acid to benzoic acid) would cause a marked increase in the urinary excretion of hippuric acid. No such

increase occurs (Table 2.9) hence it would appear that species variations in the aromatization of quinic acid may occur at some stage (or stages) during the conversion of shikimic acid to benzoic acid.

Consider the previously suggested sequence of reactions:-

$$\text{QUINIC ACID} \xrightarrow{1} \text{SHIKIMIC ACID} \xrightarrow{2} \text{BENZOIC ACID}$$

Gut bacteria are known to accomplish at least one step in the overall conversion of quinic acid to benzoic acid. If shikimic acid is an intermediate, then it is possible that gut bacteria are responsible for stage 1, the remainder of the conversion to benzoic acid taking place in the tissues.

Intraperitoneal injection of shikimic acid (150 mg./kg.) into Rhesus monkeys causes little or no difference in urinary hippuric acid levels (Table 2.10) (cf. this value with 41% increase in hippurate on oral administration). Thus it appears that gut bacteria are responsible for at least part of the conversion of shikimic acid into benzoic acid, i.e. part of stage 2.

Table 2.9. Aromatization of orally administered shikimic acid in the rat and Rhesus monkey

Animal	Normal level of hippuric acid (mg./day)	Hippuric acid level (mg./day) after feeding shikimic acid	% of dose aromatized
Rat 1	13.8	10.3	0
Rat 2	6.8	17.9	5.4
Rat 3	≏0	13.2	6.4
Rat 4	3.6	2.3	0
Rat 5	4.8	2.76	0
Rat 6	4.8	7.5	1.7
Monkey 1	69.7	763.7	55.9
Monkey 2	105.6	735.0	43.7
Monkey 1	23.4	329.4	38.1
Monkey 2	71.5	382.5	25.8

Dose to rats = 600 mg./kg.

Dose to monkeys = 300 mg./kg. (on 2 separate occasions)

Table 2.10. Aromatization of intraperitoneally-injected shikimic acid in the Rhesus monkey

Animal No.	Normal hippuric acid level (mg./day)	Hippuric acid level after i.p. injection of shikimic acid	% Aromatization
1.	40.8	53.3	3.1
2.	34.2	30	0

Dose of shikimic acid = 150 mg./kg.

SUMMARY

An investigation of the species occurrence of the aromatization of orally administered quinic acid to benzoic acid in 22 species of vertebrates has shown that, of the species examined, the reaction is peculiar to man and the Old World monkeys. In New World monkeys, lorisformes, the tree shrew, eleven species of lower mammals and one species of aves, the pigeon, the increase in excretion of urinary benzoic acid (conjugated or free) associated with the oral administration of quinic acid is either small or negligible.

Treatment with neomycin sulphate before oral administration of quinic acid, or administration of the acid by intraperitoneal injection, causes little or no increase in the urinary excretion of hippuric acid in Rhesus monkeys. Hence it seems probable that at least part of the conversion of quinic acid to benzoic acid is brought about by gut bacteria. Species variations in gut bacteria would therefore appear to be the reason for the species differences observed in the production of benzoic acid from orally administered quinic acid.

Previous work has shown that shikimic acid is an intermediate in the production of aromatic acids from quinic acid by wild-type Aerobacter and Aerobacter mutants (Davis and Weiss, 1953). Oral administration of shikimic acid to Rhesus

monkeys causes an increase in urinary hippurate comparable with that obtained on quinic acid administration. Hence it seems possible that shikimic acid is an intermediate in the *in vivo* conversion of quinic acid to benzoic acid. If shikimic acid is given to Rhesus monkeys by intraperitoneal injection, no increase in urinary hippurate is observed. Thus gut bacteria may well be responsible for at least part of the conversion of shikimic acid to benzoic acid.

In the rat, oral administration of neither quinic acid nor shikimic acid causes any significant increase in urinary hippurate levels. Thus the difference between animals which convert quinic acid to benzoic acid and those which do not may be due to the fact that the latter are unable to carry out some step (or steps) in the conversion of shikimic acid to benzoic acid.

C H A P T E R 3

METABOLISM OF [^{14}C]QUINIC ACID IN THE RAT AND RHESUS MONKEY

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INTRODUCTION

This chapter describes the results of studies on the metabolic fate of [^{14}C]quinic acid in the rat and Rhesus monkey. The Rhesus monkey has been examined as an example of a higher primate which extensively aromatizes quinic acid to benzoic acid; the rat is an example of a sub-primate species which does not carry out this conversion to any appreciable extent.

MATERIALS AND METHODS

Chemicals

[U-^{14}C]-(-)Quinic acid (specific activity 5 mC./m.mole) was purchased from the New England Nuclear Corporation, Dreieichenhain, West Germany; [U-ring-^{14}C]benzoic acid (specific activity 48.2 mC./m.mole) from the Radiochemical Centre, Amersham, Bucks; [carbonyl-^{14}C]hippuric acid (specific activity 0.022 μc/mg.) and benzoyl glucuronide (m.p. 180°C; α_D^{20} - 25.6°, c 0.7 in water) were kindly supplied by Dr. P. Millburn of this Department. Other chemicals were obtained as described in Chapter 2.

Treatment of animals

Oral administration of [^{14}C]quinic acid

Female Wistar albino rats (250 g.) were dosed orally with [^{14}C]quinic acid (350 mg./kg. containing ≏2 μC) and were kept in "Metabowl" metabolism cages (Jencons Ltd., Hemel Hempstead, Herts.) so that urine, faeces and expired CO_2 could be collected daily. Expired CO_2 was trapped in 4N-NaOH and the amount of $^{14}CO_2$ estimated as described below. After 5 days, the rats were killed by cervical dislocation. The amount of radioactivity remaining in the carcass was determined by the method described below.

Rhesus monkeys received [^{14}C]quinic acid (350 mg./kg. containing $\simeq$ 2 μC) mixed in the diet.

In each case, urine was collected for 24 hours before feeding [^{14}C]quinic acid, and subsequently for 2 days. Increases in urinary hippurate could then be estimated fluorimetrically and by radiochemical techniques.

Intravenous injection of [^{14}C]quinic acid

Three female rats were anaesthetised by intraperitoneal injection of hexobarbitone (100 mg./kg.). [^{14}C]Quinic acid (350 mg./kg.) containing 0.65 μC was injected into the femoral vein. The subsequent 24 hr. urine was collected and analysed for [^{14}C]quinic acid and its metabolites.

Intraperitoneal injection of [^{14}C]quinic acid

[^{14}C]Quinic acid (350 mg./kg. containing 0.5 μC) was dissolved in a minimal volume of sterile water, neutralised with $NaHCO_3$, and injected intraperitoneally into a Rhesus monkey. Urine was collected daily and analysed for [^{14}C]quinic acid and its metabolites.

Biliary excretion of [^{14}C]quinic acid in rats

Biliary cannulated rats were prepared according to the

method of Abou-El-Makarem, Milburn, Smith and Williams (1967). [^{14}C]Quinic acid (350 mg./kg. containing 0.36 μC) was injected intraperitoneally and bile collected for the subsequent 4 hours. In one case, the rat was kept in a restraining cage, so that urine could be collected. The ^{14}C content of the samples of bile and urine was estimated as described below.

Estimation of radioactivity in urine, bile, faeces, rat carcass and expired CO_2

Portions of urine (0.1 - 0.5 ml.) and bile (0.1 ml.) were counted in 20 ml. of scintillation fluid of the following composition: naphthalene (60 g.), 2,5-diphenyloxazole (PPO; 4 g.), 2,2-p-phenylenebis (5-phenyl oxazole) (POPOP; 200 mg.), methanol (100 ml.) and ethanediol (20 ml.) made up to 1 litre with dioxan.

^{14}C was counted in a Packard Tri-Carb scintillation spectrometer (Model 3214). The "Channels Ratio" method was used to determine the counting efficiencies of the samples. Knowing the ratio of the counts given by a sample in 2 channels of the scintillation counter, the counting efficiency and hence the absolute activity (in d.p.m.) could be determined. Faeces were homogenised in an equal volume of water. The homogenate was neutralised by addition of solid $NaHCO_3$, centrifuged at 2000 x g for 10 mins, and aliquots of the supernatant counted in the scintillation fluid (20 ml.).

Rat carcasses were suspended in 40% NaOH (1 litre) and heated to 50° for 24 hours. Samples (1 ml.) of dissolved carcass were neutralised (conc. hydrochloric acid) and counted in "Gel" scintillator (5% suspension of Cab-o-Sil, a thixotropic gelling agent supplied by Packard Instrument Co., in the scintillation fluid).

The $^{14}CO_2$ in the expired air was trapped in 4N-NaOH (400 ml.). Samples (20 ml.) were taken and $Ba^{14}CO_3$ was precipitated by adding the supernatant of a saturated solution of $BaCl_2$ to the NaOH, until no more precipitation of $Ba^{14}CO_3$ occurred. The precipitated $Ba^{14}CO_3$ was filtered, washed, dried and weighed. Suspensions of $Ba^{14}CO_3$ in 95% EtOH were layered on to a planchet to give an infinitely thick layer. Planchets were counted using a Panax end-window counter (Model No. D675, Panax Equipment Ltd., Redhill, Surrey). Results were checked by counting $Ba^{14}CO_3$ as a suspension in "Gel" scintillator as described previously.

Identification of urinary metabolites

Fluorimetry - see Chapter 2.

Chromatography

Urine (one half of the total volume excreted in 24 hrs.) was freeze dried and the residue redissolved in a minimal volume

of 90% ethanol (2-5 ml.). Aliquots (0.05 ml.) of the ethanol extraction were banded on Whatman No. 1 paper (1" band on 1½" strips), dried and chromatographed in solvents 1, 2, 3 and 4 by the descending method against authentic samples of possible metabolites (see Table 3.1.). After drying, the chromatograms were scanned for ^{14}C using a Packard Radiochromatogram scanner, Model No. 7200 (Packard Instruments, Wembley, Middlesex).

The ^{14}C content of each peak was estimated by measuring the area of the peak (height of peak x width of peak at half height). The ratio of the activity associated with each peak to the total activity on the paper could thus be determined, and expressed as a percentage of the total activity.

Shikimic acid, catechol, vanillic acid, benzoic acid hippuric acid and benzoyl glucuronide absorb u.v. light (254 nm.). When the chromatograms of these compounds were suspended in liquid nitrogen, illuminated by u.v. light and then the light source removed, the latter 3 compounds exhibited phosphorescence. Hence the position on the chromatogram of each of these compounds was confirmed.

Isotope dilutions for hippuric acid and quinic acid

Hippuric acid

The presence of [^{14}C]hippuric acid in the urine of rats and monkeys given [^{14}C]quinic acid orally was confirmed by reverse isotope dilution. Hippuric acid (0.5 g.) was added to the radioactive urine (5-20 ml.). 10N Sodium hydroxide was then added dropwise until all the hippuric acid had dissolved. The urine was adjusted to pH 2 with 10N-hydrochloric acid and extracted with ethyl acetate (3 x 40 ml.). The extract was evaporated to dryness in vacuo, and the residue recrystallized from water until constant specific activity was attained. In most cases, the results obtained by reverse isotope dilution agreed with those obtained by the fluorimetric method. However, in one case (rat C, Table 3.4), there was a discrepancy between the two methods (3% increase in hippurate when estimated fluorimetrically, compared with 12.7% when estimated by reverse isotope dilution). In this case, the p-nitrobenzyl derivative of hippuric acid was prepared. [^{14}C]Hippuric acid (0.123 g.) (obtained from reverse isotope dilution of the rat urine for hippuric acid) was suspended in water (0.5 ml.). Two drops of phenolphthalein were added as indicator and 10% sodium hydroxide added until the hippuric acid was neutralised. The solution was made slightly acid using dilute hydrochloric acid.

p-Nitrobenzyl bromide (0.123 g.) in 95% EtOH (2 ml.) was introduced and the mixture heated under reflux for 1 hour. On cooling, the product was filtered, washed with alcohol and then water. Recrystallization was carried out by dissolving the p-nitrobenzyl hippurate in hot alcohol, adding hot water to produce a turbidity, clearing the latter with a few drops of alcohol and allowing to cool, when crystallization occurred. The recrystallization procedure was repeated until constant specific activity was maintained (M.pt. 136°C). The specific activity per mole of this derivative agreed with the value obtained by reverse isotope dilution for [^{14}C]hippuric acid.

Quinic acid

The presence of unchanged [^{14}C]quinic acid in the urine of rats given [^{14}C]quinic acid intravenously and monkeys given the acid by intraperitoneal injection, was confirmed by reverse isotope dilution. Urine was centrifuged at 2000 xg. for 10 mins. Quinic acid (1 g.) was added to 1.5 ml. samples of the supernatant and dissolved by warming gently. On leaving the solution overnight at 4°C, colourless crystals of quinic acid were formed (M.pt. 162°C). The [^{14}C]quinic acid was recrystallized from small volumes of hot water, to constant specific activity.

RESULTS AND DISCUSSION

Chromatography

R_f values of quinic acid and possible urinary metabolites are shown in Table 3.1.

Fate of orally administered [^{14}C]quinic acid in the rat and Rhesus monkey

(a) Pattern of excretion

The pattern of excretion of an orally administered dose of [^{14}C]quinic acid (350 mg./kg.; $\pm$ 2 μC) in rats and Rhesus monkeys is shown in Table 3.2.

In rats, about 33% (20.2% - 43.1%) of the dose was recovered in the 0-48 hr. urine, and 22% (7.2% - 31.8%) in the 0-48 hr. faeces. $^{14}CO_2$ in the expired air accounted for 32% (19.2% - 53.6%) of the administered dose. The total amount of ^{14}C recovered, including that remaining in the carcass after 5 days, was about 90% (86.9% - 94.2%). Since the intestinal flora in the rat are responsible for at least part of the metabolism of quinic acid (i.e. the small amount of conversion to benzoic acid - see Chapter 2) then the variations observed in the pattern of excretion of orally administered [^{14}C]quinic acid may be due to individual variations in the bacterial population of the intestine of these animals. The role of

Table 3.1. R_f values of quinic acid and some possible urinary metabolites

Whatman No. 1 paper was used. The solvents were:

1. sec.-butanol:glacial acetic acid:water (14:1:5)
2. n-butanol:ethanol:water:glacial acetic acid (30:10:10:1)
3. benzyl alcohol:tert.-butanol:iso-propanol:water (30:10:10:10) containing 1.8% W/V formic acid
4. phenol:water (3:1 W/V) containing 0.9% W/V formic acid

(Descending chromatograms were run for 14 hours in all four systems.)

