THE METABOLIC FATE OF AMPHETAMINE IN MAN AND OTHER SPECIES

by

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ABSTRACT

Utilising a ^{14}C label, the metabolic fate of the sympathomimetic drug amphetamine has been studied in a number of mammalian species including man.

Administered radioactivity was excreted rapidly in all the species examined; between 60 and 70% being found in the first day's urine.

Examination of the urinary metabolites indicated that there are two main pathways for the metabolism of amphetamine, namely side-chain degradation to give benzoic acid, or hydroxylation of the aromatic ring to give 4-hydroxyamphetamine. The relative importance of the pathway varies with species. On the one hand is the rat which excretes 4-hydroxyamphetamine and its glucuronide conjugate, on the other the guinea pig which excretes largely benzoic acid and its conjugates. All the other species fall between these extremes, so that 4-hydroxyamphetamine excretion in rat $>$ mouse $>$ rabbit $>$ greyhound $>$ rhesus monkey $>$ man $>$ guinea pig and benzoic acid excretion in guinea pig $>$ mouse $>$ greyhound $>$ rabbit $\equiv$ monkey $\equiv$ man $>$ rat.

In addition to these two main urinary metabolites, norephedrine and 4-hydroxynorephedrine were identified as minor metabolites in rat urine and benzyl methyl carbinol glucuronide in rabbit urine.

A major urinary metabolite, which was found only to an appreciable extent in the rabbit, gave phenyl acetone on acid hydrolysis. This metabolite is thought to be the sulphate ester of the enol form of phenyl acetone.

Phenobarbitone administered concurrently with amphetamine sulphate was found to cause an increase in the blood level and the half life of the amine in rabbits and also to cause an increase in the excretion of 4-hydroxyamphetamine in the rat. It is suggested that the potentiation of the pharmacological effect of amphetamine-barbiturate mixtures might be caused by an inhibition of the metabolism of the amine by the barbiturate.

FOREWORD

The sympathomimetic drugs comprise one of the most widely studied groups of pharmacological agents. However, in comparison with noradrenaline, relatively little is known about the fate of amphetamine in any mammal, including man. Amphetamine has powerful central nervous system stimulant actions in addition to a peripheral sympathomimetic effect. The drug is largely employed for its central stimulant action particularly in depressive syndromes. In common with other drugs which have a potent effect on the central nervous system its use has been much abused and a tighter control over its use has become necessary.

The urinary excretion of amphetamine in dog, rat, guinea pig, rabbit, and man has been described but often large percentages of the dose remained unaccounted for, and in man only the unchanged drug has ever been identified. The work described in this thesis is an attempt to elucidate the metabolic route taken by amphetamine in a variety of mammalian species, including man, using radioactively labelled amphetamine.

I wish to express my gratitude to Professor R.T. Williams for the interest he has shown in the work and for his encouragement and invaluable supervision. I would also like to thank Dr. R.L. Smith for his practical advice coupled with helpful discussion and the other members of staff and fellow research students for the often fruitful discussions. I wish to thank

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I am grateful to Shell Research Limited for their financial support to this work and to Smith, Kline and French Laboratories, Philadelphia for the generous gift of (^{14}C) amphetamine.

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CONTENTS

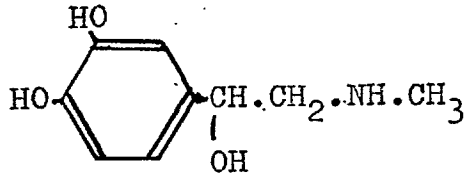
CHAPTER 1

INTRODUCTION

HISTORY OF THE PHENETHYLAMINES

Although 2-aminophenylpropane was synthesised as a chemical exercise in 1887 by L. Edeleanu, it was not until almost forty years later that its remarkable physiological properties were appreciated.

The pressor effects of suprarenal extracts were demonstrated by Oliver and Schaefer in 1895. The isolation of the active principle is accredited to Abel and Crawford in 1897 who called it epinephrine. However it was not obtained in a crystalline form until 1901 by Takamine, who proposed the elemental formula $C_{10}H_{13}O_3N$. This was found to be incorrect by several other workers one of whom, Jowett, confirmed that the formula should be $C_9H_{13}O_3N$, and proposed from chemical evidence that the formula for epinephrine (adrenaline) should be



This compound was synthesised independantly by Stolz (1904) and Dakin (1905). Already it must have been apparent that this and related compounds might have medicinal value, for the literature soon abounded in patents for the synthesis of adrenaline and related compounds.

However, there was a difference between the physiological activity of naturally ocurring laevo adrenaline and the racemic synthetic adrenaline. Cushny (1908,1909) showed that natural l-adrenaline acts approximately twice as strongly in increasing the blood pressure as synthetic racemic adrenaline and later that d-adrenaline has about one-twelfth the activity of l-adrenaline. This finding illustrates an important point about the optically active phenethylamines, namely that one optical isomer frequently has greater biological activity than another.

Following the synthesis of adrenaline by Stolz many more phenylethylamines with various substituents added to the phenyl nucleus or the aliphatic side chain were synthesised, so that by 1910 Barger and Dale were able to publish their now classical paper on the chemical structure and sympathomimetic action of amines. They were first to use the term sympathomimetic, defining it as "An action simulating that of true sympathetic nervous systems", and they were also able to define the structure activity relationships for sympathomimetic amines, the general conclusions holding to this day. They said that although aliphatic amines possess this activity, the optimum carbon skeleton for sympathomimetic activity consists of a benzene ring with a side chain of two carbon atoms, the terminal one bearing the amino group. Another optimum condition is the presence of two phenolic hydroxyl groups in 3',4' position, whereas one in the 1' position does not increase the activity; on the other hand an alcoholic hydroxyl does increase

the activity. Finally all the substances producing this action in characteristic manner are primary or secondary amines.

The idea that chemicals may act as transmitters at synapses was first proposed by Elliot in 1905 and it was suggested that adrenaline might act at the junction of sympathetic nerves and the effector organs, adrenaline being released at the sympathetic nerve endings. Later, Loewi (1921) was able to furnish experimental confirmation of this hypothesis when he showed that a substance similar to adrenaline was liberated into the fluid perfusing a frog's heart when the sympathetic fibres in the vagus were stimulated. Although this was an important step forward, it is now realised that his choice of experimental animal was unfortunate, since it caused much confusion over the identity of the sympathetic transmitter for nearly 25 years.

It is now known that the sympathetic transmitter in the frog is indeed adrenaline, but that in mammals it is noradrenaline (Euler, 1946). Despite ever increasing evidence that the transmitter was noradrenaline, the concept was not immediately accepted, but now the view is that in mammals noradrenaline is the sole sympathetic transmitter (Euler, 1959).

During the third and fourth decades of this century the various structural analogues of adrenaline were exhaustively investigated. An interesting discovery made at this time was the recognition by Chen and Schmidt (1924) of the pharmacological properties of ephedrine, isolated nearly half a century earlier by Nagai (1887).

Ephedrine (2-methylamino-1-phenylpropan-1-ol) has two configurations which each exist in a pair of optically active forms and the most active form (-) ephedrine occurs naturally in various plants (species of Ephedra). Although it is only a weak vasopressor (1% that of adrenaline) its action is ten times as long, and even more important, it is active when taken orally (Chen, Schmidt, 1930). Its most important use now is probably in asthma for the prevention of bronchospasm. This oral activity of the phenylisopropylamine nucleus led to further studies and one compound which emerged from those studies was 2-aminophenylpropane (amphetamine, "Benzedrine").

PHARMACOLOGY AND TOXICITY OF AMPHETAMINE

The pressor effects of amphetamine were first noted by Piness, Miller and Alles (1930) during a study of related phenylethylamines. The compound was given both orally and subcutaneously and was found to produce a marked rise in blood pressure for at least 8 hours. In 1933a,b, Alles observed its bronchodilator, respiratory stimulant and analeptic actions and on comparison with adrenaline he found that the cardiovascular effects were of much longer duration, but its potency only $\frac{1}{100} - \frac{1}{200}$.

Therefore the pharmacology of amphetamine can be conveniently considered under the headings of peripheral and central actions.

PERIPHERAL ACTIONS

Generally speaking, in comparison with the archetype adrenaline , the peripheral effects are not very marked. It has an affect on both the α and β-receptors on the sympathetic effector cells, (Ahlquist, 1948). These peripheral actions can perhaps best be considered by reference to the work of Gunn (1939). From results obtained using several mammalian preparations he found that relative to adrenaline the equipotent molar ratio was 75-370, and the duration of action 5-10 times as long for dl-amphetamine. The compound also had a very feeble relaxant effect on rabbit intestine the equipotent molar ratio relative to adrenaline being $>$ 10,100.

CENTRAL NERVOUS SYSTEM

Unlike its peripheral effects amphetamine's central effects are very marked. Indeed, it is probably one of the most potent stimulants of the central nervous system known and it is this property which is made use of in therapeutics wherever a stimulation of the central nervous system (C.N.S.) is required. Prinzmetal and Bloomberg (1935) were the first to use amphetamine for this purpose in the treatment of obesity, parkinsonism, poisoning by C.N.S. depressants, and in certain types of depressive illness. Of the two isomers d-amphetamine was found to have two to four times greater effect than the l-amphetamine on the C.N.S. (Prinzmetal, Alles, 1940), although either isomer had about the same effect on the peripheral nervous system (Alles, 1939).

Unfortunately this C.N.S. stimulant action soon became well known amongst the general public, particularly amongst adolescents. This has given rise to outbreaks

of "spree abuse" in Britain and other parts of the world (Connell,1958). In some cases certain individuals became compulsive users and have to obtain amphetamine wherever they can. A common source of amphetamine at one time was the amphetamine base in nasal inhalers. Such inhalers usually contained about 300mg. of amphetamine base which was consumed by addicts in the course of a few hours. When this abuse was realised the inhalers were withdrawn from the market. A considerable folk-lore has grown up around the amphetamines for which there are some very strange synonyms in Britain eg. Dexedrine; "purple hearts": Durophet; "black bombers", "French blues", "bennies". With large doses of the drug people became elated, excitable and talkative, and this may well be followed by truculence and aggressiveness. Still larger doses may give rise to delusions and temporary mental illness akin to schizophrenia (Connell,1958). Another unfortunate abuse of the amphetamines is their use to improve performance in sport. In order to prevent this, elaborate detection techniques for the drugs in urine have had to be devised (Beckett, Tucker, and Moffat, 1967).

Regular use of the drug gives rise to marked psychic dependence, but not physical dependence (Brocklehurst, 1968). Therefore people who became dependent on these drugs are said to be habituated rather than addicted.

Because of the increasing abuse particularly because it is thought in some quarters that the use of amphetamines paves the way to the use of the "harder" drugs such as heroin, morphine and cocaine in certain individuals the

total abandonment of amphetamines as therapeutic agents has been considered.(Question in Parliament to Mr. Julian Snow, Parliamentary Secretary, Ministry of Health, 30th. April 1968).

The temporary mental illness which is indistinguishable from schizophrenia is particularly interesting in the light of recent findings by Smythies, Johnston, Bradley, Morin, and Clark (1967) who found that certain methoxy derivatives of amphetamine, especially p-methoxy amphetamine have behaviour disrupting properties closely akin to LSD 25. It is not inconceivable that amphetamine may be metabolised to such methoxy derivatives in certain individuals, thus giving rise to the syndrome. The finding may also be of use in understanding schizophrenia itself.

The literature abounds with toxicity data for amphetamine in various species of mammal, but unfortunately a comparison is often difficult to make due to the varying routes of administration and the different ways of expressing the results. If only those papers which express the results in terms of either oral, or subcutaneous dosing are considered, the drugs being readily absorbed by both routes, and the toxicities are expressed in terms of an LD_{50}, then the following table can be compiled.

Animal	Route of Administration	LD_{50} mg/Kg	Author
Mouse	Oral	22	Halpern(1939)
Rat	Subcutaneous	20	Schulte et al(1941)
Rabbit	Oral	85	Reifenstein(1940)

An interesting aspect of the toxicity problem is that crowding in mice considerably increases the toxicity of amphetamine. This was originally reported by Chance (1946), and many other workers since this date have confirmed these findings (Moore, 1963).

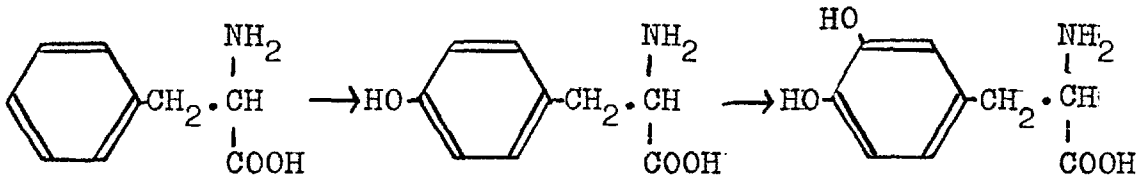
Accurate estimations of the toxicity of amphetamine in man are of necessity difficult to make. Severe reactions have occured with 30mg., yet doses of 400-500mg. have been survived. Death has followed rapid injection of 120mg. The acute toxic effects of amphetamine are usually extensions of the usual therapeutic actions. Fatal poisoning usually terminates in convulsions and coma with cerebral haemorrhages as the main pathological finding. (Goodman and Gilman, 1965).

METABOLISM OF THE PHENYLETHYLAMINES

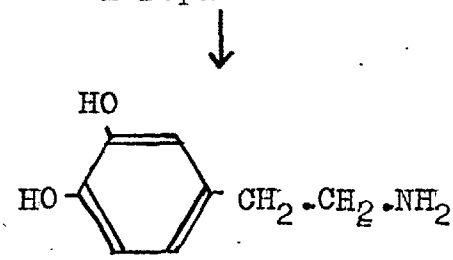
Since amphetamine is closely related to the endogenous phenylethylamines found in the mammalian body it would not seem too unreasonable to assume that amphetamine might become entrained in some of the synthetic and catabolic reactions which are undergone by these amines. Therefore a brief review of the metabolism of the phenethylamines will be undertaken and a comparison made between these known routes and the possible routes for amphetamine metabolism.

Soon after the discovery of the enzyme L-DOPA decarboxylase by Holtz, Heise and Ludtke (1938), Blaschko (1939) proposed a series of reactions by which adrenaline

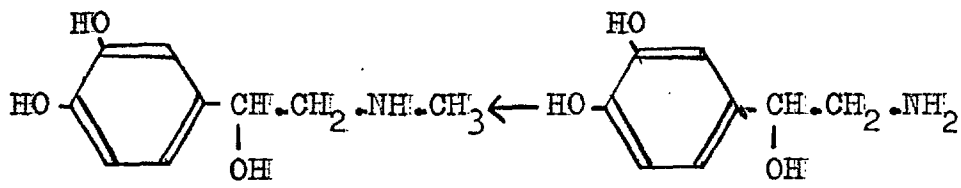
and noradrenaline would be formed from tyrosine (and thus phenylalanine) "in vivo". This is now known to be the main pathway through which catecholamines are synthesised in animal tissues.



L-Phenylalanine L-Tyrosine L-Dopa



Dopamine



L-Adrenaline L-Noradrenaline

Pathway for adrenaline synthesis proposed by Blaschko (1939)

However, the verification of this pathway took considerable time due, no doubt, in no small part to the technical difficulty of detecting the pathway intermediates which are only present in low concentrations. The first

evidence for this series of reactions came from studies of catechol amine synthesis in the adrenal medulla (Udenfriend and Wyngaarden, 1956).

It was technically even more difficult to demonstrate noradrenaline synthesis in the sympathetic nerves and it was not until the advent of refined chromatographic techniques and the availability of radio actively labelled precursors that this was possible. In 1958 Goodall and Kirshner showed that homogenates of sympathetic nerves or ganglia were able to convert labelled tyrosine to noradrenaline and more recently labelled noradrenaline, which cannot pass the blood/brain barrier has been found in the guinea pig brain after the peripheral administration of ^{14}C-tyrosine (Udenfriend, Zaltzman-Nirenberg, 1963).

ENZYMES INVOLVED IN BIOSYNTHESIS

The first enzyme in the chain, phenylalanine hydroxylase, has been extensively studied by Kaufman and his associates, who have taken particular interest in the cofactors involved. The general reaction catalysed by hydroxylases can be represented:

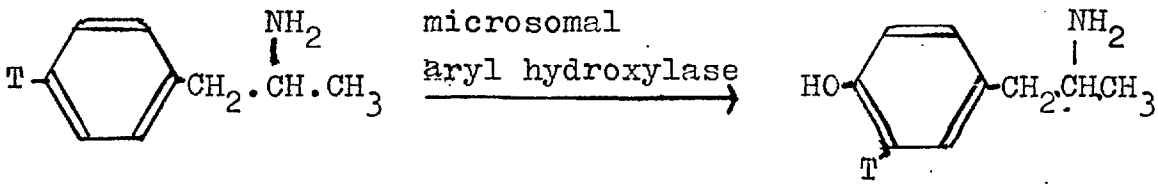
$$RH + O_2 + XH_2 \longrightarrow ROH + H_2O + X$$

where RH stands for the substrate to be hydroxylated, ROH for the hydroxylated product and XH_2 for an electron donor. Enzymes that catalyse this kind of reaction have been called mixed-function oxidases by Mason (1957) mono-oxygenases by Hayaishi (1964). They are characterised by a requirement for an external electron donor and it has been shown that the oxygen in the hydroxylated product comes from the atmosphere and not from water (Kaufman et al. 1962). A tetrahydropteridine

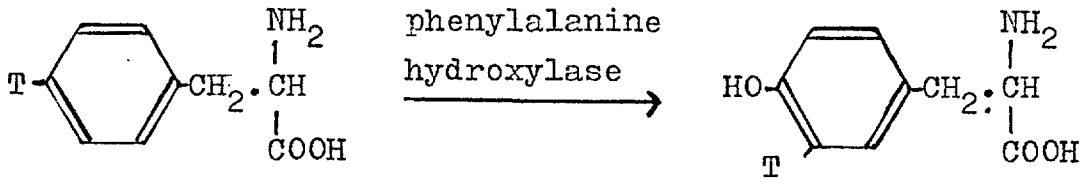
is the coenzyme in the phenylalanine hydroxylating system (Kaufman, 1966).

The hydroxylation reaction of phenylalanine serves a dual role in mammalian metabolism. It is first of all an obligatory step in the degradation of phenylalanine, there being no alternate path in mammals for cleavage of the benzene ring, and secondly it provides an endogenous source of tyrosine. Kaufman found that of all mammalian organs, a rat liver homogenate was the most active hydroxylator (Kaufman, 1957), that the activity resided in the 15,000g. supernatant fraction of the homogenate, and that the enzyme is relatively specific for L-phenylalanine (but not completely).

The mechanism of hydroxylation of aromatic compounds has been extensively studied by Guroff, et al. (1967). They observed that the hydroxylation of 4-tritioamphetamine using liver microsomal aryl hydroxylase to give 3-tritio-4-hydroxyamphetamine, yielded a retention of tritium of 90%. They reported a similar migration in the hydroxylation of 4-tritiophenylalanine using phenylalanine hydroxylase. Whether these two enzymes in this case are the same is not at present clear, but it maybe significant that the hydroxylating enzyme in both cases has its highest activity in rat liver.



4-tritioamphetamine



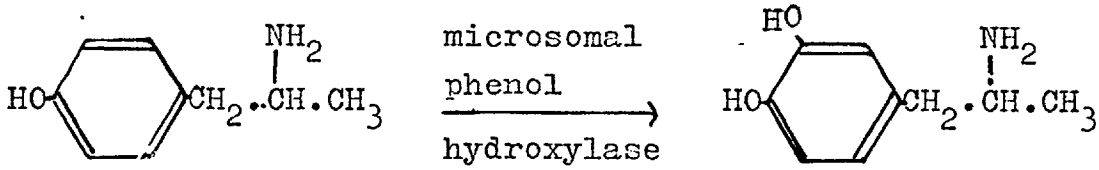
4-tritiophenylalanine

Action of microsomal hydroxylase on tritiated substrates

The conversion of L-tyrosine to L-DOPA is catalysed by the enzyme tyrosine hydroxylase. The properties of this enzyme have been described recently in a review by Udenfriend (1966). Except in the adrenal medulla the enzyme is largely associated with particles sedimenting at 15,000g. and has been found in brain and sympathetically innervated tissue. Partially purified preparations from the supernatant fraction of adrenal medullary extracts require tetrahydropteridine cofactors and are stimulated by ferrous iron. The enzyme appeared to be quite specific for L-tyrosine.