Compound	R_f value in solvent:-			
	1	2	3	4
Quinic acid[1]	0.25	0.1	0.27	0.53
Shikimic acid[2]	0.55	0.5	0.44	0.57
Benzoic acid[2]	0.93	0.85	0.95	0.86
Hippuric acid[2]	0.83	0.75	0.95	0.85
Benzoyl glucuronide[2]	0.75	0.6	-	-
Vanillic acid[2]	0.89	0.86	-	0.86
Catechol[2]	0.88	0.84	0.85	0.77

1 = R_f obtained from radiochromatogram scanning

2 = R_f obtained from u.v. absorption of compound

Table 3.2. Excretion of radioactivity by the rat and Rhesus monkey after an oral dose of [^{14}C]quinic acid

Material Examined	Day after dosing	% of dose excreted by:-							
		RAT				MONKEY			
		A	B	C	D	E^{+}	E^{+}	F	G
Urine	1	43	30.5	24.1	13.8	21	24.6	34.1	45.8
	2	0.1	9.3	5.4	6.4		1.6	28.2	2.1
Faeces	1	20	19.2	6.4	0.8	-	-	-	-
	2	0.3	9.3	0.8	31				
Expired air as $^{14}CO_2$	1 and 2	20	19.2	53.6	35.9	-	-	-	-
Carcass	5	3.5	1.3	3.9	2.9	-	-	-	-
Total ^{14}C recovered		86.9	88.8	94.2	90.8	21	26.2	62.3	47.9

+ = 2 separate experiments on the same monkey

- = not determined

Dose of quinic acid = 350 mg./kg.; $\simeq$ 2 μC.

the intestinal flora in the metabolism of quinic acid is considered in Chapter 4.

In Rhesus monkeys, the amount of activity recovered in two days' urine varied between 26.2% and 62.3% (average value 45%) of the dose. Facilities for collecting expired air and faeces from the monkeys were not available. Variations in the ^{14}C content of monkey urine were partly due to the fact that, although precautions were taken to avoid loss of urine, nevertheless, small portions of urine were probably lost either on the walls of the cages or through small leakages in the urine collection system. Another reason for the low recovery of ^{14}C in monkey urine may be that, as in rats, part of the administered dose was oxidised to $^{14}CO_2$ and part excreted in the faeces.

(b) Urinary metabolites of [^{14}C]quinic acid in the rat and Rhesus monkey

Table 3.3 shows the urinary metabolites of [^{14}C]quinic acid (350 mg./kg.; $\simeq$ 2 μC) when administered orally to the rat and Rhesus monkey.

In rats, extremely variable amounts of [^{14}C]quinic acid were excreted in the urine, between 0 and 33% of the dose appearing in the first 24 hours. Rats A, B and D aromatized less than 4% of the dose to hippuric acid, but in rat C

Table 3.3. Urinary metabolites of orally administered [^{14}C]quinic acid in the rat and Rhesus monkey

Animal	Rat				Monkey			
	A	B	C	D	E+	E+	F	G
% of dose in 0-24 hr. urine	43	30.5	24.1	13.8	21	24.6	34.1	45.8
% of dose as: (quinic acid	33	4.9	0	0	0	0	traces	traces
(hippuric acid	0	0.6	14.5	3.9	19	24.6	33	45
(unknown X	2.9	12.5	0	2.3	0	0	0	0
(unknown Y	5.2	6.2	0	4.2	2	0	0	0
(unknown Z	2.9	6.3	9.6	3.3	0	0	0	0

+ = 2 separate experiments on the same monkey

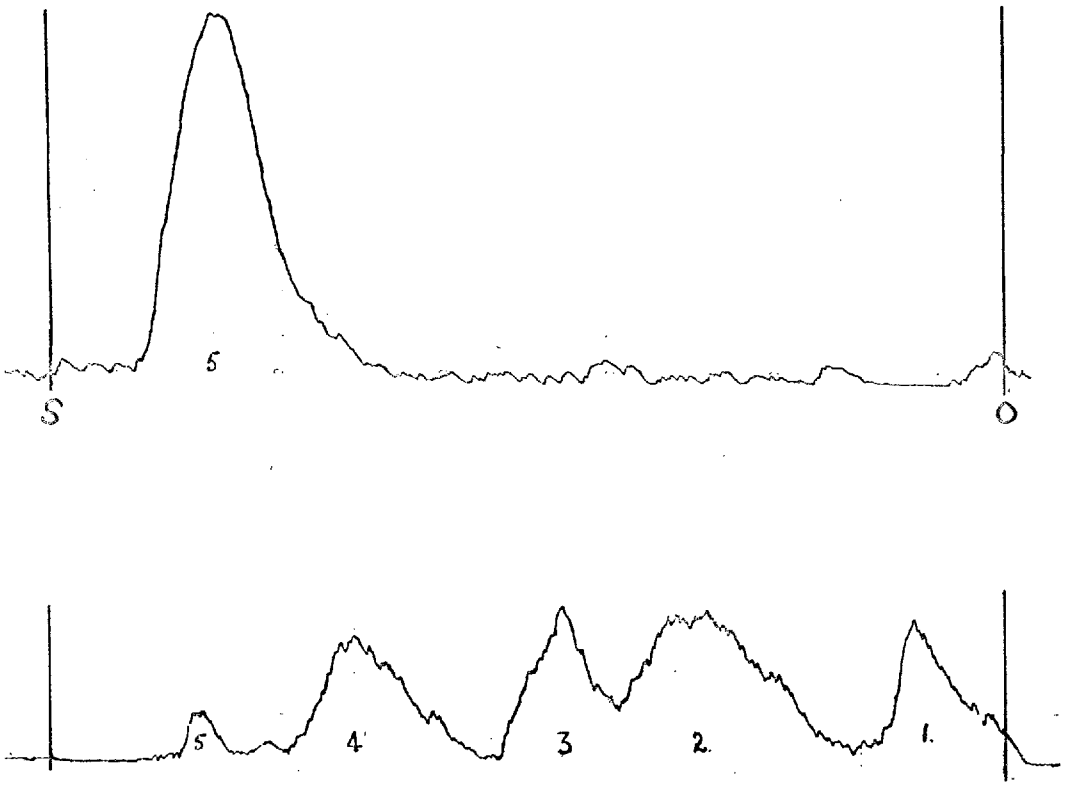
Dose of [^{14}C]quinic acid = 350 mg./kg.; ≙ 2 µC.

there was an exceptionally high degree of conversion to hippuric acid (14.5% of the dose). This value was confirmed by reverse isotopic dilution of the urine for [^{14}C]hippuric acid (see Table 3.4) and also by synthesising the p-nitrobenzyl derivative of the latter (see Methods). The urine of rats A, B and D contained 3 unknown metabolites; one of these (Y) had chromatographic properties similar to those of shikimic acid (Table 3.1; Figure 3.1). The other 2 metabolites were not identified.

Previous work by Booth, Robbins, Masri and De Eds (1960) has shown that, on acid hydrolysis of the ether-extracted urine of rats given quinic acid, catechol and vanillic acid are present. Polyhydroxy-acids such as quinic acid and shikimic acid are readily converted chemically into aromatic derivatives, e.g. shikimic acid is converted into p-hydroxybenzoic acid by the action of hydrochloric acid on a heated water bath (Posternak, 1965). Therefore, perhaps a better method of identification of the urinary metabolites of quinic acid in the rat, would be by the isolation of these metabolites.

In the present work, the chromatographic properties of the unknown metabolites X and Z were different from those of authentic samples of catechol and vanillic acid. However, the possibility that X and Z are conjugates of catechol and

Fig. 3.1. Radiochromatogram scans obtained from urines of the rat and Rhesus monkey after oral administration of [^{14}C]quinic acid



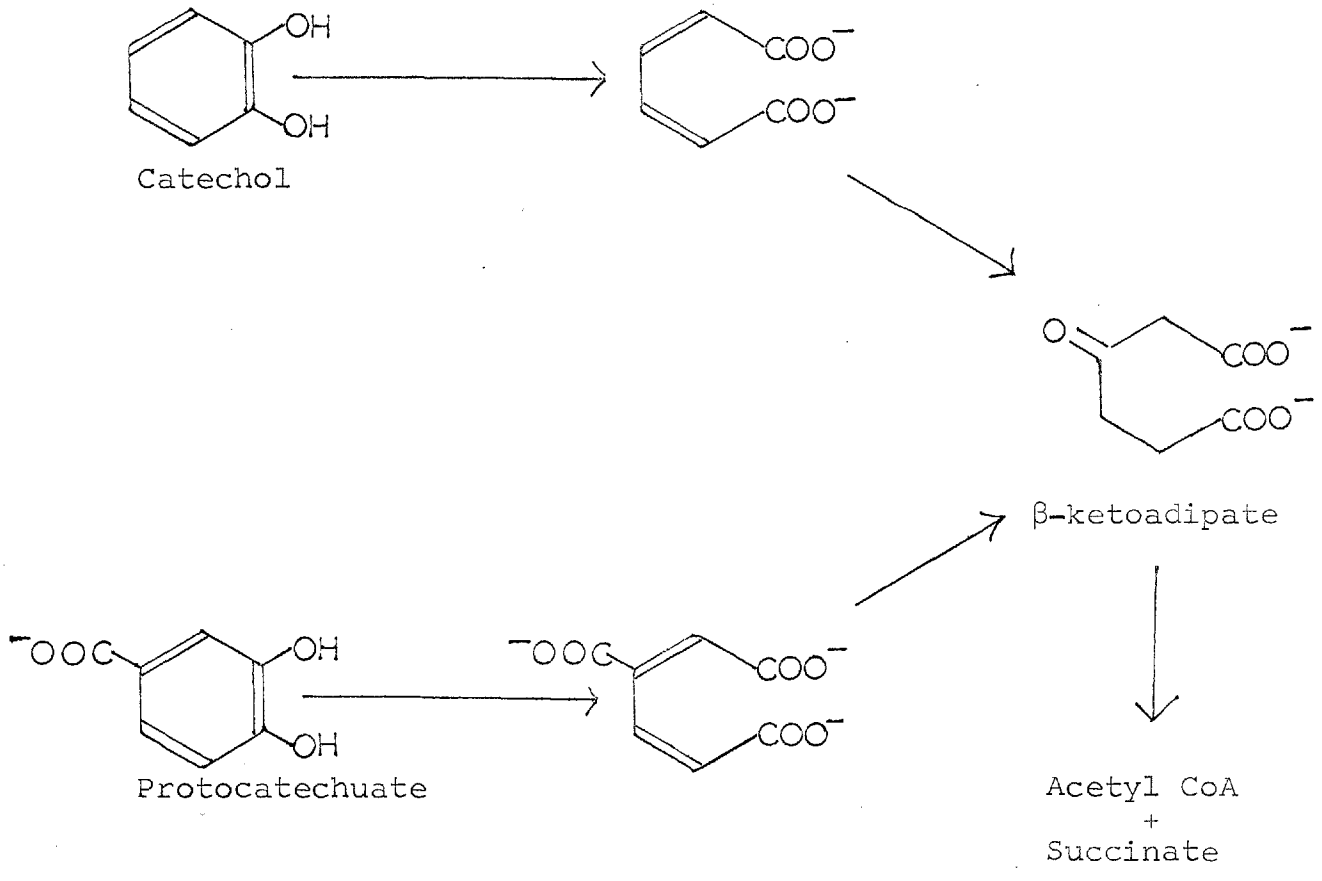
Solvent: n-Butanol:Ethanol:Water:Acetic acid

O = origin
S = solvent front

1 = quinic acid
2 = unknown X
3 = shikimic acid?
4 = unknown Z
5 = hippuric acid

vanillic acid has not been ruled out. When catechol (100 mg./kg.) is given to rabbits by stomach tube, it is excreted in urine as the monoglucuronide (70%), the monosulphate (18%), with approximately 2% excreted as free catechol, and traces of conjugated hydroxyquinol (Garton and Williams, 1948).

Scheline (1968a) has proposed that polyhydroxy acids such as quinic acid can be converted by the intestinal microflora to 3,4-dihydroxybenzoic acid (protocatechuic acid) which can be partly decarboxylated in the intestine to give catechol and partly absorbed and methylated to give vanillic acid. o-Diphenols such as catechol and protocatechuate are substrates for oxygenative ring cleavage by bacteria (Ornston and Stanier, 1964). The bacterium Pseudomonas fluorescens metabolises catechol and protocatechuate according to the scheme drawn out below:-



The presence of o-diphenols or their metabolites in rat urine would help to explain the large amount of $^{14}CO_2$ expired after oral administration of [^{14}C]quinic acid, since oxidative cleavage of these phenols would lead to the production of Acetyl CoA and succinate, and thence to CO_2.

Thus it is possible that when quinic acid is given orally to the rat, it is metabolized by the intestinal flora to

catechol and protocatechuic acid. The catechol, so produced, is partly absorbed and excreted in the urine as a conjugate; and partly oxidised to CO_2 by the intestinal flora. The protocatechuic acid is partly absorbed, methylated to vanillic acid and then excreted as a conjugate in the urine; and also partly oxidised to CO_2 by the gut flora.

In each of the Rhesus monkeys given [^{14}C]quinic acid orally, over 90% of the activity in the urine was present as [^{14}C]hippuric acid. The presence of [^{14}C]hippuric acid was confirmed by reverse isotope dilution (see Table 3.4). In the urine of 2 monkeys (F and G there were traces of unchanged [^{14}C]quinic acid; and in the urine of a third monkey (E^1) one unknown metabolite accounted for 2% of the dose. This metabolite had similar chromatographic properties to shikimic acid.

Choice of method of estimation of urinary hippuric acid

In Chapter 2, it was suggested that the fluorimetric estimation of changes in urinary hippuric acid would be an accurate determination of large increases due to the aromatization of orally administered quinic acid (as in Rhesus monkey) but that the accuracy of this method in determining smaller increases (as in rat) might be less, due to fluctuations in the "normal" level of urinary excretion of hippuric acid.

When [^{14}C]quinic acid became available, it was decided to compare the fluorimetric estimation of changes in urinary hippuric acid, after giving [^{14}C]quinic acid, with radiochemical techniques (Table 3.4).

In the case of rat urine analysis, the results obtained from the reverse isotope dilution and radiochromatogram scanning procedures agreed well. However, the fluorimetric estimation was not as accurate, possibly due to fluctuations in "normal" hippuric acid levels in rat urine.

Agreement between the values obtained from these different methods is much closer, however, in the estimation of increases in urinary hippuric acid due to the aromatization of orally administered quinic acid in Rhesus monkeys.

Thus it would appear that the fluorimetric method estimates large increases in hippuric acid with a reasonable degree of accuracy. If small increases in urinary hippuric acid, due to aromatization of orally administered quinic acid, are to be estimated accurately, however, then radiochemical techniques must be employed. Using these techniques, it can be seen that in rats, the degree of aromatization of orally fed [^{14}C]quinic acid is small but variable (0.05% to 3.6% of the dose. Rat C is probably an exception.).

Table 3.4. Comparison between radiochromatogram scanning, fluorimetry and reverse isotope dilution methods of determining increases in urinary hippuric acid after oral administration of [^{14}C]quinic acid to the rat and Rhesus monkey

Method of estimation	% of oral dose of [^{14}C]quinic acid excreted as hippuric acid in the urine of:-							
	Rat				Monkey			
	A	B	C	D	E	E	F	G
Fluorimetry	0	5	3	0	-	18	26.6	35
Reverse isotope dilution	0.05	0.6	12.7	3.6	16.6	21	26.1	39.3
Radiochromatogram scanning	0	0.6	14.5	3.9	19	24.6	33	45

For details of animals and doses, see Table 3.2

Fate of intravenously injected [^{14}C]quinic acid in the rat and intraperitoneally injected [^{14}C]quinic acid in the Rhesus monkey

When quinic acid is administered orally to rats, 4-8% of the dose is aromatized to benzoic acid. No aromatization occurs if the rats are pretreated with neomycin sulphate. Rhesus monkeys convert quinic acid to hippuric acid to a considerable extent (40% of the dose) when the acid is administered orally, but when given by intraperitoneal injection, no corresponding increase in urinary hippurate is observed (see Chapter 2). The aim of this experiment was to determine

(1) the fate of [^{14}C]quinic acid in the rat, when injected intravenously (350 mg./kg. containing 0.65 μC).