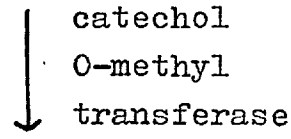
Apart from this specific tyrosine hydroxylase less specific enzymes which hydroxylate a variety of monophenols to catechols have been found to be localised in the microsomes of the liver (Axelrod J., 1963, Daly, Inscoe, Axelrod, 1965). Since the catechol metabolites formed would be unstable and the amounts are too small to measure, advantage was taken of the unique property of these compounds to be O-methylated with catechol O-methyl transferase. Thus incubation of a monophenol with the hydroxylating enzyme, catechol O-methyl transferase and C^{14}-methyl-S-adenosyl methionine would give a product

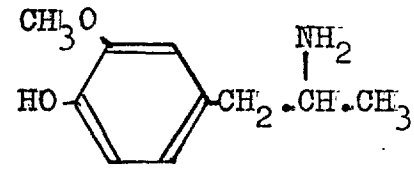
with a radioactive methyl group if any catechol were formed. In fact a number of monophenolic biogenic amines were produced including 3-methoxy-4-hydroxyamphetamine from p-hydroxyamphetamine.



p-hydroxyamphetamine

3,4-dihydroxyamphetamine





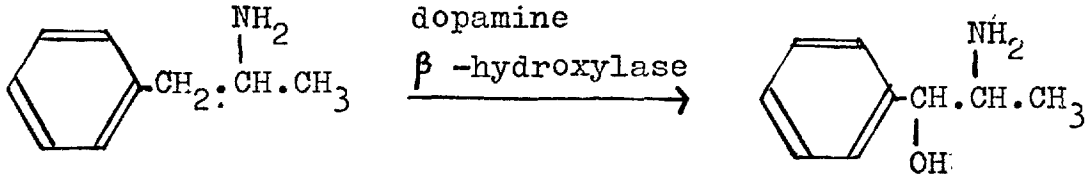
3-methoxy-4-hydroxy amphetamine

Enzymatic formation of O-methylated catechol of amphetamine

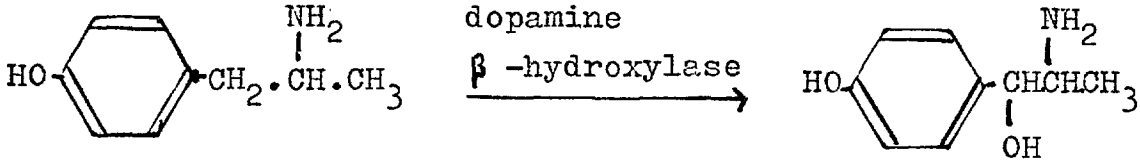
The decarboxylation of L-DOPA is well documented (Sourkes, 1966) and since amphetamine does not possess a carboxyl group the enzyme "DOPA decarboxylase" will not be further considered here.

The final stage in the biosynthesis of noradrenaline

involves the β-hydroxylation of dopamine, catalysed by the enzyme dopamine β-hydroxylase. The enzyme has been purified from ox-adrenal glands by Levin and Kaufman (1961) and Potter and Axelrod (1963) reported its association with catecholamine storage particles obtained from rat heart homogenates. In "in vitro" studies on the substrate specificity of this enzyme, Goldstein and Contrera (1962) reported that not only dopamine was hydroxylated but also α-methyl dopamine to α-methyl noradrenaline, and significantly p-hydroxyamphetamine to p-hydroxynorephedrine and amphetamine to norephedrine. Creveling, et al. in the same year, pointed out that since many well known sympathomimetic drugs are substrates for the enzyme, these drugs may owe some of their activity to the corresponding metabolites. In an extension of his study Goldstein (1964) showed that d-amphetamine but not the laevo isomer was β-hydroxylated.



Amphetamine — Norephedrine



p-Hydroxyamphetamine — p-Hydroxynorephedrine

"In vitro" Beta hydroxylation of amphetamine and p-hydroxy-amphetamine by dopamine β-hydroxylase

The first "in vivo" demonstration of β-hydroxylation was by Sjoerdsma and von Studnitz (1963) who administered parahydroxyamphetamine orally to human subjects and they were able to identify parahydroxynorephedrine in the urine to the extent of about 5% of the dose.

In the adrenal medulla the further conversion of noradrenaline to adrenaline is catalysed by the enzyme phenylethanolamine N-methyl transferase (Axelrod, 1962). The enzyme in common with other methyl transferases requires S-adenosyl methionine as a methyl donor and is only found to any significant extent in the supernatant fraction of adrenal homogenates. The enzyme will methylate a number of phenylethanolamines including norephedrine although the enzyme was three times more active towards noradrenaline.

Another enzyme which can N-methylate noradrenaline to adrenaline has been found in the rabbit lung. It again requires S-adenosyl methionine to carry out the methylation. One striking feature of this enzyme is its lack of specificity, although serotonin is the best substrate. Both ephedrine and amphetamine have been found to act as substrates for this enzyme. (Axelrod, 1962).

METABOLIC DEGRADATION OF PHENYLETHYLAMINES

Studies on the metabolism of amines with the phenylethylamine nucleus were initiated in 1910 by Ewins and Laidlaw, who found that the administration of phenylethylamine and tyramine to dogs, cats and rabbits resulted in the excretion of large amounts of phenylacetic and p-hydroxyphenylacetic acid. Guggenheim and Loeffler

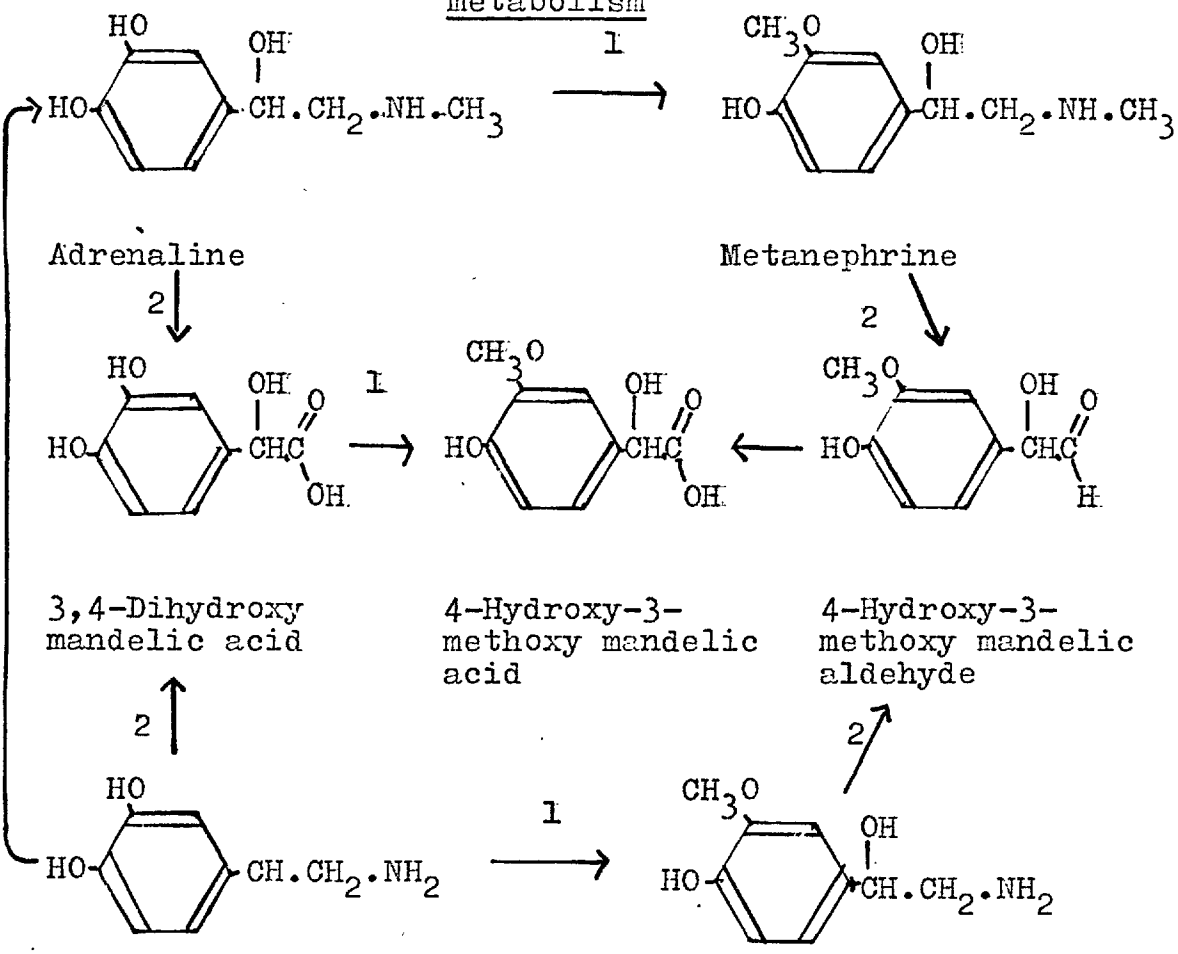
(1915) confirmed these findings in rats and mice. These observations suggested the presence of an enzyme that could deaminate these amines "in vivo".

An enzyme which oxidised tyramine in liver was described by Hare in 1928. That this liver enzyme in rats, guinea pigs and rabbits could also destroy adrenaline was shown by Blaschko, Richter and Schlossman (1937). This enzyme was called "amine oxidase" by Richter (1938) who showed that the enzyme was also capable of attacking a large variety of amines eg. dopamine, tyramine, β-phenylethylamine. However, phenylethylamines with an α-methyl substituent are not attacked by the enzyme, but act as competitive inhibitors eg. amphetamine ephedrine, and α-methylnoradrenaline. The enzyme is distributed widely in animal tissues where it is exclusively localised in mitochondria (Hawkins, 1952).