(2) its fate in the Rhesus monkey when injected intraperitoneally (350 mg./kg. containing 0.5 μC).

The rats and Rhesus monkey were injected with [^{14}C]quinic acid and the urine collected and analysed as described under "Methods".

On chromatography in systems 1, 2, 3 and 4 (see Table 3.1) and subsequent radiochromatogram scanning, one peak of activity was observed for the urines of the rats and monkey. In each

case, the R_f value corresponded with that of authentic [^{14}C]quinic acid. In order to confirm the identity as quinic acid, reverse isotope dilutions were carried out, as described under "Methods", on one sample of rat urine and the monkey urine. In each case 96-97% of the activity in the urine was found to be present as [^{14}C]quinic acid (see Table 3.5).

Thus it would appear that when quinic acid is given by a route which avoids the gastro-intestinal tract, it is excreted unchanged in the urine of both rats and Rhesus monkeys.

If the sequence of reactions leading from quinic acid to benzoic acid is as suggested in Chapter 2:-

$$\text{QUINIC ACID} \xrightarrow{1} \text{SHIKIMIC ACID} \xrightarrow{2} \text{BENZOIC ACID}$$

and if it can be assumed that the gut bacteria are implicated in stage 2 (Chapter 2), then the fact that intraperitoneally injected quinic acid is excreted unchanged indicates that the intestinal flora must also be involved in stage 1 of the transformation.

Biliary excretion of intraperitoneally injected [^{14}C]quinic acid in the rat.

If an orally administered compound is to reach the lower part of the gastro-intestinal tract, there are 2 possibilities:-

Table 3.5. Urinary metabolites of intravenously injected [^{14}C]-quinic acid in the rat, and intraperitoneally injected [^{14}C]quinic acid in the Rhesus monkey

Animal	% of dose excreted in 0-24 hr. urine	% of dose of [^{14}C]quinic acid (350 mg./kg.) as unchanged acid
Rat 1	49.2	49.2*[1]
Rat 2	64.6	64.6
Rat 3	29.2	29.2
Rhesus monkey	66.8	66.8*[2]

*[1] Reverse isotope dilution gave value of 47.7%

*[2] Reverse isotope dilution gave value of 64.3%

(1) little or no absorption from the stomach and small intestine and passage into the lower gut,

(2) absorption from the gastrointestinal tract followed by secretion into the digestive tract.

As the most important secretion into the enteric tract is the bile (Smith, 1966) the biliary excretion of intraperitoneally injected [^{14}C]quinic acid (350 mg./kg.) was examined in the rat (see Table 3.6). Less than 1% of the dose was excreted in the bile in 4 hours. During this period, the [^{14}C] content of the urine of rat 1 accounted for 73% of the dose. Thus it appears that quinic acid is not significantly excreted in the bile. Quinic acid has a molecular weight of 192. Thus the insignificant biliary excretion of this compound supports the theory that, in order that a compound is excreted in the bile in appreciable amounts in the rat, it must have a molecular weight not less than 300 (Millburn, Smith and Williams, 1967).

Quinic acid is excreted unchanged when given by a route of administration which avoids the gastro-intestinal tract. Furthermore, biliary excretion of this compound is insignificant. Thus it would appear that on <u>oral</u> administration, quinic acid is not appreciably absorbed from the stomach and small intestine, but passes directly into the lower gut.

Table 3.6. Biliary excretion of intraperitoneally injected [^{14}C]quinic acid in the rat

Rat No.	% of dose recovered in bile in 4 hr.	% of dose recovered in urine in 4 hr.
1	0.47	73
2	0.17	Not determined
3	0.15	Not determined

Dose of quinic acid = 350 mg./kg.; 0.36 μC.

SUMMARY

On oral administration of [^{14}C]quinic acid to the rat, approximately 33% of the dose is excreted in the urine, 22% in the faeces and 32% expired as $^{14}CO_2$. The pattern of urinary excretion shows large individual variations, 0-33% of the dose is excreted unchanged, and 0-14.5% of the dose is aromatized to benzoic acid, which is then conjugated and excreted as hippuric acid. The remaining 3 metabolites have not been identified, although one of these has similar chromatographic properties to those of shikimic acid. It is suggested that the other 2 unidentified metabolites may be conjugates of catechol and vanillic acid.

Rhesus monkeys given [^{14}C]quinic acid orally, excrete approximately 32% of the dose in the urine. More than 90% of the urinary ^{14}C content is present as [^{14}C]hippuric acid. Small amounts of unchanged [^{14}C]quinic acid and one other metabolite (chromatographically similar to shikimic acid) account for the remainder of the activity in the urine.

When [^{14}C]quinic acid is administered to rats or Rhesus monkeys by a route avoiding the gastro-intestinal tract, it is excreted in the urine as unchanged [^{14}C]quinic acid. This observation, together with the finding that biliary excretion

of [^{14}C]quinic acid in the rat occurs to a very small extent (<1% of the dose) indicates that on oral administration, quinic acid is not absorbed from the stomach or upper intestine, but passes directly into the lower intestine, where it is metabolized.

C H A P T E R 4

INVESTIGATION OF THE AROMATIZATION OF QUINIC ACID BY GUT BACTERIA

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INTRODUCTION

Metabolism by the intestinal microflora

In the preceding two chapters it has been shown that the aromatization of quinic acid is, in part at least, dependent on the gut bacteria. The metabolic activity of the bacteria in the gut is potentially equal to that of the liver (Drasar, Hill and Williams, 1971). Several articles have reviewed the reactions known to be carried out by the intestinal flora (Smith, 1966; Scheline, 1968a; Drasar *et al.*, 1971). These reactions can be divided into 3 main groups:-

(a) hydrolytic reactions,
(b) removal or modification of substituent groups,
(c) modification of the basic skeleton of the compound (see Fig. 4.1).

(a) Hydrolytic reactions

Compounds known to be hydrolysed in the gut include glucuronides, glycine conjugates, glycosides, glutathione conjugates, ethereal sulphates, amides, acetyl conjugates and esters.

(i) Glucuronides: β-glucuronidase activity has been found in the alimentary tract of the sheep, cow, horse, rabbit,

rat, pig, cat and fowl (Marsh, Alexander and Levvy, 1952). Several glucuronides have been fed orally, and it has been shown that hydrolysis occurs in the gastrointestinal tract, e.g. phenylglucuronide is partly hydrolysed in the gut to free phenol which is then oxidized to polyphenols (Garton and Williams, 1949).

(ii) Glycine conjugates: Relatively little is known about the metabolism of glycine conjugates by intestinal flora, although the peptide bond in glycocholic acid is hydrolysed on incubation with rat faecal suspensions; and rat caecal extracts, when incubated anaerobically with p-aminohippuric acid, produce p-aminobenzoic acid (Scheline, 1968b).

(iii) Glycosides: Phenyl-β-D-glucoside undergoes extensive hydrolysis on aerobic incubation with rat caecal extract, the aglycone being the only metabolite detected (Scheline, 1968b).

(iv) Glutathione conjugates: Glutathionase, the enzyme responsible for the hydrolysis of glutathione conjugates, is present in the small intestine of rats. It rapidly breaks down S-(p-chlorobenzyl)glutathione in vitro; one of the

products of hydrolysis of this conjugate is S-(p-chlorobenzyl)-L-cysteine (Bray, Franklin and James, 1959). Glutathione conjugates which are excreted in the bile, may be broken down in the gut to the cysteine conjugates. These are then absorbed and N-acetylated to mercapturic acids (Smith, 1966).

(v) Ethereal sulphates: Since rat and human gut contain significant amounts of arylsulphatase activity (Dodgson, Spencer and Thomas, 1953; Dodgson, Spencer and Wynn, 1956) some hydrolysis of sulphate esters might be expected to occur in the intestine. Certain ethereal sulphates, e.g. 1- and 2-naphthyl sulphate, are hydrolysed to only 4-12% of the orally administered dose (Hawkins and Young, 1954). Sodium oestrone sulphate, however, is extensively hydrolysed on oral administration (Hanahan and Everett, 1950). It should be pointed out that hydrolysis of the latter conjugate occurs to a similar extent when it is administered intravenously or subcutaneously. Furthermore, of the many tissues examined, only liver homogenate contained an enzyme capable of hydrolysing the sulphate ester.

(vi) Amides: Phthalylsulphathiazole and succinylsulphathiazole are intestinal antiseptics which owe

their activity to the liberation of sulphathiazole in the large intestine, as a result of bacterial hydrolysis (Scheline, 1968a). Rat caecal microorganisms, when incubated with succinylsulphathiazole, bring about this hydrolysis (Scheline, 1968b).

(vii) Acetyl conjugates: Rat caecal preparations were found to deacetylate p-acetamidobenzoic acid in 2 out of 6 experiments, although no deacetylation was detected with N^4-acetylsulphanilamide (Scheline, 1968b).

(viii) Esters: Succinic acid esters of chloramphenicol undergo intestinal hydrolysis prior to absorption (Scheline, 1968a). Carbenoxolone, the hemisuccinate of β-glycyrrhetic acid is principally hydrolysed in the gastrointestinal tract (Iveson, Parke and Williams, 1966). It was assumed that intestinal lipases were bringing about these hydrolyses. However, methyl gallate is hydrolysed to gallic acid by rat caecal microorganisms; and chlorogenic acid, the quinic acid ester of caffeic acid, is hydrolysed and further metabolized to m-hydroxyphenylpropionic acid when incubated with rat intestinal microorganisms. Hence it seems possible that bacterial esterases may be responsible for the intestinal hydrolysis of esters (Scheline, 1968b).

(b) Removal or modification of substituent groups

This category of reaction carried out by the intestinal flora includes dehydroxylation, decarboxylation, O-demethylation, reduction and dehalogenation.

(i) Dehydroxylation: This reaction appears to occur only in the gut of mammals and does not occur in other tissues (Smith, 1966). Dehydroxylation occurs with compounds containing alcoholic or phenolic hydroxyl groups.

In the rat, the main product of dehydroxylation of caffeic acid (3,4-dihydroxycinnamic acid) is m-hydroxyphenylpropionic acid (Booth, Emerson, Jones and De Eds, 1957). This metabolite is found in the urine whether the acid is administered orally or by intraperitoneal injection. However, rats maintained on a purified diet containing caffeic acid (2 mg./g. diet) and neomycin (2 mg./g. diet) do not excrete m-hydroxyphenylpropionic acid in the urine. Addition of the intestinal antiseptic "Dimol" to the rat diet also prevents dehydroxylation of caffeic acid (Booth and Williams, 1963a). The possibility that the intestinal flora are responsible for the dehydroxylation was confirmed when incubates of rat or rabbit caecal contents, or sheep rumen contents with caffeic acid were found to contain 3,4-dihydroxyphenylpropionic acid, m-hydroxyphenylpropionic acid and m-coumaric acid. Further

studies on dehydroxylation have led to the isolation from rat faeces, of a strain of Pseudomonas species which has the ability to dehydroxylate caffeic acid, yielding both m-hydroxyphenylpropionic acid and m-coumaric acid (Perez-Silva, Rodrigues and Perez-Silva, 1966).

(ii) Decarboxylation: The metabolism of many phenolic acid derivatives by rat caecal microorganisms has been studied. It appears that a free p-hydroxy group is essential for decarboxylation. For example, p-hydroxybenzoic acid is extensively metabolized to phenol, whereas o- and m-hydroxybenzoic acids are unaffected (Scheline, 1968a).

(iii) O-Demethylation: Many phenolic acids containing methoxy groups undergo demethylation on incubation with rat caecal microorganisms. The reaction has been found to occur with methoxy derivatives of benzoic, phenylacetic, phenylpropionic and cinnamic acids (Scheline, 1968a).

(iv) Reduction: Intestinal bacteria are known to bring about the reduction of nitro-groups. Studies on the metabolism of chloramphenicol in the rat have shown that the nitro-group can be reduced by tissue enzymes, but the greater

amount of reduction takes place in the intestinal tract. Large quantities of chloramphenicol glucuronide are excreted in the bile of rats. The glucuronide is poorly absorbed from the small intestine. In the caecum and large intestine it is converted into chloramphenicol which is then reduced to aromatic amines by bacterial action (Glazko, Dill and Wolf, 1952).

The reduction of azo-compounds is also brought about by the intestinal flora. Radomski and Mellinger (1962) studied the metabolism of the disulphonated dyes amaranth [1-(4-sulpho-1-naphthylazo)-2-naphthol-3,6-disulphonic acid trisodium salt], Ponceau SX, [2-(5-sulpho-2,4-xylylazo)-1-naphthol-4-sulphonic acid disodium salt], and sunset yellow (1-p-sulphophenylazo-2-naphthol-6-sulphonic acid disodium salt) in rats. They found that the liver azo-reductase system is relatively unimportant and that the dyes were reduced to the sulphonated amines mainly by the intestinal bacteria.

Finally, the reduction of double bonds by intestinal micro-organisms has been studied in the metabolism of unsaturated fatty acids. Hydrogenation of α-linolenic acid can be brought about by a mixed population of sheep rumen microorganisms (Wilde and Dawson, 1966).

(v) Dehalogenation: The insecticide DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)-ethane] is partly metabolized to DDD

[1,1-dichloro-2,2-bis(p-chlorophenyl)ethane] when given orally to rats, but not when given by intraperitoneal injection. Aerobacter aerogenes and Escherichia coli isolated from rat intestine dechlorinate DDT (Mendel and Walton, 1966). Hence it appears that, in the rat, DDT is converted to DDD by the intestinal flora.

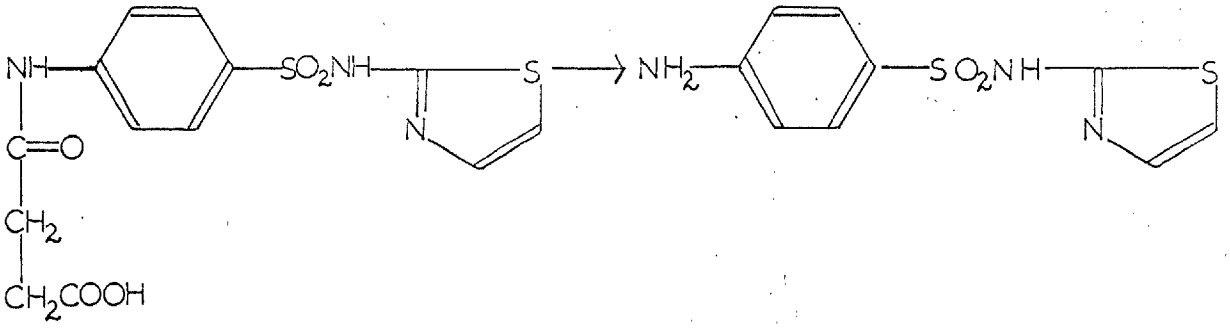
(c) Modification of the basic skeleton of compounds

Ring fission of heterocyclic oxygen compounds is a common reaction of gut bacteria. Booth and Williams (1963b) found that rutin (the glycoside of the flavonol quercetin [3,5,7,3',4'-pentahydroxyflavone]) was converted to m-hydroxyphenylpropionic acid by rat intestinal microorganisms. Scheline (1968b) showed the presence of another ring fission product, namely, m-hydroxyphenylacetic acid, on incubation of rutin with rat caecal extracts. Both of these meta-hydroxylated acids are found in the urine of rats given quercetin orally (Nakagawa, Shetlar and Wender, 1965). However, they are not found in the urine of rats pretreated with neomycin. Thus it is probable that ring fission of flavonols in rats is achieved by the gut bacteria.