An enzyme which considerably deactivates adrenaline and noradrenaline by O-methylation to the 3-methoxy compound was discovered by Axelrod (1957). This enzyme catechol-O-methyl transferase, has already been mentioned in the consideration of the "in vitro" production of 3, 4-dihydroxyamphetamine by the non-specific phenol microsomal hydroxylase. In this it was shown that the enzyme could methylate the dihydroxyamphetamine to give 3-methoxy, 4-hydroxyamphetamine. The enzyme requires S-adenosyl methionine as a methylating agent, Mg^{2+} ions for activity, occurs widely in the cell sap of animal tissues, and will O-methylate most catechol substrates.

The relative importance of O-methylation and oxidative deamination "in vivo" is still in doubt, although it has been adequately demonstrated that these are important reactions "in vivo". Experiments by Axelrod and his co-workers (Axelrod, 1966) showed that O-methylation was the major metabolic pathway for adrenaline and noradrenaline. The O-methylated compounds are subsequently deaminated to yield the major urinary excretion product, 3-methoxy, 4-hydroxy mandelic acid.

Summary of the major pathways for adrenaline and noradrenaline metabolism



1=catechol-O-methyl transferase; 2=monoamine oxidase; 3=phenylethanolamine N-methyl transferase. (From Axelrod, 1965)

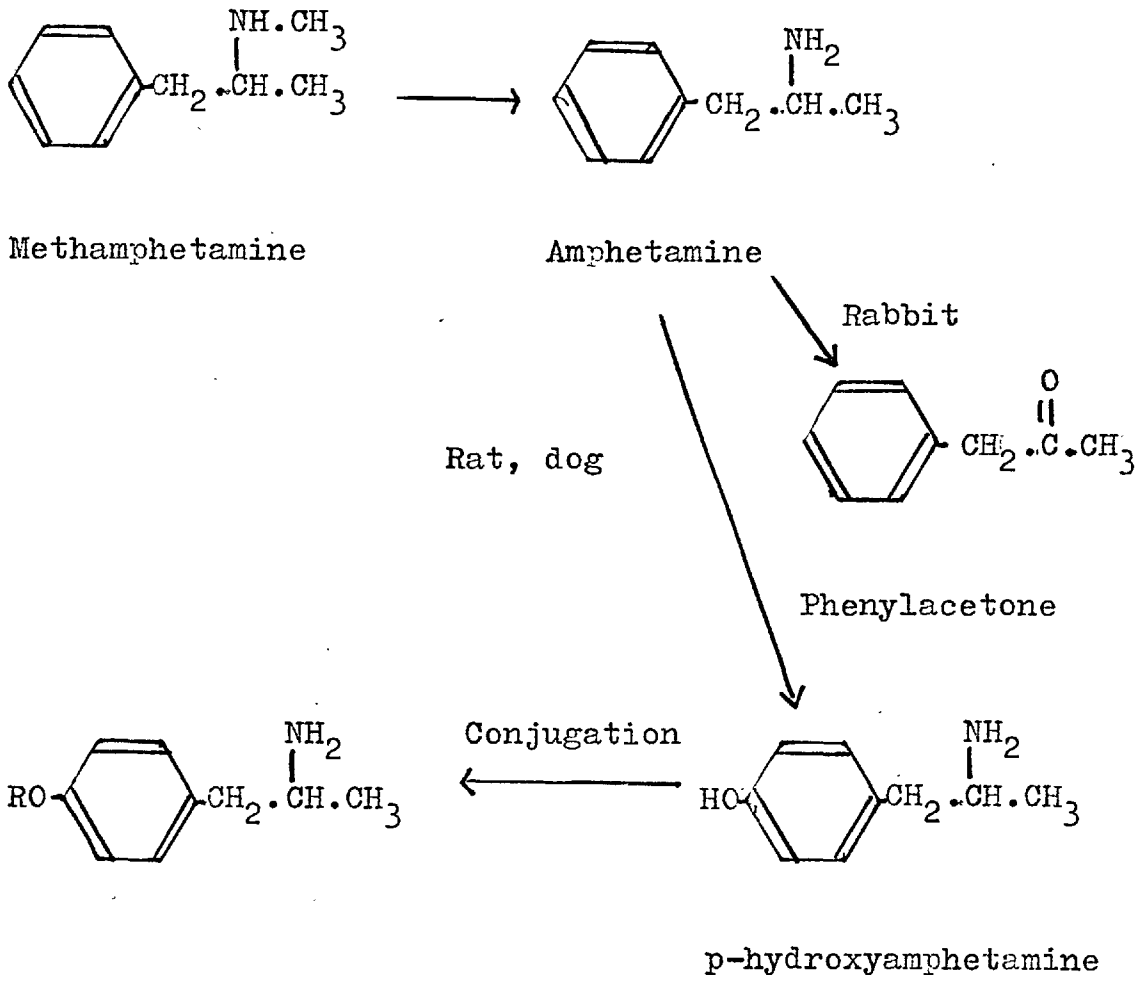
METABOLISM OF PHENYLISOPROPYLAMINES

Initial attempts at studying the phenylisopropylamines were thwarted for similar reasons to those which had prevented a detailed study of the metabolism of the phenylethylamines during the same period, namely, lack of sensitive and specific methods for their estimation. However a start was made in the late thirties, and by the time the most useful method, that of the use of radioactively labelled compounds, came to be applied to the problem, a considerable body of knowledge had already accumulated.

Richter (1938) reported that when the phenylisopropylamines, amphetamine and ephedrine were administered to man they were excreted almost entirely unchanged. However Beyer and Skinner (1940) and later workers were not able to corroborate these findings. They found that about half the given dose in dogs was excreted unchanged in 48 hours. Beyer in later papers (1941 a,b and 1942) proposed that the amphetamine which was not excreted unchanged was deaminated in the liver. The compound resists oxidative deamination by amine oxidase, which accounts in part for its long action; indeed amphetamine is a strong inhibitor of amine oxidase more so than is ephedrine. He was able to show "in vitro" that an ascorbic acid-dehydroascorbic acid system together with phenol oxidase was able to deaminate amphetamine. This he tentatively suggested was the system which occurred in the body. This was substantiated "in vivo" for amphetamine when it was reported that the excretion of

the amine in dogs could be markedly diminished by subcutaneous injections of large amounts of ascorbic acid.

A further ten years was to elapse before the problem of amphetamine metabolism was again tackled, this time by Axelrod (1954). The following scheme summarises his findings

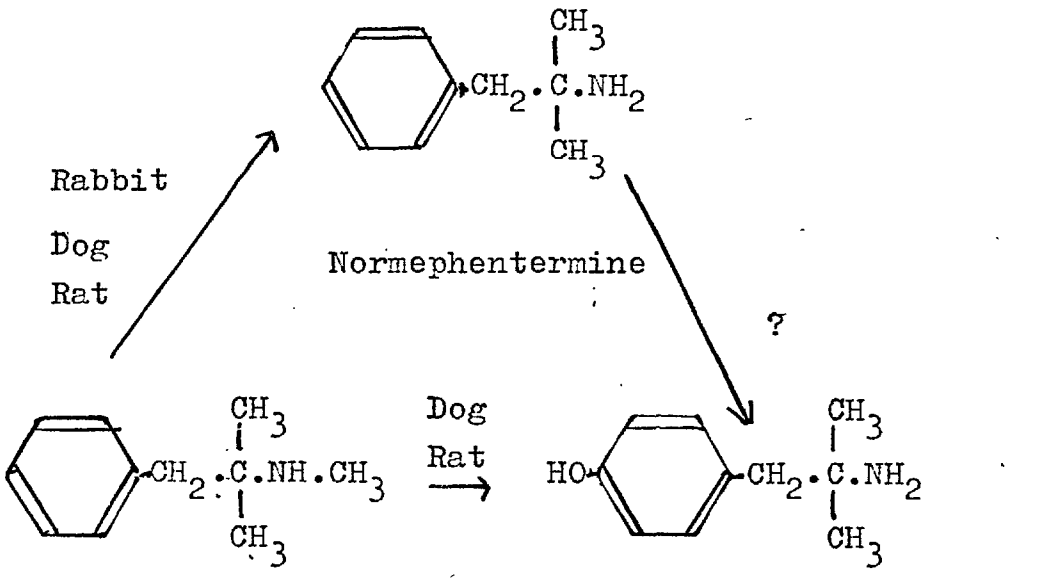


The metabolism of amphetamine and N-methyl amphetamine

He suggested that the main route of metabolism of d-amphetamine in the dog involves hydroxylation to p-hydroxy-amphetamine, an active pressor agent. In the dog the d-amphetamine disappears more slowly than its p-hydroxylated metabolite, suggesting that the major part of the pharmacological effect of the drug, is due to the parent compound. The plasma protein binding of d-amphetamine is negligible, but it is highly localised in most organ tissues including the brain, suggesting that amphetamine readily passes the blood/brain barrier in contrast to the catecholamines which do not.(Weil-Malherbe, Axelrod, Tomchick, 1959). Parahydroxyamphetamine is excreted in both free and conjugated forms in dog urine.

In the same paper, he showed that there are marked differences in the metabolism of amphetamine in various species. Dogs and rats hydroxylate considerable amounts of the amine whereas in rabbits and guinea pigs hydroxylation occurs to a negligible degree. After administration of methamphetamine to the dog about half is demethylated to amphetamine a part of which is hydroxylated to p-hydroxyamphetamine. This demethylation reaction has also been demonstrated in man using gas chromatographic detection techniques (Beckett, Rowland, 1965). They found that more (+) than (-) isomer was demethylated and this was confirmed by Gunne (1967). Similar species differences have been found with the metabolism of mephentermine (2-methyl N-methylamphetamine) by Walkenstein, Chumakow and Shifter (1955). The principal if not sole metabolites in dog and rat were normephentermine and p-hydroxynormephentermine. Although the major metabolite excreted by the rabbit was normephentermine

small amounts of parahydroxymephentermine and an unidentified substance were also excreted.