Fig. 4.1.

Some reactions brought about by gut bacteria

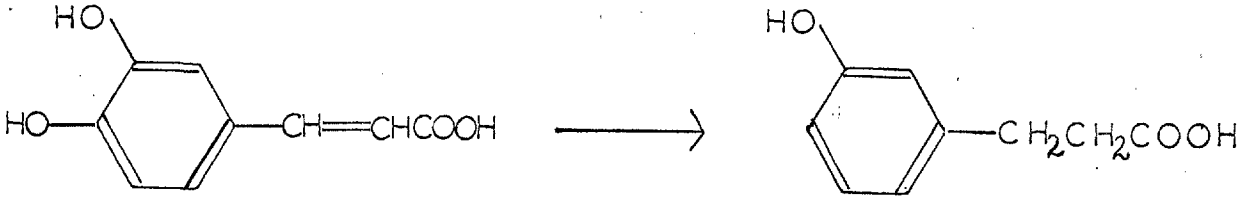
(1) Hydrolysis



Succinylsulphathiazole

Sulphathiazole

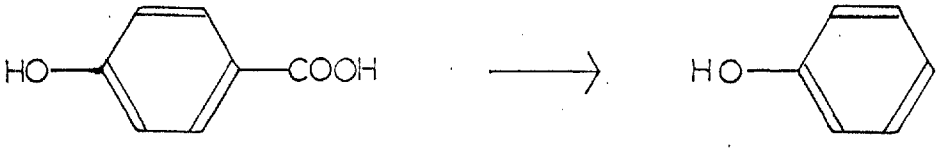
(2) Dehydroxylation



Caffeic acid

m-Hydroxyphenylpropionic acid

(3) Decarboxylation

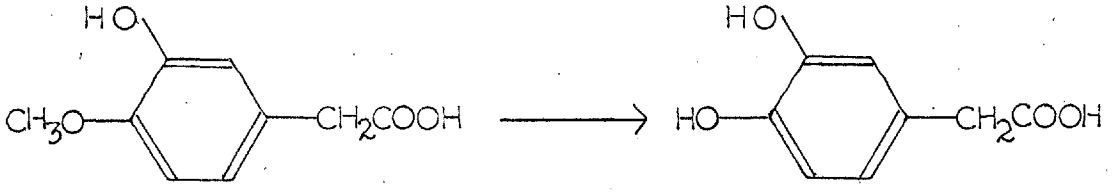


p-Hydroxybenzoic acid

Phenol

Fig. 4.1. (cont'd)

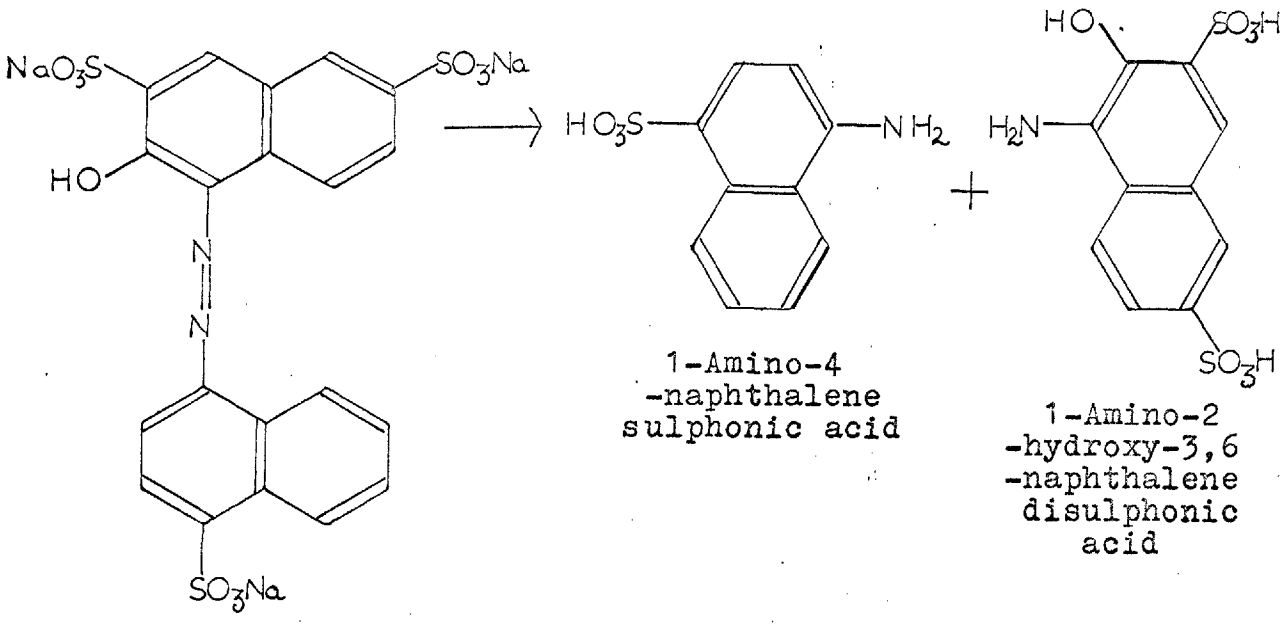
(4) O-Demethylation



3-Hydroxy-4-methoxy-phenylacetic acid

3,4-Dihydroxyphenylacetic acid

(5) Azo-reduction

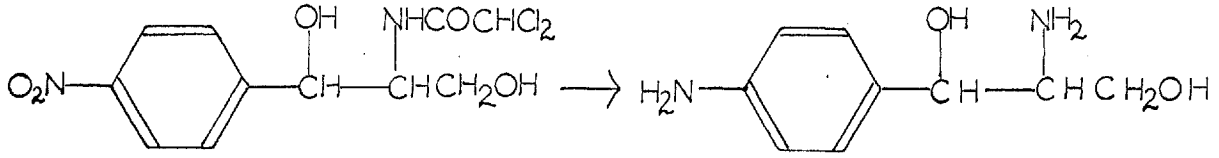


Amaranth

1-Amino-4-naphthalene sulphonic acid

1-Amino-2-hydroxy-3,6-naphthalene disulphonic acid

(6) Nitro-reduction

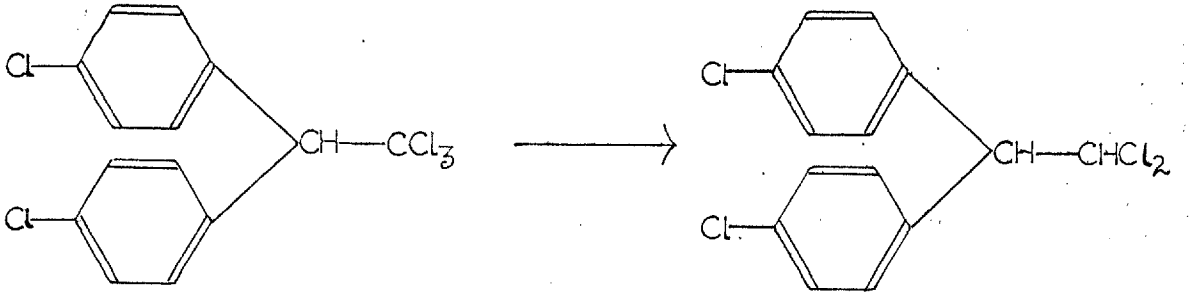


Chloramphenicol

Aryl amine

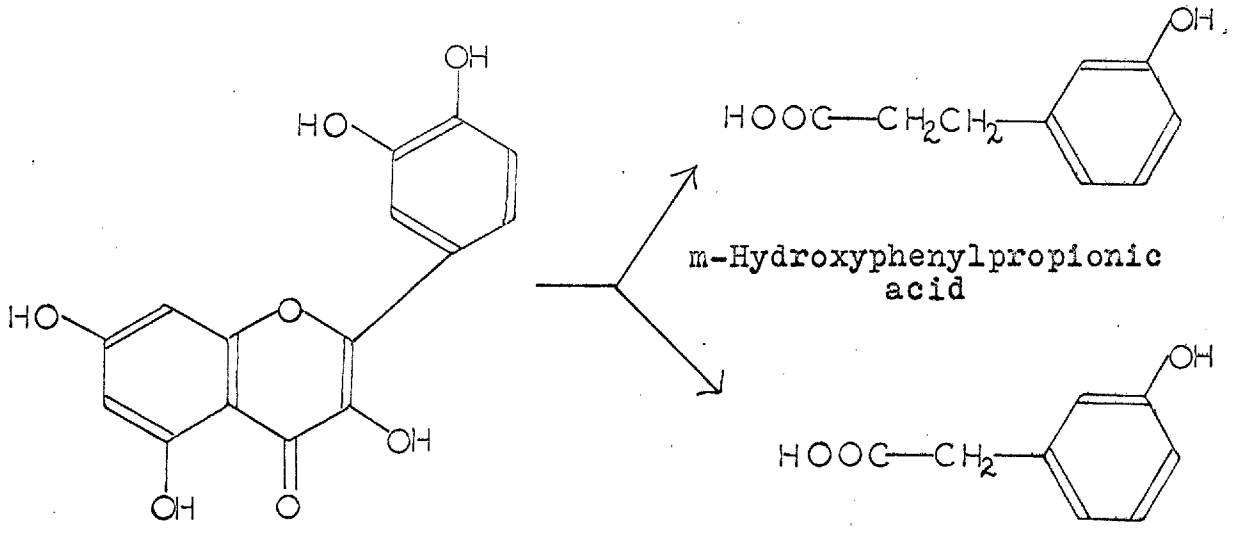
Fig. 4.1. (cont'd)

(7) Dehalogenation



D.D.T. D.D.D.

(8) Ring fission



Quercetin

Species variations in the intestinal microflora

The normal gastrointestinal flora of man and the common laboratory animals has not been extensively studied in the past. Recently the subject has received more attention, because of the realization of the potential of the intestinal flora to carry out many metabolic transformations. The complexity of such a study is partly due to the fact that (1) over 60 different species of bacteria have been isolated from the intestinal tracts or faeces of healthy animals including man (Donaldson, 1964), and partly (2) that many of the dominant bacteria in human and animal faeces are oxygen sensitive, hence requiring great care in their cultivation (Drasar, 1967).

Williams-Smith (1965) compared the viable counts of bacteria and yeasts in different parts of the stomach and small intestine, caecum, colon and faeces of 15 homothermic animals fed on conventional diets. The flora were found to be qualitatively similar, but great variations occurred in the extent of the population in various regions of the alimentary tract. Large numbers of organisms were found in the anterior compartment of the stomach where the pH was sufficiently high to permit bacterial multiplication, and much lower numbers in the posterior compartment, due to the bactericidal effect of the low pH. The numbers of bacteria increased progressively

from the duodenum to the ileum, and the highest numbers occurred in the large intestine. Lactobacilli, Escherichia coli, streptococci, Clostridium welchii and veillonellae all followed this pattern. Yeasts were found in similar numbers in all regions of the alimentary tract and bacteroides were usually confined to the large intestine. These groups formed the majority of the alimentary flora in the animals studied. The rabbit was found to be somewhat exceptional since the stomach and small intestine were, in most cases, sterile; also, the flora of the large intestine consisted almost entirely of bacteroides.

It is suggested that variations in the diet probably account for some of the differences between the flora of the various species, while the physiology of the stomach and feeding habits account for others.

More recently, the intestinal flora of man and some common laboratory animals have been compared using stringent methods of cultivation (Drasar, Hill and Williams, 1971). The results are tabulated below.

Region of G.I. tract	Log_{10} total number of viable organisms per gram of sample wet weight				
	Man	Rabbit	Guinea pig	Rat	Mouse
Stomach	0-5	0-6	5-6	7-9	7-9
Proximal intestine	0-5	0-5	5-6	6-8	7-9
Distal small intestine	6-7	6-7	6-7	7-8	7-8
Large intestine	7-10	8-9	8-9	8-9	9-9
Rectum and faeces	10-11	9-10	9-10	9-10	9-10

0 -N indicates a range from zero to N

In man, guinea pig and rabbit, the stomach and upper intestine are almost sterile, whereas in rat, and mouse ~~and guinea pig~~, they are heavily colonised. The lower small intestine, large intestine, rectum and faeces of all the species investigated appear to be colonised to a similar extent. Bacteroides and bifidobacteria are the dominant organisms in the faeces of all these species. However, there are more lactobacilli and streptococci in the faeces of rats and mice than in other animals, and fewer enterobacteria in rabbits and guinea pigs.

The investigations described in this chapter were designed to try and elucidate the role of the intestinal flora in the metabolism of quinic acid.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained as described in Chapter 3.

Chromatography - for details see Chapter 3.

Reverse isotope dilution for benzoic acid

On incubation with [^{14}C]quinic acid, both rat faecal homogenates and human faecal homogenates produced a single metabolite with chromatographic properties corresponding to benzoic acid (see "Results"). The method of reverse isotope dilution was used to try and confirm this identity. Homogenates were centrifuged at 2000 xg for 10 mins., and samples of the supernatant (1 ml.) were treated as follows: Benzoic acid (500 mgm.) dissolved in $NaHCO_3$ solution (10 ml.) was added to the supernatant and mixed thoroughly. The pH of the solution was adjusted to $\simeq$ 2 by the addition of concentrated hydrochloric acid and then the solution extracted using 3 x 40 ml. portions of ether. The extract was evaporated *in vacuo* and the residue recrystallized from water. In each case, the activity associated with benzoic acid was negligible.

Investigation of the aromatization of [^{14}C]quinic acid by faecal homogenates

A 1 ml. portion of 10% w/v suspension of freshly voided human or rat faeces in Todd-Hewitt broth (Mackie and McCartney, 1965) was added to a solution of the sodium salt of [^{14}C]quinic acid (2 mg. containing 0.2 μC in 1 ml.). Incubations were carried out at 37°C, for 18 hours in a sealed tube. Samples (0.05 ml.) of the supernatant were banded on Whatman No. 1 paper, chromatographed and the ^{14}C associated with various metabolites was estimated as described previously.

Investigation of the aromatization of [^{14}C]quinic acid by specific strains of human intestinal flora

Strains of bacteria representing the major species present in human intestinal flora were isolated by the method of Drasar (1967).

Incubation of each strain with [^{14}C]quinic acid were carried out as follows:

A 1 ml. portion of the washed cell suspension (containing approximately 10^{10}_{Λ} cells/ml.) of the strain under investigation was added to a solution of the sodium salt of [^{14}C]quinic acid (2 mg. containing 0.2 μC in 1 ml.). Incubations were carried out at 37°C for 18 hours aerobically or anaerobically depending on the species under investigation. Samples

(0.05 ml.) of the supernatant were banded on Whatman No. 1 paper, chromatographed and the ^{14}C content of any metabolites estimated as described previously.

RESULTS

Investigation of the aromatization of [^{14}C]quinic acid by faecal homogenates

Incubations of [^{14}C]quinic acid with faecal homogenates were carried out using samples of rat and human faeces (see "Methods"). In each case, chromatography of the supernatant and subsequent scanning revealed one peak of activity. The product had similar chromatographic properties to benzoic acid in 5 solvent systems (A, B, C, D and E - see Table 4.4 in Part II of this Chapter). Attempts were made to confirm this identity by using the technique of reverse isotope dilution. However, it was found that after one recrystallization, little or no activity remained associated with the benzoic acid. Hence it was assumed that the product was not benzoic acid.

The second part of this chapter deals with an investigation of the nature of this product.

Investigation of the aromatization of [^{14}C]quinic acid by specific strains of human intestinal flora

Incubations of [^{14}C]quinic acid with specific strains of intestinal bacteria were carried out as described in "Methods". In each case, the supernatant was chromatographed in 5 solvent systems (A, B, C, D and E - see Table 4.4 in Part II of this

Table 4.1. Metabolism of [^{14}C]quinic acid by specific strains of human intestinal bacteria

Bacterial species	Conditions of incubation	No. of strains tested	No. of strains which metabolized quinic acid
Clostridia	Anaerobic	2	None
Enterococci	Aerobic	2	None
Enterobacteria	Aerobic	12	2
Bacteroides	Anaerobic	1	None

Chapter), dried and scanned for radioactivity.

Enterobacteria appeared to be the only species, of those investigated, which metabolized quinic acid. The product, on incubation of the two strains of enterobacteria which metabolized quinic acid, had chromatographic properties identical with those of shikimic acid in 4 solvent systems (see Table 4.4). Suspensions of this product were then reincubated with whole human faeces homogenate in Todd-Hewitt broth (1 ml.) for a further 24 hours. The supernatant was chromatographed and the chromatograms dried and scanned for radioactive metabolites. In both cases, the product had chromatographic properties corresponding with those of benzoic acid in 5 solvent systems. However, this identity was not confirmed by reverse isotope dilution for benzoic acid.

It seemed possible that if enterobacteria are responsible for the first stage of the conversion of quinic acid to an aromatic ring, then a different species of bacteria may be responsible for the next stage.

Therefore, suspension of both strains of enterobacteria, which had already metabolized [^{14}C]quinic acid were reincubated with various other species of human gut flora for 24 hours. The supernatant was then chromatographed and scanned for radioactive metabolites (Table 4.2).

None of the bacterial species investigated was able to

Table 4.2. Action of specific strains of human intestinal bacteria on the enterobacterial metabolite of [^{14}C]quinic acid

Bacterial species	Conditions of incubation	No. of strains tested	No. of incubations where further metabolism occurred
Clostridia	Anaerobic	5	None
Bacteroides	Anaerobic	3	None
Bifidobacteria	Anaerobic	2	None
Enterococci	Aerobic	2	None

metabolize the compound (possibly shikimic acid) produced when certain strains of enterobacteria were incubated with [^{14}C]quinic acid.

A thorough investigation into the role of intestinal flora in the metabolism of [^{14}C]quinic acid would entail carrying out incubations of the acid with several strains of each species of the intestinal bacteria. Since a limited amount of [^{14}C]quinic acid was available at the time of this work, incubations could be carried out only with the more common bacterial species. Further investigation into this topic may show that either one particular bacterial species can convert quinic acid into benzoic acid; or that the conversion of quinic acid to benzoic acid occurs in several stages, one particular strain of bacteria being responsible for one particular stage. As it has already been shown that two strains of enterobacteria metabolized quinic acid into a compound that can be further metabolized by whole faeces, the latter theory appears to be the more probable.

PART 2

Investigation of the nature of X, the product obtained on incubation of [^{14}C]quinic acid with human faeces

In order to justify certain investigations carried out here, a brief survey of what was known of quinic acid metabolism both on oral administration to certain mammalian species and on incubations with faeces or with certain species of bacteria, is presented.

Oral administration of quinic acid to man and Old World monkeys causes an increase in urinary hippurate (Adamson, Bridges and Williams, 1966). Lower species of mammals do not appear to convert quinic acid to hippuric acid to any appreciable extent (Chapter 2), but the fate of the administered acid in these species has not been investigated.

The urine of rats given quinic acid orally contains increased amounts of hippuric acid and conjugates of catechol and vanillic acid (Booth, Robbins, Masri and De Eds, 1960). It has been proposed (Scheline, 1968a) that quinic acid can be converted by the intestinal flora to 3,4-dihydroxybenzoic acid which can be partly absorbed and methylated to give vanillic acid, and partly decarboxylated in the intestine to produce catechol. This theory is supported by the observations that (1) quinic acid is converted to catechol when incubated with rat faecal extracts (Booth and Williams,

1963b) and (2) both quinic and shikimic acid are converted into catechol on incubation with rat caecal extracts, shikimic acid also being partly converted into protocatechuic acid (Scheline, 1968b). However, it should be pointed out that only 1 out of 4 of the incubations with shikimic acid gave rise to aromatic products; and 1 out of 6 incubations with quinic acid produced catechol.

It has been known for some time that wild-type Aerobacter, as well as Aerobacter mutants, can utilize quinic acid in aromatic biosynthesis (Davis and Weiss, 1953). Incubations of wild-type Aerobacter aerogenes with quinic acid in iron-deficient media give rise to the following aromatic products: catechol, 2,3-dihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid and anthranilic acid. In iron-sufficient media only the latter 2 compounds accumulate (Ratledge, 1967). Finally, investigations into the origin of salicylic acid in Mycobacterium smegmatis have shown that, on incubating this strain with quinic acid, salicylic acid, anthranilic acid, 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid and p-hydroxyphenylacetic acid are released into the medium (Ratledge and Winder, 1966). Mycobacterium smegmatis, however, is not an intestinal organism.

Hence it can be seen, there is a wide variety of

possibilities for the identity of the unknown product, X, of faecal incubations with quinic acid (Table 4.3).

The first step in the identification of X was the elimination of some of these possibilities from consideration of the chromatographic properties of X (Table 4.4).

Investigation

(a) Chromatography

The chromatographic properties of X, the unknown compound, were compared with those of the authentic compounds listed in Table 4.4. 20 µl. of 1% solutions of the authentic compounds were used for chromatography on Whatman's No. 1 paper. Descending chromatography was carried out in solvents A, B, C, D (14 hrs.) and E (6 hrs.) (Table 4.4). All the compounds examined, except quinic acid, absorb u.v. light at 254 n.m., hence the R_f values could be determined. The R_f values for quinic acid were determined by chromatographing [^{14}C]quinic acid and subsequently scanning the chromatograms for radioactivity.

Benzoic acid and o-hydroxyphenylacetic acid have similar chromatographic properties to X (Table 4.4). However the possibility of X being benzoic acid had already been ruled out by carrying out reverse isotope dilutions. Insufficient o-hydroxyphenylacetic acid was available to perform a reverse isotope dilution for this compound. Hence, larger scale

Table 4.3. Aromatic metabolites of quinic acid

Condition	Products
Oral administration of quinic acid to man and Old World monkeys	Hippurate
Oral administration of quinic acid to rats	Hippurate, conjugated catechol, conjugated vanillate
Rat faecal incubations with quinic acid	Catechol
Rat caecal incubations with quinic acid	Catechol
Incubations of quinic acid with *Aerobacter aerogenes*	
(i) iron deficient	Catechol, p-hydroxybenzoate, anthranilate, 2,3-dihydroxybenzoate, 3,4-dihydroxybenzoate
(ii) iron sufficient	p-Hydroxybenzoate, anthranilate
Incubations of quinic acid with *Mycobacterium smegmatis*	Salicylate, anthranilate, p-hydroxyphenylacetate, 3,4-dihydroxybenzoate, p-hydroxybenzoate

Table 4.4. R_f values of X and possible metabolites of quinic acid

Compound*	R_f value in solvent system:-				
	A	B	C	D	E
Quinic acid[1]	0.25	0.10	0.27	0.53	
Shikimic acid	0.55	0.50	0.46	0.58	
Benzoic acid	0.87	0.85	0.90	0.86	0.53
Hippuric acid	0.83	0.75	0.90	0.80	0.51
Anthranilic acid				front	0.38
Vanillic acid	0.89	0.86		0.86	origin
o-Hydroxybenzoic acid				front	0.62
m-Hydroxybenzoic acid				0.79	0.35
p-Hydroxybenzoic acid	0.92	0.90	0.90	0.79	0.28
3,4-Dihydroxybenzoic acid				0.52	origin
3,5-Dihydroxybenzoic acid				0.46	origin
2,3-Dihydroxybenzoic acid				0.76	0.40
Catechol	0.88	0.84	0.85	0.77	0.68
o-Hydroxyphenylacetic acid	0.89	0.87	0.87	0.86	0.55
p-Hydroxyphenylacetic acid	0.90	0.80	0.86	0.80	0.35
X	0.90	0.85	0.91	0.83	0.54

A. sec.-Butanol:Glacial acetic acid:water (14:1:5)

B. n.-Butanol:Ethanol:Water:Glacial acetic acid (30:10:10:1)

C. Benzyl alcohol:t.-Butanol:i-Propanol:Water (3:1:1:1) (containing 1.8% w/v formic acid)

D. Phenol:Water (3:1 w/v) containing 0.9% w/v formic acid

E. i-Propanol:Ammonia:Water (20:1:2)

Descending chromatograms were run on Whatman no. 1 paper

* 20 µl. of 1% solutions were used for chromatography. All compounds except quinic acid absorb u.v. light at 254 n.m.

1 R_f value determined by chromatographing [^{14}C]quinic acid and subsequent radiochromatogram scanning

incubations with [^{14}C]quinic acid were carried out in an attempt to isolate X. Incubations were performed as described under "Methods", using 50 ml. portions of 10% w/v suspensions of human faeces and a solution (50 ml.) of the sodium salt of 100 mgm. of [^{14}C]quinic acid containing 2.5 μC. After incubation, the homogenate was acidified to pH 2 (concentrated hydrochloric acid), ether extracted (3 x 100 ml.) and the ether extract concentrated under reduced pressure. Approximately 50% of the activity was present in the ether extract. Attempts to isolate X were unsuccessful. It was therefore decided to attempt to convert X into a derivative which could be crystallized.

(b) Preparation of the benzylthiuronium derivative of X

A 25 ml. portion of a large scale incubation with [^{14}C]quinic acid was acidified to pH 2 with concentrated hydrochloric acid and ether extracted (3 x 100 ml.). The ether extract was evaporated and the residue redissolved in water (2.5 ml.). Two drops of phenolphthalein were used as indicator and N/10 NaOH added dropwise until a pink colour was seen. The solution was then acidified with 2N-hydrochloric acid (1 drop). Benzylthiuronium chloride (100 mg. in 0.5 ml. H_2O) was added to the above solution and the mixture cooled in ice. On recrystallization from ethanol containing 10% water,

the crystalline product had a m.p. of 138°C. An infra-red spectrum of the product was recorded.

The benzylthiuronium salts of o-hydroxyphenylacetic acid and benzoic acid were prepared from o-hydroxyphenylacetic acid (0.37 g.) or benzoic acid (0.3 g.) and benzylthiuronium chloride (0.5 g.). The benzylthiuronium salts of o-hydroxyphenylacetic acid and benzoic acid had melting points of 156°C and 167°C respectively (cf. 138°C for the benzylthiuronium salt of X). The infra-red spectra were different from that of the benzylthiuronium salt of X. Hence it would appear that X is not o-hydroxyphenylacetic acid or benzoic acid.

No m-hydroxyphenylacetic acid was available at the time of this investigation.

(c) Colour reactions of X with certain spray reagents

Since it seemed possible that X is a phenolic acid, chromatograms of X were sprayed with various reagents including those known to react with phenolic acids (Table 4.5). X gave a positive colour reaction with only one reagent, viz. p-nitraniline. p-Nitraniline reagents yield stronger colours with many phenolic compounds than sulphanilic reagents. Thus it appears that X may contain a phenolic group.

Table 4.5. Colour reactions of X with certain spray reagents*

Spray reagent	Colour reaction
Ferric chloride	None
Ninhydrin	None
p-Nitraniline reagent I	Reddish pink
Sulphanilic reagent	None

*Spray reagents were made as described in "Chromatographic and Electrophoretic Techniques", Vol. I (1960) by I. Smith. William Heinemann Medical Books Ltd., London.

DISCUSSION

Previous work (see Chapters 2 and 3 of this thesis) has shown that the aromatization of quinic acid is dependent, at least partly, on the intestinal flora. It was hoped that comparative studies on the metabolism of [^{14}C]quinic acid by faecal homogenates of the rat and human might help to elucidate the reason for the differences in the urinary excretion of metabolites when [^{14}C]quinic acid is administered orally to these species.

However, it has been found that both rat and human faecal homogenates, when incubated with [^{14}C]quinic acid, produce one metabolite, X, with identical chromatographic properties in each case. Consideration of some physico-chemical properties of X indicates that X is probably not benzoic, hippuric, anthranilic, vanillic, o-hydroxybenzoic, m-hydroxybenzoic, p-hydroxybenzoic, 3,4-dihydroxybenzoic, 3,5-dihydroxybenzoic, 2,3-dihydroxybenzoic, o-hydroxyphenylacetic, p-hydroxyphenylacetic acid or catechol. One possible identification for X, viz. that of m-hydroxyphenylacetic acid, could not be investigated as this acid was not available at the time of this work.

The information obtained on X points to the fact that X is an ether soluble carboxylic acid, and is probably a phenolic acid.

Although incubations of rat and human faecal homogenates with [^{14}C]quinic acid both produced the same metabolite, this may not necessarily be the case *in vivo*. It is possible that a reaction will occur readily in the incubate due to the artificial environment which favours the growth of a bacterium which may be only a minor inhabitant of the gastrointestinal tract.

The difference between animals which aromatize quinic acid to benzoic acid and those that do not may be that

(1) completely different metabolic pathways are found in the two types of animals, or
(2) the initial stages in the transformation of quinic acid are the same in both cases but the sequence of reactions is longer in one type of animal than in the other. Animals which do not aromatize quinic acid to benzoic acid may be unable to carry out the final stage(s) in the production of benzoic acid.

Alternatively, animals which do not aromatize quinic acid to benzoic acid may be carrying the reaction one stage further than the production of benzoic acid. The observation that rats given benzoic acid orally (50 mg./kg.) excrete 97-100% of the

dose as hippuric acid (Bridges, French, Smith and Williams, 1970) does not entirely rule out the latter possibility, since orally administered benzoic acid (pKa = 4.2) would probably be absorbed from the stomach and upper intestine and thus would not come into contact with the bacterial population of the lower intestine (Schanker, Shore, Brodie and Hogben, 1957).