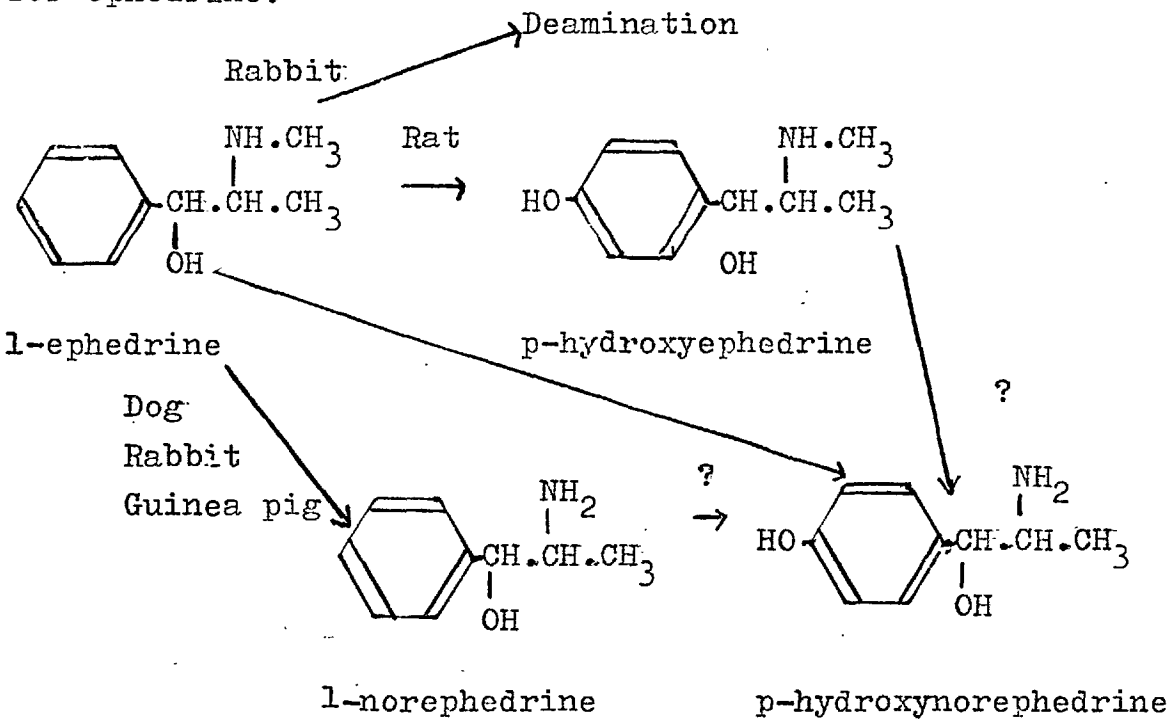
The metabolism of mephentermine

Axelrod (1955) further investigated the fate of amphetamine in the rabbit and he confirmed, using a rabbit liver preparation, the production of phenyl acetone and ammonia which had been predicted by Beyer using an "in vitro" phenol oxidase system. The enzyme requires TPNH and atmospheric oxygen and was one of the first known examples of what appears to be a general cofactor requirement for a group of non-specific microsomal hydroxylating enzymes which have become known as "mixed function oxidases". He suggested that species differences in the metabolism of amphetamine may be explained in part by an inhibitory factor in the microsomes of dog

and rat. This he demonstrated by showing that rat liver microsomes reduced the ability of rabbit liver microsomes to deaminate amphetamine. However he did not exclude the possibility that the rat microsomes might be competing for the substrate and metabolising it by a route other than deamination. The enzyme which deaminates amphetamine differs from other deaminating enzymes such as amine oxidase, D-amino acid oxidase, L-amino acid oxidase and glutamic acid dehydrogenase with respect to its substrate specificity, intra cellular localisation and cofactor requirements.

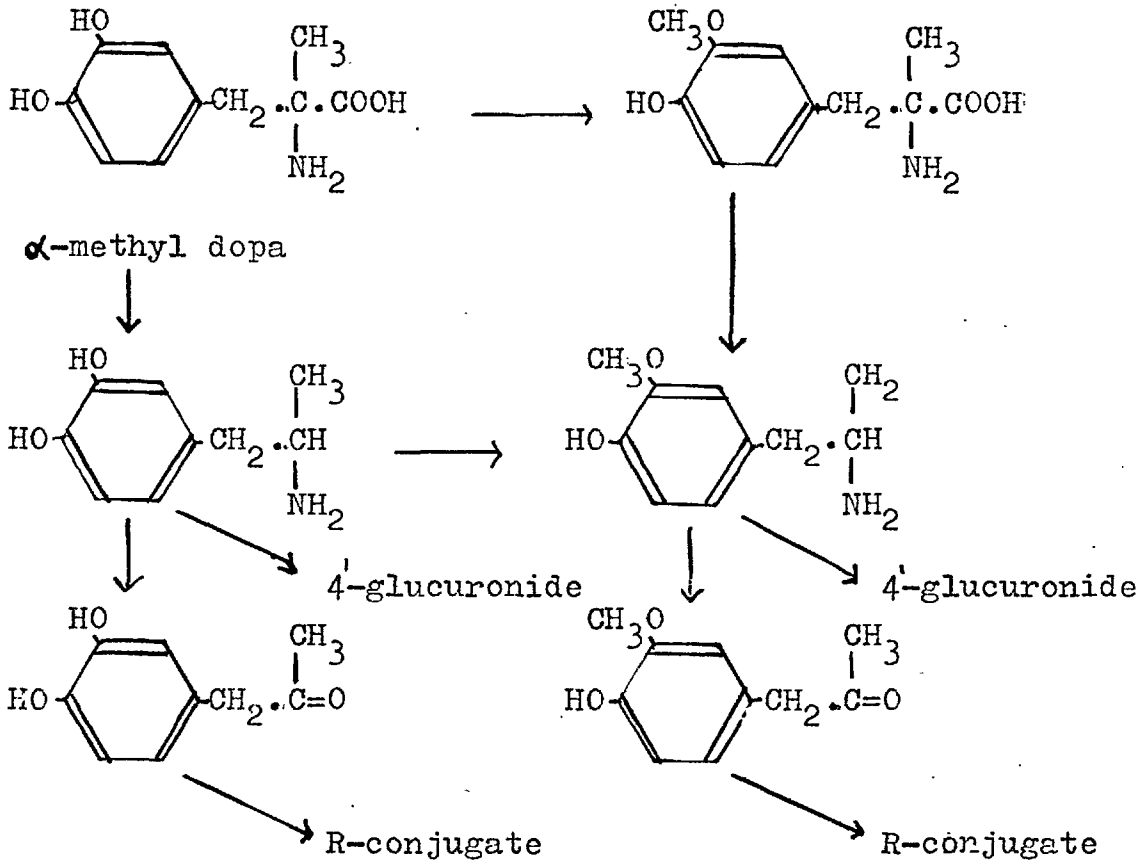
A similar state of affairs was found by Axelrod (1953) with the biotransformation of ephedrine in the dog which involves N-demethylation to norephedrine, a stable and potent pressor agent. Other minor routes of metabolism involve hydroxylation of both ephedrine and norephedrine to yield the corresponding p-hydroxy derivatives, both of which are potent pressor agents. There are again differences in the metabolism of ephedrine in other mammalian species. Like the dog, the guinea pig demethylates rapidly, but on the other hand the rat demethylates but slowly, and the p-hydroxy derivatives are more important; total hydroxylated metabolites accounting for nearly 13% of the dose in the first 24 hours, whereas they are only 1% in the dog. As with amphetamine, p-hydroxylation was not found to be an important route in the rabbit, and since very little unchanged ephedrine was recovered from the urine, pathways other than p-hydroxylation must be utilised. In fact Axelrod (1955) found that ephedrine was an even better substrate than amphetamine for the rabbit liver

microsomal deaminating system, so it seems reasonable to assume that deamination is the primary pathway in rabbit for ephedrine.



Metabolism of ephedrine. (after Axelrod 1953)

From the above work it would seem that the rat is unable to deaminate phenylisopropylamines to any extent but this would not appear to be the case, since Porter and Titus (1963) in studies on the metabolism of α - methyl dihydroxy phenylalanine in the rat found 3-methoxy -4-hydroxy and 3,4-dihydroxy phenylacetone in the urine. Presumably these were metabolites of the decarboxylated intermediate, 3,4-dihydroxy amphetamine.



R- may be sulphate

Pathways for the metabolism of α-methyl DOPA in the rat

Several methods have been used for the determination of amphetamine in the urine of man and most of them are non-specific. Richter (1938), Jacobsen and Gad (1940), and Harris, Searle and Ivy (1947) all used variations on a picric-acid dye complexing method. The methyl orange method of Brodie and Udenfriend (1945) has been used with modification by several workers until recently (Keller, Ellenbogen, (1952); Axelrod, (1954); Utena, Ezo, Kato, (1955); Connell, (1958); Asatoor,

Galman, Johnson, and Milne, (1965)). Yet another method that of coupling amphetamine with diazotised p-nitro aniline has been tried (Beyer, Skinner, 1940) and Alles and Wiesgarver (1961) claimed that by suitable modification this chemical method could be made to work.

All of these chemical methods however had several drawbacks in that they were non-specific and as a consequence not reproducible. Other amines present in the urine could react and it has even been shown that tobacco smoking can influence the apparent amine excretion, presumably due to the nicotine in the urine. (Beckett, Rowland and Triggs, (1965)).

Only recently has a specific and sensitive method been devised by Beckett and his co-workers. His method, which is a modification of the method of Cartoni and Stefano (1963), makes use of gas-liquid chromatography for the separation and estimation of amphetamine and related bases (Beckett, Rowland 1964, 1965). With this method he has been able to show the rhythmic diurnal pattern of excretion of amphetamine which closely follows urinary pH (Beckett, Rowland, 1964). This pH dependance of excretion rate was also demonstrated by Asatoor, Galman, Johnson and Milne (1965) using Axelrod's methyl orange method (1954). These results would appear to agree with the theory of non-ionic diffusion of bases in the distal portion of the kidney tubules(Milne, Scribner, Crawford (1958); Beckett (1965) was also able to add further support to the finding of Alles and Wiesgarver (1961) that more laevo than dextro amphetamine was excreted in the urine after giving similar doses of

either isomer. The sensitivity of the method also enabled Beckett and Tucker (1966) to confirm the findings of Rosen, Tannenbaum, Ellison, Free and Crossley (1965) that a 15mg. capsule of amphetamine in a prolonged release form was equivalent to taking three 5mg. tablets three times a day. Rosen and his co-workers had resorted to the use of radioactive drug. The sensitivity of gas chromatography was also of use in a study of the excretion and N-demethylation of N-methylamphetamine (Beckett, Rowland, 1965). They found that of the order of 20-40% of the dose was excreted unchanged in 24 hours, and that about 5% of the dose was excreted as amphetamine, the excretion being greater after administration of d-N-methylamphetamine.

Few studies on the total metabolism of amphetamine have been persued since the initial work of Axelrod in the mid-fifties. Recent workers have utilised for the first time a radio chemical "label". The first to do this was Alleva (1963), who confirmed Axelrod's findings that hydroxylation was the main metabolic route in the rat and he also found a new metabolite, hippuric acid.

THEORIES OF THE MECHANISM OF THE CENTRAL NERVOUS STIMULANT EFFECT OF AMPHETAMINE

Amphetamine has a most striking stimulant effect on the central nervous system. Several hypotheses have been put forward to explain these effects. Among those most frequently given are

(a) Inhibition of monoamine oxidase

(b) Direct action on serotonin receptors
(c) Direct action on catecholamine receptors
(d) Indirect action by release of catecholamines

INHIBITION OF MONOAMINE OXIDASE

Early theries assumed that amphetamine produced its effect on the C.N.S. by inhibiting monoamine oxidase (MAO) and thus retarding the destruction of the catecholamines (Blaschko, Richter and Schlossman, 1937). Since that time evidence for and against the MAO inhibition theory has been published. It is well known that d-amphetamine has a greater C.N.S. stimulatory effect than l-amphetamine. Therefore if the MAO idea is to stand up there should be some correlation between activity and MAO inhibition. Pratesi and Blaschko (1959) showed that the rabbit liver enzyme is equally inhibited by both isomers and Grana and Lilla (1959) could find no correlation between central stimulation and inhibition of MAO in the rat. On the other hand, Fuller and Walters (1965) and Parmar (1966) did find that the d-isomer inhibited MAO to a greater extent than the l-isomer. "In vivo" work by Glowinski, Axelrod and Iversen (1966) showed a decrease in catechol deaminated metabolites in the brain, on the other hand there was no decrease in the O-methylated deaminated metabolites as occurs with one of the hydrazine MAO inhibitors. In general the stiking effect on the C.N.S. produced by amphetamine does not seem to be wholly accounted for by MAO inhibition.

DIRECT ACTION ON SEROTONIN RECEPTORS

Amphetamine has been found to produce a facilitation of self stimulation in rats which have had electrodes

implanted in rewarding areas in their brain (Stein, 1964). If amphetamine did have a direct action on serotonin receptors, a similar facilitation should be observed with serotonin or tryptamine. However, no such facilitation is observed (Cook, Weidley, 1957), but serotonin does penetrate easily into the brain and both it and tryptamine are readily metabolised by MAO. Vane (1960), the originator of the serotonin hypotheses, has suggested that this hypotheses could be further tested by administering α-methyl tryptamine, since this is resistant to MAO. Although amphetamine-like stimulation was observed in rats after α-methyl tryptamine, no facilitaion of self stimulation could be detected (Stein, 1964). These results suggest that although amphetamine may produce excitement by an action on serotonin or tryptamine receptors, it does not facilitate behaviour by this means.

DIRECT ACTION ON CATECHOLAMINE RECEPTORS

It has been shown that amphetamine still exerts its behavioural effects in reserpine treated animals (John, Wenzel, and Tschirgi, 1958). On the basis of these experiments it has been suggested that in the C.N.S., amphetamine acts primarily as a directly acting sympathomimetic amine (van Rossum, van der Schoot, and Hurkmans, 1962).

On the other hand Stein (1964) found a decrease in facilitation of self stimulation in rats 24 hours after large doses of reserpine. Indeed sucessive doses of amphetamine after reserpine treatment tend to have a decreased effect whereas ordinarily the reverse is true.

The findings do not lend support to the hypothesis that amphetamine facilitates behaviour by a direct action on catecholamine receptors. In fact the diminishing effect of reserpine on a dose of amphetamine led Burn and Rand (1958) to propose that amphetamine exerted its peripheral sympathomimetic effects indirectly by a local release of catecholamine.

INDIRECT ACTION BY LIBERATION OF CATECHOLAMINE

In addition to the findings with reserpine, there is gradually accumulating a great deal of evidence to support the catecholamine release hypothesis.

Firstly it has been shown by Moore (1963) and several other investigators that amphetamine lowers the level of noradrenaline in the brain; indeed Moore showed that d-amphetamine was 3-5 times more effective as a depleting agent than l-amphetamine. This activity coincides with the C.N.S. stimulatory potency of the two isomers. The toxicity of amphetamine, especially the d-isomer, is greatly increased in grouped animals, and this is mirrored in the greater loss of brain noradrenaline (Moore, 1964).

Secondly, it has been demonstrated that the facilitating effect of amphetamine on self-stimulation is strongly potentiated by MAO inhibitors (Stein, 1962). This would be difficult to explain if it were assumed that amphetamine acts directly, since it is not attacked by MAO. On the other hand if we assume that it acts by releasing a substance which is destroyed by the enzyme then the potentiation is easily explained.

The common objections to this release theory are that it fails to explain why amphetamine still has a stimulatory action after depletion of amine stores with reserpine (and may even have an enhanced effect). These observations may not be incompatible with the theory for several reasons. First, even large doses of reserpine do not entirely deplete the brain of amines. Second, the supersensitivity of the peripheral neurones to noradrenaline has been recognised after prolonged reserpine treatment (Trendelenburg, 1963), so it is not unreasonable to suppose a similar effect is operative in the brain too.

WHICH SUBSTANCE(S) DOES AMPHETAMINE RELEASE?

The most likely candidates are considered to be noradrenaline and dopamine. This has been further investigated recently by several workers using α-methyl tyrosine. This substance is known to be an inhibitor of the synthesis of noradrenaline at the tyrosine hydroxylase step which is thought to be the rate-limiting step in noradrenaline synthesis (Spector, Sjoerdsma, Udenfriend, (1965)). Significantly α-methyl paratyrosine abolishes stereotyped behaviour and lowers motor activity when given before a dose of amphetamine (Randrup, Munkvad, (1966)). However a dose of DOPA rapidly restores the classical amphetamine effects, thus it is apparent that DOPA or a related catecholamine is probably responsible for the amphetamine response. Dingell, Owens, Norvich, and Sulser (1967) showed that l-α-methyltyrosine did not alter the levels of ^{3}H-amphetamine in the brain, so the blockade of the amphetamine response by α-methyl tyrosine, cannot be attributed to the interference in

the metabolism of amphetamine or its uptake by the brain.

Using diethyl dithiocarbamate Randrup and Scheel-Kruge (1966) were able to antagonise the hypermotility, but not the stereotyped behaviour exhibited by rats. The fact that diethyl dithiocarbamate inhibits the synthesis of noradrenaline from dopamine suggests that dopamine is associated with stereotyped behaviour, and hypermotility with noradrenaline.

OTHER ACTIONS

In addition to all the other mechanisms so far quoted two other properties of amphetamine should be mentioned.

The decrease of brain noradrenaline produced by amphetamine could be partly due to the prevention of the re-uptake of physiologically released noradrenaline. The accumulation of exogenous noradrenaline is impaired by amphetamine in both peripheral sympathetically innervated tissue (Burgen, Iversen, 1965) and "in vivo" in the rat brain (Glowinski and Axelrod, 1965).

It has also been found that the conversion of ^{3}H-dopamine to ^{3}H-noradrenaline in certain brain areas is inhibited by amphetamine, suggesting that amphetamine is inhibiting dopamine β-hydroxylase (Glowinski, Iversen, Axelrod, 1966). This has been shown to be the case "in vitro", but only at high concentrations (Goldstein, Contrera, 1961).

Amphetamine thus has a highly complex action on

the brain and the peripheral sympathetic nervous system. At present it is by no means clear which of these actions is of the greatest importance and it may even be wrong to attempt to ascribe the powerful behavioural effects of the drug to any one biochemical locus of action. In fact it may be that the effects of the drug are a culmination of several points of interaction of the drug with the chemistry of the brain.

THE AMPHETAMINE-BARBITURATE MIXTURE

Amphetamine-barbiturate drug mixtures have been widely used in psychiatry for some time for the treatment of anxious or depressed neurotic patients. Several commercial preparations are available and most contain dexamphetamine sulphate and amylobarbitone, usually in the ratios 1:6.5 or 1:13 by weight. Although amphetamine and the barbiturates tend to have opposite pharmacological effects in both man and animals, when given in conjunction, their clinical effects can be "mutually corrective" in that the advantages of both drugs remain and the side effects antagonise each other (Myerson, 1939). Since then further clinical findings have supported this statement, yet it was not until a relatively short time ago that the pharmacology was studied in detail. Unfortunately this valuable mixture, which is of great use in psychiatric treatment due to the beneficial feeling it produces (Nowlis, Nowlis, 1956), has for similar reasons been taken for non-medical reasons by young people who unfortunately then become dependent upon the drug (Leader, B.M.J., 17 August 1963).

Legge and Steinberg (1962) found that a mixture of 15mg. amphetamine sulphate and 300mg. of cyclobarbitone in man impaired the efficiency of performance of tasks much less than did the barbiturate alone; it produced almost as big arise in the pulse rate as did amphetamine and it produced reports of feeling "elated" from many more subjects than either drug alone. In a series of experiments on rats Rushton and Steinberg (1963) showed that such mixtures could stimulate exploratory activity in rats to a much greater degree than either drug alone and this they regarded as a true potentiation. More recently Dickens, Lader and Steinberg (1965) showed that the 1:6.5 ratio of dexamphetamine sulphate:amylobarbitone sodium (Drinamyl; Smith, Kline and French) was potentially more useful than the mixture used by Legge and Steinberg in 1962. Therefore Steinberg and her co-workers have shown the amphetamine-barbiturate mixture to have a pharmacologically sound basis for its continued use and yet little is known about the biochemical basis of the phenomenon.

If this mutual potentiation is in fact founded on a biochemical basis rather than a neurological one (eg. a change in the proportion of facilitation to inhibition in the C.N.S.), it could be that the barbiturate has an influence on the metabolism of the amphetamine (or vice versa). Several workers have shown that various drugs competitively inhibit each others metabolism. Often this effect is diphasic (Serrone, Fujimoto, 1961) and (Kato, Chiesara, and Vasanelli, 1964) one drug inhibiting the metabolism of a second drug and then 36-48 hours later stimulating the metabolism of the

second drug. Kato and co-workers surveyed a variety of drugs and he found that phenobarbitone was a weak inhibitor of metabolism and yet a strong stimulator. Rubin, Tephly and Mannering (1964a,b) reasoned that since the metabolism of drugs by hepatic microsomes is thought to proceed through a coupled reaction involving NADPH oxidase and one or more "hydroxylases" it could be predicted that numerous drugs would competitively inhibit the metabolism of another drug employed as a substrate for the microsomal system. This they showed to be the case "in vitro" but not always so "in vivo".