Quinic acid can be converted into aromatic acids by wild-type Aerobacter and Aerobacter mutants (Davis and Weiss, 1953). This conversion takes place in several steps and the enzymes catalysing some of these steps have now been isolated. In the present study, two strains of enterobacteria converted quinic acid into a product with similar chromatographic properties to those of shikimic acid. When this incubation mixture was further incubated with whole human faecal homogenate, X was produced.

Thus it seems possible that specific strains of bacteria are responsible for specific stages in the metabolism of quinic acid.

SUMMARY

Comparative studies have been carried out on the metabolism of [^{14}C]quinic acid by faecal homogenates of the rat and human, in order to elucidate the differences observed in the pattern of urinary metabolites after oral administration of the acid. Both rat and human faecal homogenates produced one metabolite with identical chromatographic properties in each case. The information obtained on this metabolite indicates that it is an ether soluble carboxylic acid, and is probably a phenolic acid.

On consideration of results obtained from the study of the ability of various strains of intestinal flora to metabolize quinic acid, it would appear that specific strains of bacteria may be responsible for specific stages in the transformation.

C H A P T E R 5

SPECIES VARIATIONS IN THE CONJUGATION OF 3-INDOLYLACETIC ACID

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INTRODUCTION

The conjugation of arylacetic acids is a reaction that shows marked species differences. Phenylacetic acid, 3-indolylacetic acid and 3,4-dihydroxy-5-methoxyphenylacetic acid are excreted by man as conjugates with glutamine (Thierfelder and Sherwin, 1914; Jepson, 1956; Harley-Mason, Laird and Smythies, 1958) whereas most of the common laboratory and domestic animals such as horse, dog, rabbit, sheep and the rat excrete arylacetic acids conjugated with glycine (Shiple and Sherwin, 1922; Erspamer, 1955). Glycine conjugates of phenylacetic acid have also been found in the urine of turtles and frogs fed phenylacetic acid and the housefly is known to conjugate this acid with glycine (Komori, Sendju, Sagara and Takamatsu, 1926; Smith, 1964). In the hen arylacetic acids are conjugated with ornithine to produce the diphenylacetylornithine conjugates (Smith, 1964).

The conjugation of phenylacetic acid with glutamine in man was discovered by Thierfelder and Sherwin in 1914, when they isolated phenylacetylglutamine and phenylacetylglutamine - urea from the urine of subjects who had ingested phenylacetic acid. Sherwin (1917) found that a female monkey (Macacus Rhesus) when given phenylacetic acid orally, excreted 51% of the dose as phenaceturic acid, that is phenylacetylglycine.

Later work, however, has shown thet indolylacetylglutamine is present in normal urine of a Macaca Rhesus monkey, and in increased amounts on feeding the monkey with 3-indolylacetic acid (Williams, 1964). Glutamine conjugation has also been found to occur in the chimpanzee, a member of the Anthropoid apes, by isolating phenylacetylglutamine from urine collected after feeding the animal with phenylacetic acid (Power, 1936).

The urine of patients suffering from Hartnup disease contains indolylacetylglutamine (Jepson, 1956). During investigations into this disease, normal subjects were given single doses of 3-indolylacetic acid, and the urinary excretion of indoles examined (Jepson, 1958). 40% of a 500 mg. dose of the acid is excreted as the glucuronide and the remainder as free acid and indolylacetylglutamine.

The occurrence of glutamine conjugation of 3-indolylacetic acid in members of the Cercopithecoidea (Old World monkeys) and of the Prosimii has been investigated (Patel and Crawford, 1963). The baboon (Papio anubis vigilis), the red monkey (Erythrocebus patas) and the common African monkeys (Cercopithecus aethiops and Cercopithecus neglectus) conjugate 3-indolylacetic acid with glutamine; that is, Old World monkeys conjugate with glutamine, whereas the Prosimii investigated, Lemuriform Lemur (Lemur catta) and Lorisform Lemur (Galago senegalensis) do not conjugate 3-indolylacetic

acid with glutamine. Further evidence of glutamine conjugation in the Rhesus monkey has been supplied by the identification of one of the diphenhydramine urinary metabolites in this species, as the glutamine conjugate of diphenylmethoxyacetic acid, although this is not an arylacetic acid (Drach and Howell, 1968).

Thus it would appear that glutamine conjugation of arylacetic acids is confined to the Old World monkeys and the Anthropoid apes. (For details of classification of mammals, see Appendix.)

The conjugation mechanism of phenylacetic acid with glutamine, probably consists of 3 stages:-

Phenylacetic acid + ATP ⟶ Phenylacetyl AMP + pyrophosphate

Phenylacetyl AMP + CoASH ⟶ Phenylacetyl CoA + AMP

Phenylacetyl CoA + L-glutamine ⟶ Phenylacetylglutamine + CoASH

Homogenates of human liver and kidney, human liver mitochondrial preparations, and supernatant fractions of human liver and kidney homogenates obtained by high speed centrifugation, catalysed the formation of phenylacetylglutamine from phenylacetyl CoA + L-glutamine. This cell sap acylating preparation also catalysed the formation of benzoylglycine from benzoyl CoA + glycine and, at a relatively low rate, the

synthesis of phenylacetylglycine from phenylacetyl CoA + glycine. Indolylacetyl CoA, however, was inactive in the acylation of glycine and glutamine. A rat liver cell-sap preparation catalysed the formation of phenylacetylglycine, and a beef liver mitochondrial preparation catalysed the activation of phenylacetate, but neither preparation was capable of forming phenylacetylglutamine. The reaction between phenylacetyl CoA and glutamine is apparently specifically catalysed by human tissues (Moldave and Meister, 1957).

The difference in specificity between the acylating systems of human tissues and that of other mammals may be due to

(a) a qualitative difference in the acylating enzymes

(b) human liver and kidney possessing an acylating enzyme not found in other species.

In the present investigation, [^{14}C]indolylacetic acid has been administered to 17 species of mammals and the urinary metabolites examined. The mammals chosen include 9 primate species:

(a) man (Hominidae)

(b) Rhesus monkey, green monkey and baboon [Cercopithecidae (Old World monkeys)]

(c) squirrel monkey, capuchin and owl monkey [Cebidae (New World monkeys)]

and (d) the bush baby and slow loris (Prosimii).

MATERIALS AND METHODS

Chemicals

3-Indolyl(acetic acid-2-C14) (specific activity 47.2 mC/mmole) was purchased from the Radiochemical Centre, Amersham, Bucks. 3-Indolylacetic acid (M.Pt. 164°C), glycine (M.Pt. 234-236°C) and D-glucuronic acid (M.Pt. 156°C) were supplied by Hopkin and Williams Ltd., and L-glutamine (M.Pt. 184°C) by Koch-Light Laboratories Ltd. Indolylacetylglycine and indolylacetylglutamine were prepared by the method of Wieland and Hörlein (1955).

Synthesis of glutamine and glycine conjugates of 3-indolylacetic acid

(1) Indolylacetylglutamine

3-Indolylacetic acid (1.75 g.) was dissolved in sodium-dried tetrahydrofuran (30 ml.) containing triethylamine (1.4 ml.) and the mixture cooled to 0°. Ethyl chloroformate (0.96 ml.) was added dropwise with shaking, and shaking continued until the smell of the acid chloride disappeared. Immediately, a solution of L-glutamine (1.46 g.) in water (20 ml.) was added, and the mixture shaken as it was allowed to reach room temperature. The tetrahydrofuran was evaporated *in vacuo* at room temperature and then the aqueous

solution was acidified with 2N hydrochloric acid until no more precipitate was formed. The precipitate was treated with ethyl acetate (10 ml.) to remove excess 3-indolylacetic acid. The less soluble indolylacetylglutamine was filtered off, boiled with decolourising charcoal, and recrystallized from water (m.p. 186°C).

(2) Indolylacetylglycine

A similar method to that described for the preparation of indolylacetylglutamine was followed, the weight of glycine being 0.79 g. The method of purification of the product was different as the glycine conjugate is more soluble in ethyl acetate than the glutamine conjugate. After precipitation of the product from the aqueous solution, all the precipitate was redissolved in ethyl acetate. On addition of petroleum ether (40° - 60°) a turbidity was seen, and after cooling on ice and scratching the surface of the container with a glass rod, pink crystals of indolylacetylglycine were produced. Recrystallization from water gave a product with a melting point of 78°C.

Chromatography and colour reactions

R_f values of 3-indolylacetic acid, indolylacetylglycine and

indolylacetylglutamine were measured in three solvents and the results are given in Table 5.1. The solvent systems used were:-

Solvent 1: methyl ethyl ketone:water:2N-acetic acid (200:100:1 by vol.)

Solvent 2: benzene:glacial acetic acid:water (4:1:1 by vol.)

Solvent 3: n-butanol:glacial acetic acid:water (12:3:5 by vol.)

Descending chromatograms were run on Whatman No. 1 paper for 3 hours in solvents 1 and 2 and 13 hours in solvent 3.

Ehrlich Reagent

Chromatograms were sprayed with p-dimethylaminobenzaldehyde (2% solution in 2N-hydrochloric acid). 3-Indolylacetic acid and its conjugates yielded blue colours at room temperature over a period of about 20 minutes, but colours developed immediately if chromatograms were heated.

Naphthoresorcinol Test

Chromatograms were sprayed with 2% naphthoresorcinol in 33% w/v trichloroacetic acid, allowed to stand for five minutes,

then heated to 110°C for a few minutes. Indolylacetylglucuronide showed up as a blue spot on a pink background.

Administration of [^{14}C]indolylacetic acid

Doses of [^{14}C]indolylacetic acid (100 mg./kg.) were administered by intraperitoneal injection of a solution of the sodium salt of the acid (2 μC/kg. for animals weighing more than 1 kg., 5 μC/kg. for animals less than 1 kg.). In the case of man, the dose (500 mg.; 5 μC) was administered orally as a solution of the sodium salt of the acid.

Urine was collected for 2 days after feeding the dose, acidified to pH 5 using glacial acetic acid, and stored at 0°C.

Estimation of ^{14}C metabolites in urine

(a) Quantitative

Urine (0.1 - 0.5 ml.) was counted in 20 ml. of Dioxan scintillator in a Packard Tri-Carb scintillation spectrometer, as described in Chapter 3. Thus the amount of activity excreted in 2 days' urine could be calculated.

(b) Qualitative

Urine containing $\simeq$0.004 μC was banded (1" band) on a strip (1.5" wide) of Whatman No. 1 paper. These strips were

chromatographed in solvents 1, 2 and 3 by the descending technique, against reference spots of 3-indolylacetic acid, indolylacetylglycine and indolylacetylglutamine. Chromatograms were scanned using a Packard Radiochromatogram Scanner (Model No. 7200). The ^{14}C peaks corresponding to 3-indolylacetic acid, indolylacetylglycine and indolylacetylglutamine were identified by comparison of their R_f values with those of authentic samples.

Indolylacetylglucuronide was identified as follows:-

(a) by spraying chromatograms with naphthoresorcinol reagent,

(b) by incubation of the urine with a preparation of β-glucuronidase (Ketodase, William R. Warner & Co. Ltd., Eastleigh, Hampshire).

Samples of urine (1 ml. adjusted to pH 5) were incubated with Ketodase (1 ml. containing 5,000 units of β-glucuronidase derived from beef liver) at 37°C for 24 hours. Control incubations consisted of urine (1 ml.) + boiled enzyme (1 ml.). Aliquots (0.5 ml.) of both sets of incubations were chromatographed on thick layer plates (fluorescent silica) in solvent 3 (n-butanol:glacial acetic acid:water; 12:3:5) and scanned for activity. Ketodase hydrolysed the indolylacetylglucuronide conjugate to free 3-indolylacetic acid

and glucuronic acid (detected by naphthoresorcinol).

The ^{14}C related to each peak was estimated by measuring the areas of each peak (height of peak x width of peak at half height). The proportion of each peak to the total activity on the paper could thus be determined, and expressed as a percentage of the total activity recovered in the urine.

RESULTS AND DISCUSSION

Chromatography

Table 5.1 gives the R_f values of 3-indolylacetic acid and its conjugates with glycine, glutamine and glucuronic acid. The chemical formulae of these compounds are shown in Fig. 5.1.

Excretion of intraperitoneally injected [^{14}C]indolylacetic acid and its metabolites by some lower mammals

The sub-primate species examined, including members of Rodentia (rat, mouse, guinea pig and hamster), Carnivora (cat, dog and ferret) and Lagomorpha (rabbit) all excreted intraperitoneally administered [^{14}C]indolylacetic acid as the free acid and as its glycine conjugate. The glutamine conjugate was not detected in the urine of any of these lower mammals, and the glucuronide was found in only one species, namely, the ferret. In this species, indolylacetylglucuronide accounted for 52% of the urinary ^{14}C content. The ferret is known to use glucuronic acid extensively as a conjugating agent and excretes 22% of a 50 mg./kg. dose of benzoic acid as the glucuronide, and 44% of a 200 mg./kg. dose of benzoic acid (Bridges et al., 1970). The percentages of the various urinary metabolites of [^{14}C]indolylacetic acid in lower mammals are given in Table 5.2. Figs. 5.2 and 5.3 are

Table 5.1. R_f values of 3-indolylacetic acid and conjugates of this acid

Whatman No. 1 paper was used. The solvents were:-

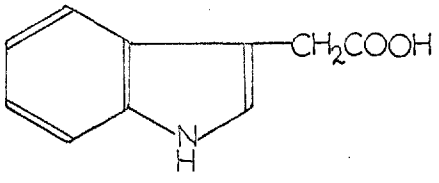
1. methyl ethyl ketone:water:2N acetic acid (200:100:1)
2. benzene:glacial acetic acid: water (4:1:1)
3. n-butanol:glacial acetic acid:water (12:3:5)

Descending chromatograms were run for 3 hours in solvents 1 and 2, and for 13 hours in solvent 3.

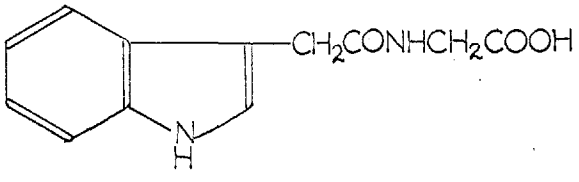
Compound	R_f value in solvent:-		
	1	2	3
3-Indolylacetic acid	0.97	0.7	0.91
Indolylacetylglycine	0.77	Origin	0.82
Indolylacetylglutamine	0.38	Origin	0.74
Indolylacetylglucuronide	0.05	Origin	0.5

Fig. 5.1.

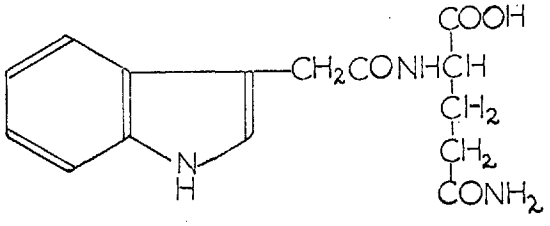
3-Indolylacetic acid and its metabolites



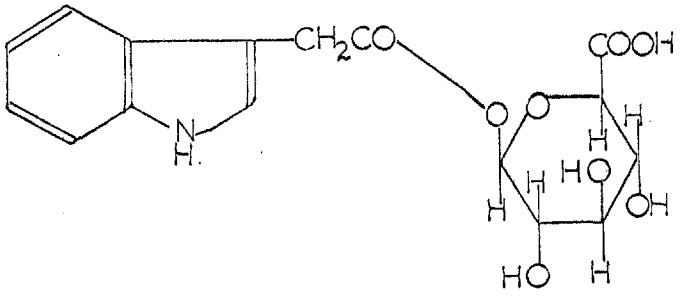
3-Indolylacetic acid



Indolylacetylglycine



Indolylacetyl-glutamine



Indolylacetyl-glucuronide

Table 5.2. Excretion of intraperitoneally injected [^{14}C]indolylacetic acid and its metabolites by sub-primate species

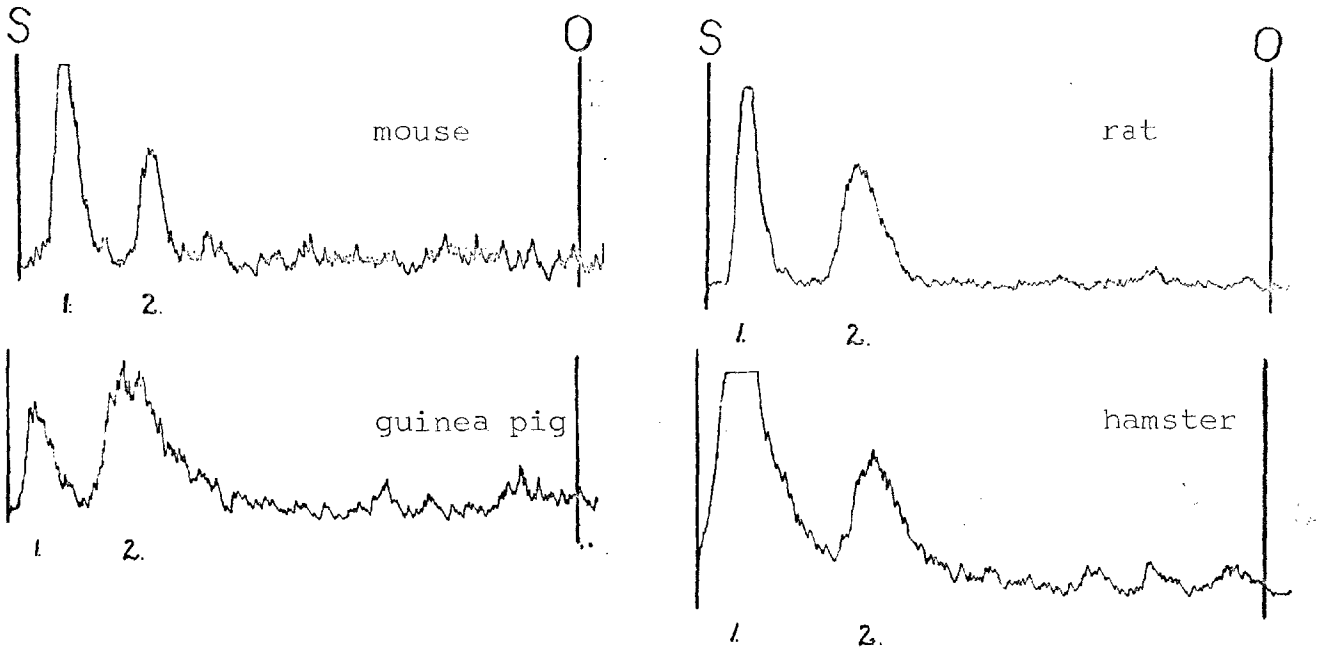
Mammalian Order	Species	% of dose found in 0-48 hr. urine	% of ^{14}C content of urine as:-			
			Free acid	Glycine conjugate	Glutamine conjugate	Glucuronide
Rodentia	^{3}Rat	76.1 (68-90.9)	42.9 (33.6-49.3)	57.1 (50.7-66.4)	N.D.	N.D.
"	^{3}Mouse	63.7 (46.6-91.2)	52.5 (38.4-66.5)	47.5 (33.5-61.6)	N.D.	N.D.
"	^{3}Guinea pig	96.4 (91.6-101.4)	45.0 (36.7-54.5)	55.0 (45.5-63.3)	N.D.	N.D.
"	^{3}Hamster	83.2 (67.5-91.8)	75.4 (68.6-80.2)	24.6 (19.8-31.4)	N.D.	N.D.
Carnivora	^{2}Cat	92.5 (87.3-97.6)	24.5 (17.8-31.3)	75.5 (68.7-82.2)	N.D.	N.D.
"	^{2}Ferret	57.2 (46.4-68)	37.3 (29.1-45.4)	10.5 (5.7-15.2)	N.D.	52.5 (48.9-55.7)
"	^{1}Dog	83.5	30	70	N.D.	N.D.
Lagomorpha	^{3}Rabbit	92.4 (88.8-99)	42.5 (22.0-56.4)	57.5 (43.6-78)	N.D.	N.D.

N.D. = not detected

Superscripts indicate the number of animals used.

Dose = 100 mg./kg.

Fig. 5.2. Radiochromatogram scans obtained from the urines of a number of rodents given [^{14}C]indolylacetic acid

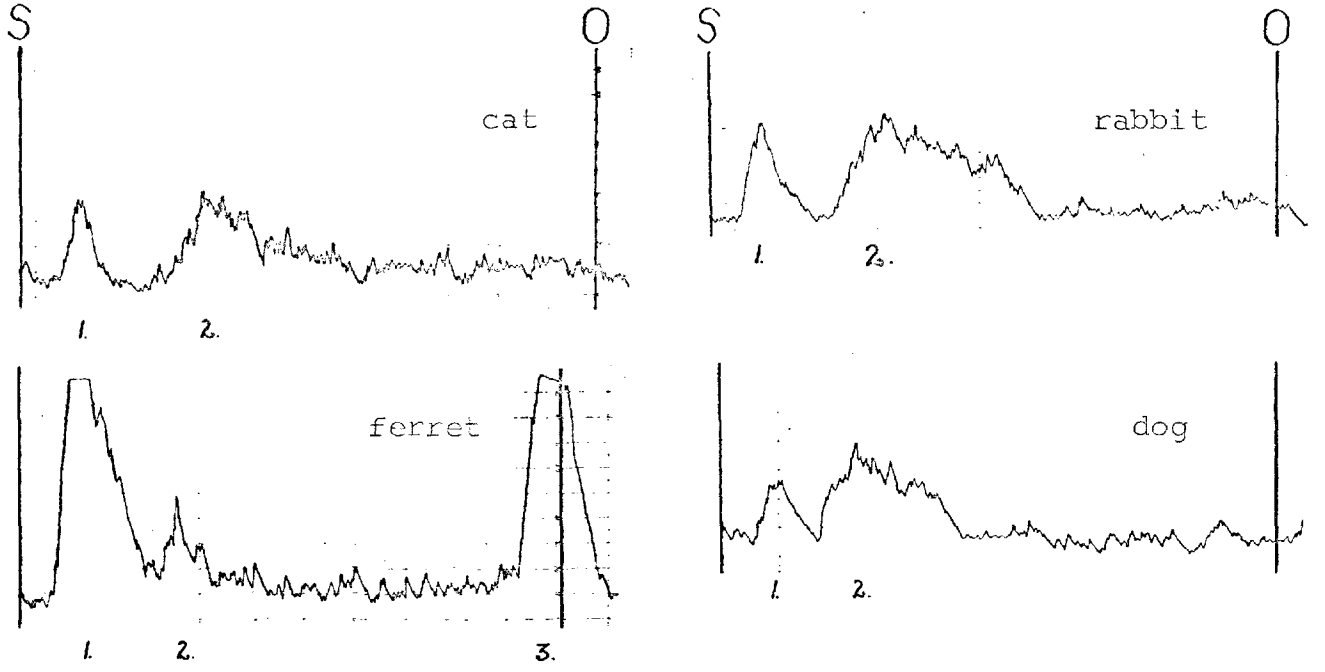


Solvent: Methyl ethyl ketone:Water:Acetic acid

O = origin
S = solvent front

1 = 3-Indolylacetic acid
2 = Indolylacetylglycine

Fig. 5.3. Radiochromatogram scans obtained from urines of a number of lower mammals given [^{14}C]indolylacetic acid



Solvent: Methyl ethyl ketone:Water:Acetic acid

O = origin
S = solvent front

1 = 3-Indolylacetic acid
2 = Indolylacetylglycine
3 = Indolylacetylglucuronide

photographs of the radiochromatogram scans obtained from the animal urines.

Excretion of [^{14}C]indolylacetic acid and its metabolites by certain primate species

The nine primates given 3-indolylacetic acid showed an interesting pattern in the urinary metabolites of this compound. Human urine contained the free acid and its glutamine and glucuronide conjugates; the relative proportions of these compounds, however, varied considerably from one individual to another (see below). The three Old World monkeys (Rhesus monkey, green monkey and baboon) excreted 3-indolylacetic acid partly free and partly conjugated with glutamine; the green monkey forming a considerable amount of glucuronide as well (38%). No indolylacetylglycine could be detected in the urine of the Old World monkeys or man. Of the New World monkeys examined, the squirrel and owl monkey excreted free 3-indolylacetic acid, the glutamine and glycine conjugates, the squirrel monkey also excreting a large proportion of the glucuronide conjugate (50%). The capuchin monkey was injected with [^{14}C]indolylacetic acid on two separate occasions. On the first, only the free acid and glucuronide were excreted in the urine. However, on repeating the injection several months later, both glycine and glutamine

conjugates were detected in the urine, as well as free [^{14}C]indolylacetic acid and its glucuronide (see Table 5.3 and Figs. 5.4 and 5.5).

Table 5.3. Excretion of intraperitoneally injected [^{14}C]indolylacetic acid and its metabolites by primate species

Sub-Order	Family	Species	% of dose found in 0-48 hr. urine	% of ^{14}C content of urine as:-			
				Free acid	Glycine conjugate	Glutamine conjugate	Glucuronide
Anthropoidea	Hominidae (Anthropoid Apes)	*Man	85.4	16.2	N.D.	20.4	63.4
"	Cercopithecidae (Old World Monkeys)	^{2}Rhesus monkey	92.2 (90.6-93.9)	68.3 (67.1-69.6)	N.D.	31.7 (30.4-32.9)	N.D.
		^{1}Green monkey	46.1	19.9	N.D.	42.3	37.8
		^{1}Baboon	53.1	47.7	N.D.	52.3	N.D.
"	Cebidae (New World Monkeys)	^{3}Squirrel monkey	59.9 (41.0-76.2)	15.8 (9.4-24.2)	7.2 (3.1-12.8)	26.7 (21.3-36.4)	50.3 (48.7-51.1)
		^{2}Owl monkey	41.3 (37.1-45.5)	54.7 (50.9-58.6)	26.9 (21.7-32.1)	18.3 (17-19.7)	N.D.
		^{2}Capuchin	72.5 (66.5-78.5)	36.8 (13.6-60)	5.6 (0-11.2)	16.7 (0-33.4)	40.9 (40-41.8)
Prosimii	Lorisidae	^{2}Bush baby	33.9 (17.6-50.3)	51.4 (40.2-60.6)	48.6 (37.4-59.8)	N.D.	N.D.
		^{1}Slow loris	27.7	85.5	14.5	N.D.	N.D.

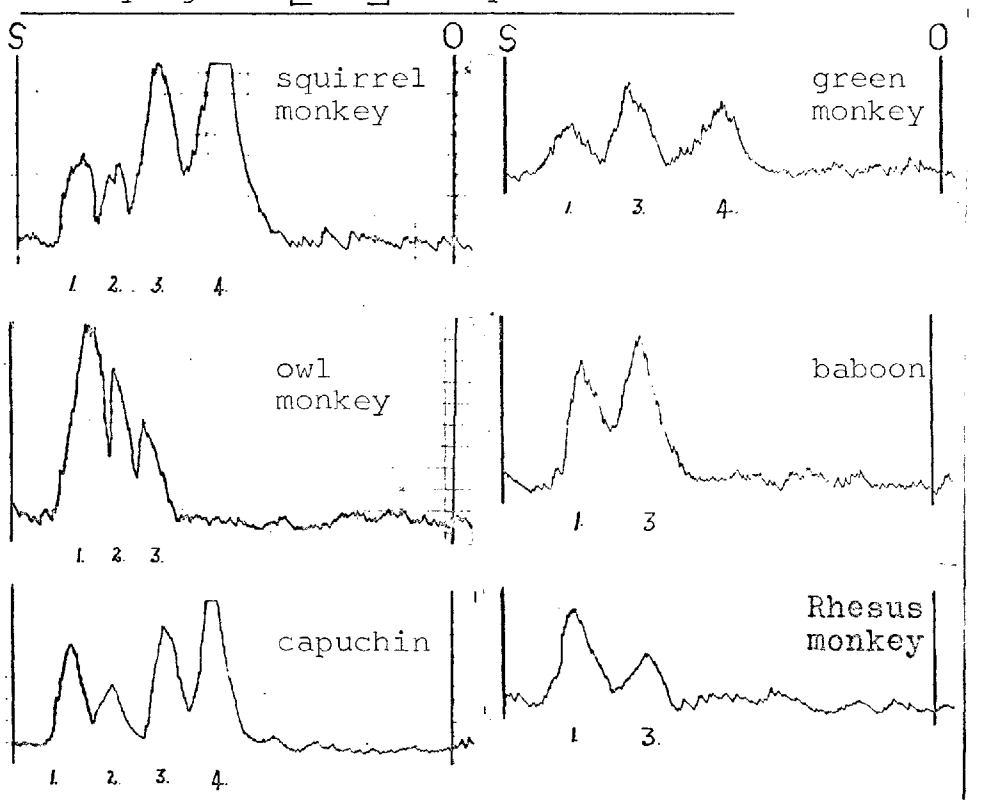
N.D. = not detected

Superscripts indicate the number of animals used.

Dose = 100 mg./kg. except for man (500 mg.)

* Result quoted for subject H.D. The acid was given orally to humans.