The effect of one drug on the metabolism of another is by now so well founded that the antagonism of the metabolism of amphetamine by the barbiturate in the mixture is a distinct possibility, the converse having already been shown to be true by Klissiunis, Várónos, Tzitzi, (1965). He gave amphetamine and pentobarbitone together orally and showed that amphetamine increased the blood level of pentobarbitone.

SCOPE OF THE PRESENT WORK

In the preceding review it has been shown how previous workers have demonstrated two possible routes for the metabolism of amphetamine. These routes are aromatic hydroxylation to p-hydroxyamphetamine and deamination to benzyl methyl ketone, and the extent to which these two reactions occur is species dependant. (Axelrod,1954 a,b; Alleva, 1963). In addition it has been shown that the pharmacology of the (+) and (-) isomers is

markedly different (Prinzmetal and Alles, 1940) and that the mixing of a barbiturate with a dose of amphetamine causes much less inhibition of the stimulant effect than would have been predicted (Myerson, 1939; Legge and Steinberg, 1962).

The following chapters are an account of an investigation in detail into the metabolic fate of dl-amphetamine and its two optical isomers in man and several other mammals in order to confirm and extend knowledge of its metabolic fate and interspecies variation in metabolism of the drug and to see if there is a difference in the metabolism of the two isomers. Finally, with this information to hand, the effect of barbiturates, if any, on the metabolism of amphetamine was studied to see if this could explain the unexpected pharmacology of the mixture.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

(±)-2-Amino-1-phenylpropane (amphetamine) sulphate, m.p. 302° (decomp.) and its (+)- and (-)-forms, m.p. 308° (decomp.) and 300° (decomp.), respectively, (±)-2-amino-1-(4'-hydroxyphenyl) propane (paredrine) hydrobromide, m.p. 190-192°, (±)-2-methylamino-1-phenylpropane (methamphetamine) hydrochloride, m.p. 132-134°, and (±)-2-amino-1-phenylpropanol (norephedrine) hydrochloride, m.p. 190-194°, were the gifts of Smith, Kline and French Laboratories, Philadelphia. (±)-2-Amino-1-(3',4'-dihydroxyphenyl) propanol (cobefrine) hydrochloride, m.p. 176-178°, was the gift of Bayer Products Ltd., Surbiton, Surrey. (±)-Benzyl methyl carbinol, b.p. 105°/20mm., was prepared by lithium aluminium hydride reduction of benzyl methyl ketone (Smith, Smithies and Williams, 1954). Other known compounds used were purchased and purified.

(±)-, (+)-, and (-)-(1-^{14}C) Amphetamine sulphate (see Blackburn and Burghard, 1965) of specific activity 16.4, 2.92 and 6.0 μC/mg., respectively were the gifts of Smith, Kline and French Laboratories.

4-METHOXYBENZYL METHYL KETONE

The method used was similar to that of Kratzl and Schweers, (1956). To glacial acetic acid (300ml.), heated to 80°, was added continuously, in a nitrogen atmosphere, during 1½ hours, anethol (135g.) and lead peroxide (235g.). The lead peroxide was added in 40-50g. lots.

The temperature was maintained at 90-95° during the addition, and when this was complete the reaction mixture was stirred at this temperature until a clear solution was obtained, and then for a further two hours. To the reaction mixture was added water (150ml.) followed by sodium hydroxide solution (36g. in 110ml.). The temperature of the mixture was adjusted to 50-60° and the upper organic layer removed and refluxed with dilute sulphuric acid (28ml. conc. H_2SO_4 in 230 ml. H_2O) for four hours. The upper organic layer was again removed, cooled, and washed with 2 N-sodium carbonate solution until no longer acid, and finally with water (2x50 ml.). Finally, after drying over Na_2SO_4, the product was distilled at reduced pressure, unchanged anethol came over first (114° C, 14 mm.), and the product which distilled over at 140°/15 mm. was the p-methoxybenzyl methyl ketone (le Brazidec, 1922, gives 139°/13 mm.). Yield of the ketone was 80g.

p-HYDROXYBENZYL METHYL KETONE

This was prepared by the demethylation of p-methoxybenzyl methyl ketone using hydrogen bromide in glacial acetic acid (le Brazidec, 1922), 18g. of the p-methoxy ketone yielding 8.5g. of the phenol b.p. 147°/4-5 mm., semicarbazone m.p. 213°(lit. 213°).

(±)-2-AMINO-1-(4'-HYDROXY-3'-METHOXYPHENYL) PROPANE

Vanillin benzyl ether (60g.; 0.25 mol.; from Vanillin by the method of Spaethe, Orechoff, and Kuffner, 1934), nitroethane (25g.; 0.33 mol.), piperidine (1.9ml.) and n-butylamine (1.25ml.) in benzene (125ml.) were refluxed for 40 hours using a Dean and Stark trap to remove the water formed. The mixture was washed with N-HCl (2x200ml.)

and then water (3x200ml.). The benzene solution was dried (anhydr. Na_2SO_4) and the solvent removed at reduced pressure. Recrystallisation of the residue from ether/benzene (8:1; 450ml.) gave yellow crystals (33g.) of 1-(4'-benzyloxy-3'-methoxyphenyl)-2-nitroprop-1-ene m.p. 90.5-91.5° (Fodor, 1943 gives m.p. 92°). This (29.9g.) was reduced with lithium aluminium hydride (11.4g.) in ether (500ml.) by using a soxhlet extraction apparatus to add the relatively insoluble nitro compound to the ethereal solution of lithium aluminium hydride. When the mixture became colourless (4 hr.) the mixture was cooled and treated with water. Then 2-N-NaOH was added to dissolve insoluble material and the ether layer was separated and evaporated. The residue was taken up in 2-N-HCl (200ml.), made alkaline with 2.5N-NaOH and extracted with ether (200ml.). The extract was evaporated to an oil which solidified overnight at 0°. This solid (15g.) in acetic acid (225ml.) was treated during 10min. with 10N-HCl (52ml.) and the whole heated for 1 hr. at 100°. The solution was evaporated to dryness, and the residual solid washed with acetone and then recrystallised from aqueous ethanol to give fine white needles (3.7g.; m.p. 254-255° decomp.) of (±)-2-amino-1-(4'-hydroxy-3'-methoxyphenyl) propane hydrochloride (Robinson, Lowe and I.C.I., 1940 give m.p. 254-255° decomp.)

(±)-2-AMINO-1-(3',4'-DIMETHOXYPHENYL) PROPANE

Veratraldehyde (42g.; 0.25 mol.), nitroethane (24ml.; 0.33 mol.), piperidine (1.9ml.) and n-butylamine (1.25ml.) were refluxed together for 18 hr. in toluene (125ml.) in a Dean and Stark apparatus. The reaction mixture

was processed as described above for the preparation of 1-(4'-benzyloxy-3'-methoxyphenyl)-2-prop-1-ene. The isolated material was recrystallised from ethanol to give pale yellow crystals (36.5g.) of 1-(3',4'-dimethoxyphenyl)-2-nitro-prop-1-ene m.p. 70-71° (Bu'Lock and Harley-Mason, 1951 quote 73° and Alles, (1932) gives 70-71°). The latter (22.3g.; 0.1 mol.) was reduced with $LiAlH_4$ as described above. After the reduction, the ethereal solution was evaporated to dryness and the oil obtained was dissolved in acetone (5ml.) and made slightly acid by the addition of dry hydrogen chloride gas dissolved in absolute ethanol. The solvents were removed by evaporation under reduced pressure, the residual oil was treated with acetone and the crystalline (±)-2-amino-1-(3',4'-dimethoxyphenyl) propane hydrochloride that separated was filtered off and washed with acetone. Yield 3.8g., m.p. 144° (Mannich and Jacobsohn, 1910 quote 144°).

(±)-2-AMINO-1-(3',4'-DIHYDROXYPHENYL) PROPANE HYDROCHLORIDE

This, m.p. 190° was prepared by demethylation of 2-amino-1-(3',4'-dimethoxyphenyl) propane with HI as described by Mannich and Jacobsohn,(1910).

(±)-2-AMINO-1-(4'-METHOXYPHENYL) PROPANE

This, m.p. 207° was prepared by methylation of (±)-2-amino-1-(4'-hydroxyphenyl) propane with diazomethane in methanol (lit. 210°, Mannich and Jacobsohn, 1910; 208°, Hoover and Hass, 1947).

(±)-2-AMINO-1-(4'-HYDROXYPHENYL) PROPANOL

The hydrochloride of this compound is described in U.S. Patent No. 1,957,092 (1932). However, in common

with many patents, although the route was outlined, the details of the synthesis, including intermediates were not given. The following synthesis uses this route, but the details and intermediates are recorded for the first time.

p-Hydroxypropiophenone (m.p. 148°) was prepared from phenol and propionic acid by a modified Fries reaction using the method of Hartung, Munch, Miller and Crossley (1931). The benzyloxy derivative (m.p. 100-101°) of p-hydroxypropiophenone was made using the general method of Spaethe, Orechoff, and Kuffner, (1934). To a solution of p-benzyloxypropiophenone (48g., 0.2 mol.) in methylene dichloride (250ml.) solid $CaCO_3$ (25g.) was added. The mixture was heated under reflux with stirring and bromine (32g., 0.2 mol.) was added dropwise at a rate (1.5 hr.) that kept the reaction mixture a pale yellow colour. After cooling 2N-HCl was added to dissolve insoluble material and the methylene chloride layer was separated, washed with $NaHCO_3$ (5%; 125ml.) and then water (2x100ml.) and dried over anhydrous Na_2SO_4. Evaporation of the solvent at reduced pressure gave an oil which crystallised at 0°. Recrystallisation from ethanol gave white platelets of (±)-2-bromo-1-(4'-benzyloxyphenyl) propanone (24g.), m.p. 77-78°. (Found: C, 60.2; H, 4.7; Br, 25.3%: $C_{16}H_{15}O_2Br$ requires C, 60.2; H, 4.7; Br, 25.0% . N.M.R. spectrum confirms presence of phenyl group τ =2.6 (4.9), a disubstituted benzene ring τ =1.86, 2.05 (2.0) and 2.86, 3.05 (2.0), -CO.CH.CH_3 gives a quartet at τ =4.6, 4.68, 4.78, 4.90 and $PhCH_2$-O- at τ =4.84 (integral for group 3), and a doublet for the methyl group centred around τ =8.1

(3). Integrals in brackets. Spectrum in $CDCl_3$).