Fig. 5.4. Radiochromatogram scans obtained from the urines of certain monkeys given [^{14}C]indolylacetic acid

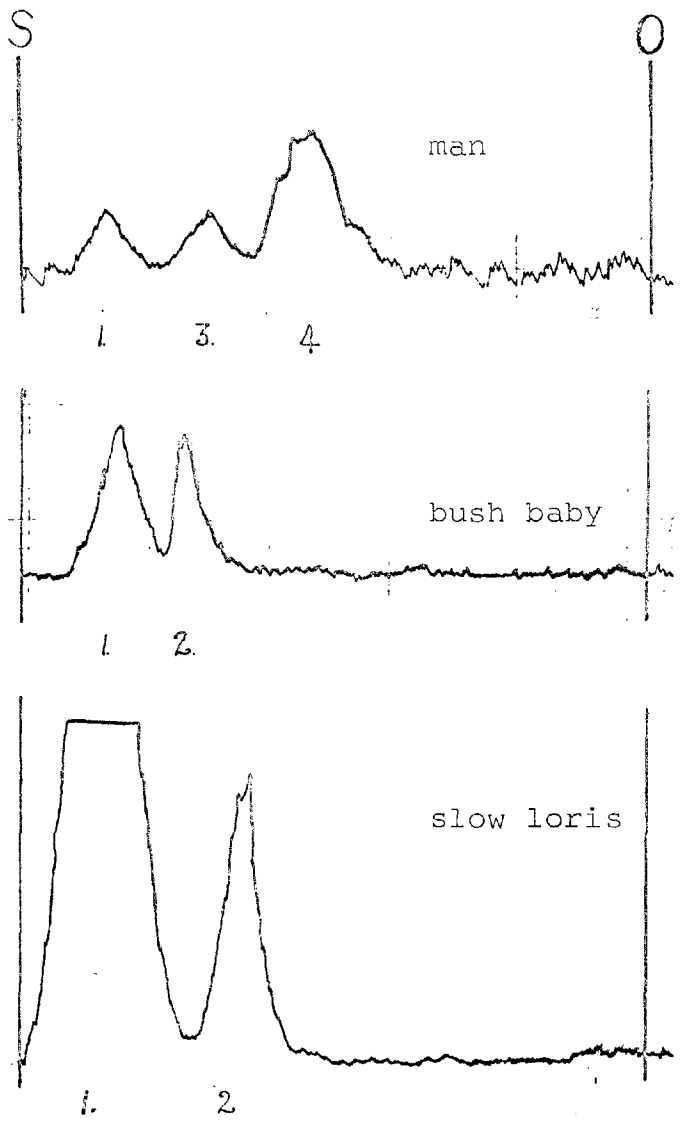


Solvent: n-Butanol:Acetic acid:Water

O = origin
S = solvent front

1 = 3-Indolylacetic acid
2 = Indolylacetylglycine
3 = Indolylacetylglutamine
4 = Indolylacetylglucuronide

Fig. 5.5. Radiochromatogram scans obtained from the urines of lorisform lemurs and man given [^{14}C]indolylacetic acid



Solvent: n-Butanol:Acetic acid:water.

O = origin
S = solvent front
1 = 3-Indolylacetic acid
2 = Indolylacetylglycine
3 = Indolylacetylglutamine
4 = Indolylacetylglucuronide

In man, approximately 84% of the administered radioactivity was excreted in 2 days' urine. Subject H.D. (in the first hour's urine) excreted 16% of the activity as the free acid, 20% as the glutamine conjugate, and 63% as the glucuronide. A similar pattern was observed for the urine collected over the second and third hours. Subjects M.E.E. and R.L.S. excreted large amounts (56-90%) of free acid, with only traces (<5%) of the glutamine conjugate, although subject M.E.E. also excreted 38% of the activity as the glucuronide. It is interesting to note that, in the case of R.L.S., over 65% of the dose had been excreted after 6 hours, this subject excreting predominantly free [^{14}C]indolylacetic acid. Subject H.D. excreted only about 35% of the dose in this period, with mainly the glucuronide present in the urine (see Table 5.4 and Fig. 5.6).

The influence of different dose levels on the extent and type of conjugation has not been investigated here, but previous work has shown that there is not a direct relationship between the amount of 3-indolylacetic acid ingested or produced and the pattern of its metabolites in urine (Armstrong, Shaw, Gortatowski and Singer, 1958).

On consideration of the mammals investigated, it would appear that the lower mammals conjugate 3-indolylacetic acid with glycine, man and the Old World monkeys conjugate this acid

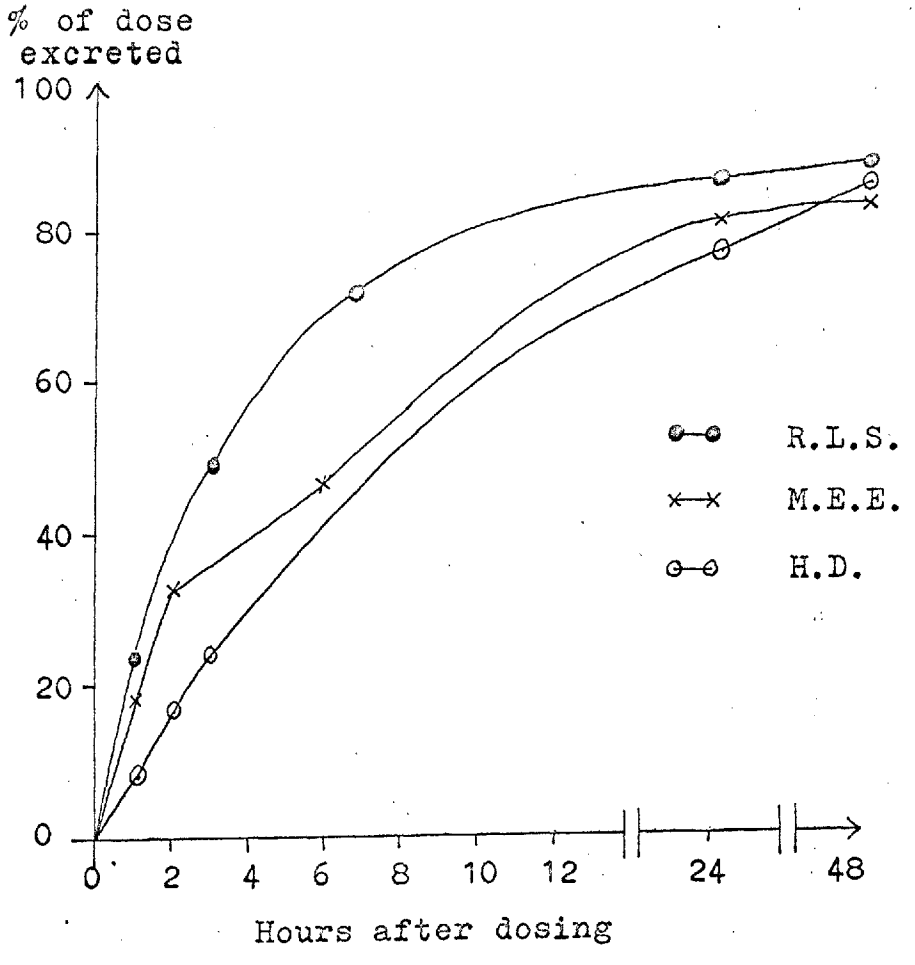
Table 5.4. Urinary metabolites of [^{14}C]indolylacetic acid in man

Subject	% of dose found in 0-48 hr. urine	% of urinary activity excreted in 1 hour as:-		
		Free acid	Glutamine conjugate	Glucuronide
H.D.	85.4	16.2	20.4	63.4
M.E.E.	82.1	56.5	< 5	38.5
R.L.S.	86.2	90	< 5	< 5

Dose = 500 mg.; 5 μC, administered orally

Fig. 5.6.

Rate of excretion of ^{14}C activity by man after ingestion of [^{14}C] indolylacetic acid



with glutamine, whereas in the New World monkeys there is a transition in the conjugation mechanism where both glycine and glutamine conjugates are formed. Indolylacetylglucuronide excretion showed no particular pattern with regard to its occurrence in different species. Of the lower mammals investigated, only the ferret excreted this conjugate; whereas four out of the nine primates investigated excreted indolylacetylglucuronide.

SUMMARY

The metabolism of [^{14}C]indolylacetic acid has been investigated in 17 species of vertebrates, including 8 sub-primates and 9 primates.

The sub-primates all conjugate 3-indolylacetic acid with glycine, this conjugate being excreted in the urine together with the free acid. The ratio of free acid to conjugate varies extensively from one species to another. The ferret is the only lower mammal which excretes indolylacetylglucuronide as well as the free acid and the glycine conjugate.

An extremely interesting pattern is observed in the conjugation mechanisms employed by the various primates examined. Old World monkeys and man form the glutamine conjugate of 3-indolylacetic acid; the Prosimii (bush baby and slow loris) conjugate this acid with glycine, whereas the New World monkeys (squirrel monkey, owl monkey and capuchin) excrete both the glycine and glutamine conjugates of 3-indolylacetic acid. Indolylacetylglucuronide is excreted by four species of primates (man, green monkey, squirrel monkey and capuchin) but the occurrence of this conjugate shows no particular "species pattern".

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

The work described in this thesis has been concerned with species variations in the metabolism of 2 anutrients. The aromatization of quinic acid to benzoic acid has been taken as an example of a phase I reaction, and the conjugation of 3-indolylacetic acid as an example of a phase II reaction.

The extent of aromatization of an orally administered dose of quinic acid was measured in 22 species: 10 primates, including man, and 12 sub-primates. Man and the Old World monkeys (Rhesus monkey, green monkey and baboon) converted over 40% of the dose to benzoic acid. The New World monkeys (spider monkey, squirrel monkey and capuchin) and members of the Prosimii (bush baby, slow loris and tree shrew) aromatized very small amounts of the dose of quinic acid. Members of 5 orders of lower mammals (Carnivora, Lagomorpha, Rodentia, Insectivora and Chiroptera) and the pigeon were also unable to aromatize quinic acid to benzoic acid to any appreciable extent. There is thus a marked difference between, on the one hand, man and the Old World monkeys, that are able to convert quinic acid to benzoic acid, and, on the other, the New World monkeys, lower mammals and birds, that are unable to carry out this conversion. Thus, in this instance, a biochemical classification of mammals

would follow the pattern of the existing taxonomical classification based on the physiological and anatomical characteristics.

Evidence has been presented which strongly suggests that the intestinal flora are responsible for the aromatization of quinic acid.

The results obtained from the study of incubation of [^{14}C]quinic acid with faecal homogenates or with specific strains of intestinal flora indicate that the conversion of quinic acid to benzoic acid takes place in several steps. Specific strains of bacteria may be responsible for certain stages in the chain of reactions. One of the intermediates in the chain may be shikimic acid.

It was hoped that comparative studies in the metabolism of [^{14}C]quinic acid by homogenates of rat and human faeces might elucidate the reason for the differences in the urinary excretion of metabolites when the acid is administered orally to these species. The results suggest 2 possible reasons for the difference between animals that aromatize quinic acid to benzoic acid and those that do not. Firstly, different metabolic pathways may be found in the 2 types of animals; or secondly, the chain of reaction in the metabolism of quinic acid may be longer in one type of animal than in the other. Whether the "longer" chain is associated with the production

of benzoic acid or a metabolite of benzoic acid could not be assessed on the information available.

In order to ascertain the reason for the species variations observed in the aromatization reaction, it is suggested that future work should be directed towards investigating the ability of many strains of intestinal bacteria from many species of animals to carry out this conversion. It may be that certain strains of bacteria are found only in certain animal species.

Renwick and Williams (1969) have shown that the conversion of cyclamate into cyclohexylamine in the rat is carried out by the gut flora, which acquire this ability when the diet contains cyclamate. This ability is largely lost on removing cyclamate from the diet. In such a case as this, it is apparent that, during the course of screening tests in lower mammals, the importance of chronic toxicity testing must be emphasized.

Another important application of further studies into the role of the intestinal flora in the metabolism of foreign compounds is the possibility of modifying the flora in lower mammals to resemble those in humans. The intestinal flora could be modified either by the introduction or removal of certain strains of bacteria.

The conjugation mechanism employed in the metabolism of 3-indolylacetic acid showed an interesting pattern in the 17 species studied. The 8 sub-primates (members of the Carnivora,

Lagomorpha and Rodentia orders) all conjugated this acid with glycine. Of the primates examined, man and the Old World monkeys (Rhesus monkey, green monkey and baboon) conjugated 3-indolylacetic acid with glutamine, the Prosimii (bush baby and slow loris) used glycine, whereas the New World monkeys (squirrel monkey, owl monkey and capuchin) utilized both glycine and glutamine for conjugation.

Thus, in both the occurrence of the aromatization of quinic acid to benzoic acid and that of the conjugation mechanism employed for the excretion of 3-indolylacetic acid, the biochemical pattern observed amongst the species examined appears to follow the existing taxonomical classification.

It is hoped that, when sufficient data has been accumulated on the metabolism of a wide variety of chemical compounds in a large number of animal species, it may be possible to classify the species on a biochemical basis. The applications of such a classification in the medical-scientific sphere would be many.

The major difficulty in trying to predict species differences in metabolism, in the past, has been the lack of adequate data on which to base these predictions. In addition to inaccuracies arising from poor experimental methodology, a number of other variables contribute to the observed differences

in metabolism. These include the dose level and route of administration, the strain of animals used, their age, sex and health, their diet and the presence of anutrient chemicals. However, with improved, highly sensitive chemical techniques and awareness of the many factors involved in species variations in metabolism, the projection of biochemical, pharmacological and toxicological data from animals to man will become more precise.

Many ideas on this subject have been put forward in recent years - the answer may lie in using animal species appropriate to certain chemical groupings; placing more reliance upon plasma levels; carrying out thorough investigations into metabolism in the higher non-human primates; modifying animals so that their response is comparable with that of man; finally, classifying our own species on a biochemical basis. Only further research will provide us with the answer.

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APPENDIX

CLASSIFICATION OF SPECIES

USED IN METABOLIC STUDIES

(a) Lower mammals and birds

Species	Class	Order	Family	Generic name
Dog	Mammalia	Carnivora	Canidae	Canis familiaris
Cat	"	"	Felidae	Felis catus
Ferret	"	"	Mustelidae	Mustela putorius
Rabbit	"	Lagomorpha	Leporidae	Oryctolagus cuniculus
Rat	"	Rodentia	Muridae	Rattus norvegicus
Mouse	"	"	"	Mus musculus
Guinea pig (English)	"	"	Caviidae	Cavia porcellus
Hamster (Golden)	"	"	Cricetidae	Mesocricetus auratus
Lemming (Steppe)	"	"	"	Lagurus lagurus
Hedgehog	"	Insectivora	Erinaceidea	Erinaceus europaeus
Fruit bat (Indian)	"	Chiroptera	Pteropidae	Pteropus giganteus
Pigeon	Aves		Columbidae	Columba livia

(b) Primates

Species	Sub-Order	Family	Generic name	Geographical distribution	Salient features
Man	Anthropoidea	Hominidae	Homo sapiens	World wide	Voluminous brain-case, flat face, eyes directed forwards, shrunken external ear, possession of a "hand", erect posture.
Rhesus monkey (Bengal)	"	Cercopithecidae (Old World monkeys)	Macaca mulatta	Widely distributed over the Old World	Horizontal posture, presence of ischial callosities and cheek pouches, rudimentary tail, forelimbs not much longer than hind limbs
Green monkey	"	"	Cercopithecus aethiops		
Baboon	"	"	Papio cynocephalus		
Spider monkey	"	Cebidae (New World monkeys)	Ateles	Widely distributed over the New World	Horizontal posture, no ischial callosities, prehensile tails generally, arboreal in habit
Squirrel monkey	"	"	Saimiri sciureus		
Capuchin	"	"	Cebus apella		
Owl	"	"	Aotus trivirgatus		
Bush baby	Prosimii	Lorisidae	Galago crassicaudatus	Southern and Eastern Africa	Arboreal, mainly nocturnal; tail is reduced or absent.
Slow loris	"	"	Nycticebus coucang	Southern Asia, Sumatra Java and Borneo	
Tree shrew	"	Tupaiidae	Tupaia glis	"	Most primitive primate, closely resembles Insectivores