To a solution of the above bromo compound (16g.; 0.05 mol.) in warm ethanol (100ml.), dibenzylamine (19.9g. 0.1 mol.) was added during 10min. and the solution refluxed with stirring for 4.5 hr. After keeping at room temp. overnight, excess dibenzylamine hydrochloride was filtered off, and washed with ether, and the washings added to the filtrate. The latter was evaporated at reduced pressure to a yellow solid which was extracted with ether. The ethereal solution was washed with water, dried (anhydrous Na_2SO_4) and evaporated to dryness. The resulting yellow solid was recrystallised from methanol (300ml.) to give white rhombic plates (23g.) of 2-(N,N-dibenzylamino)-1-(4'-benzyloxyphenyl) propan -1-one, m.p. 94-95.5°.(Found: C, 82.4; H, 6.7; N, 3.35%. $C_{30}H_{29}NO_2$ requires C, 82.7; H, 6.7; N, 3.2%).

The latter compound (8g.) in warm ethanol (50ml.) was neutralised with dry HCl in ethanol. Palladium/ Charcoal (10%; 3g.) was added and the mixture hydrogenated at 60 lb./sq. in. for 13 hr. The reaction mixture was filtered and the solvents evaporated at reduced pressure. The residual oil was dissolved in acetone and neutralised with an alcoholic solution of dry HCl, and again evaporated to dryness and left in a vacuum desiccator overnight. The partially crystallised residue was triturated with a little acetone, and on standing for a further 48 hr. the remainder crystallised. Recrystallisation from ethanol/ether gave white crystals of (±)-2-amino-1-(4'-hydroxyphenyl) propanol hydrochloride (2.4g.) m.p. 191-192° (d). (Van Dijk and Moed, 1959 give m.p. 193-

194°).

N-ACETYLAMPHETAMINE

This, m.p. 66-67° (64°, 93° on standing; Hey, 1930), was prepared by the acetylation of amphetamine with acetic anhydride.

ANIMALS

Female Wistar albino rats (200±20g. body wt.), English guinea pigs (0.7±0.1kg.), New Zealand White rabbits (3.5±0.5kg.), greyhound dogs (23±2kg.), Rhesus monkeys (4±0.1kg.), and S.A.S/I.C.I. mice (22±2g.) were used.

ADMINISTRATION OF COMPOUNDS

Amphetamine sulphate was administered orally by stomach tube as an aqueous solution to the rat, mouse and rabbit. In the case of the greyhound and the guinea pig the drug was given by intraperitoneal injection in isotonic saline and the drug for the monkey as the accurately weighed solid concealed in a banana. Human volunteers took the compound dissolved in water.

COLLECTION OF EXCRETA

The animals were kept in metabolism cages with free acess to food and water except in the case of the rodents where food was withheld for the first 24 hrs. to avoid contamination of the urine. Urine and faeces were collected daily for periods up to 5 days.

RADIOCHEMICAL TECHNIQUES

^{14}C in urine and faeces was determined with a

Packard Tri-Carb scintillation spectrometer (model 3214) as described by Bridges, Davies and Williams (1967). The amounts of amphetamine and its metabolites were determined either by isotope dilution (see below) or in some cases towards the end of the project, where the activity of the urine was great enough, by scanning paper chromatograms of urine (0.01-0.15ml.) using a Packard Radiochromatogram Scanner (model no. 7200). The ^{14}C peaks were identified by either comparison of their R_F values with those for authentic samples or in some cases by co-chromatography. The ^{14}C related to each peak was then estimated using either disc integrator values or by planimetry. In this way, knowing the amount of activity in the urine expressed as a percentage of the original dose, the proportion of each peak to the total activity on the paper could be determined and hence expressed as a percentage of the original dose.

ISOTOPE DILUTION PROCEDURES

Unless otherwise stated one tenth of the volume of the urine collected for 24 hr. after dosing was used.

(a) Amphetamine. For free amphetamine (±) amphetamine sulphate (1.363g.) was dissolved in urine which was then adjusted to pH12 by the addition of 10N-NaOH and extracted with ether (3x100ml.). The ether was evaporated and the residue treated with 5% NaOH (20ml.) and benzoyl chloride (2ml.) and the mixture shaken vigourously for 5 min. The precipitate of N-benzoylamphetamine was filtered off and recrystallised from 50% aqueous ethanol to constant specific activity, m.p. 128°.

For total (free + conjugated) amphetamine the urine was boiled for 2 hr. under reflux with an equal volume of 10N-HCl. Carrier amphetamine sulphate was added and the benzoyl derivative prepared as before.

(b) 4-Hydroxyamphetamine. The HBr salt of 4-hydroxy-amphetamine (1.536g.) was dissolved in the urine which was adjusted to pH13 with 10N-NaOH and extracted with ether (1x50ml.). This extract (containing amphetamine) was discarded. The urine was adjusted to pH9.5-10 with 10N-HCl and extracted with ether (3x100ml.). The bulked ether extracts were evaporated to dryness. The residue was dissolved in 5% sodium hydroxide (70ml.) and benzoylated with benzoyl chloride (5ml.). The O,N-dibenzoyl derivative of 4-hydroxyamphetamine was filtered off, washed with 5% NaOH and water, and recrystallised from 50% aqueous ethanol to constant activity (m.p. 143°).

(±)-2-Benzamido-1-(4'-benzoyloxyphenyl)-propane appears to be a new compound and a sample for analysis was prepared as above from (±)-4-hydroxyamphetamine hydrobromide. It formed fine white needles from aqueous ethanol (Found: C, 77.1; H, 5.8; N, 4.0%. $C_{23}H_{21}NO_3$ requires C, 76.9; H, 5.9; N, 3.9%).

Total 4-hydroxyamphetamine was determined after acid hydrolysis of urine as described for amphetamine.

(c) Benzoic Acid. Carrier benzoic acid (1g.) was added to urine and dissolved with the aid of the minimum of 10N-NaOH. After acidification of the urine with

10N-HCl, the liberated benzoic acid was extracted with ether (3x100ml.). The bulked ether extracts were evaporated to dryness and the benzoic acid recrystallised from hot water to constant specific activity (m.p. 122°).

Total benzoic acid was determined by hydrolysing urine by boiling equal volumes of urine and conc. nitric acid under reflux for 2 hrs. Carrier was then added to the mixture which was then treated as above.

(d) Hippuric Acid. Hippuric acid (1g.) was dissolved in the urine with aid of a little NaOH. The urine was acidified to pH2 with conc. HCl and the hippuric acid extracted with ethyl acetate (3x100ml.). The bulked ethyl acetate extracts were evaporated to dryness and the crude hippuric acid recrystallised to constant specific activity from hot water (m.p. 187°). In calculating the amount of hippuric acid derived from amphetamine allowance was made for the endogenous acid in the urine. This was determined by the fluorimetric method of Ellman, Burkhalter and La Dou (1961). However, compared with the amount of carrier added, the endogenous acid was quite small and when allowance was made for the endogenous acid the percentage excretion was only increased by about one percent.

(e) Benzyl Methyl Ketone. The ketone (1g.) was added to portions of urine or acid hydrolysed urine (see amphetamine) and dissolved by addition of the minimum quantity of ethanol. The urine was diluted with water and extracted with ether (3x100ml.). The bulked ether extracts were evaporated and the residual oil in ethanol (5ml.) was treated with a solution of semicarbazide

hydrochloride (1g.) and anhydrous sodium acetate (0.9g.) in water (5ml.). The semicarbazone which separated after heating the mixture on a water bath for 15 min. was filtered off, washed with water and recrystallised to constant specific activity from ethanol (m.p. 194°).

(f) Benzyl Methyl Carbinol. The (±)-carbinol (1g.) was added to untreated, acid-hydrolysed and enzyme-hydrolysed urine. (The acid hydrolysis as for amphetamine.) The enzyme hydrolysis was carried out with β-glucuronidase preparation since previous work had indicated that the carbinol was excreted as the glucuronide (Smith, Smithies and Williams, 1954). A suitable volume of urine brought to pH5 with glacial acetic acid was incubated with an equal volume of β-glucuronidase solution (Ketodase; Warner-Chilcott) for 24 hrs. at 37°. Two controls were set up, one with boiled enzyme, the other containing in addition phenolphthalein glucuronide to ensure that the enzyme was in fact working.

The carbinol was extracted from the untreated or processed urines with ether (3x100ml.) and the bulked ether extracts dried with anhydrous Na_2SO_4 and evaporated. The residual oil was dissolved in light petroleum (b.p. 100-120°; 10ml.) and treated with phenyl isocyanate (1.2ml.). After heating on a boiling water bath for 2 hrs. under reflux the solution was filtered while hot. On cooling, crystals of the phenylurethane separated. These were filtered off and recrystallised from light petroleum (b.p. 100-120°) to constant specific activity (m.p. 89°).

(g) 2-Amino-1-Phenylpropanol. The amine hydrochloride (1.235g.) was dissolved in acid hydrolysed urine (see

amphetamine) which was then adjusted to pH12 with 10N-NaOH and extracted with ether (3x100ml.). The ether extracts were evaporated and the residue treated with 3N-NaOH (10ml.) and acetic anhydride (3ml.), shaken and left to stand at 0° overnight. The crystals of 2-acetamido-1-phenylpropanol that separated were filtered off and recrystallised from an ethyl acetate/n-hexane mixture to constant activity (m.p. 134°).

(h) 2-Amino-1-(4'-Hydroxyphenyl) Propan-1-ol. The urine was hydrolysed as for amphetamine. The carrier (±)-p-hydroxynorephedrine hydrochloride (0.304g.) was added, and the mixture neutralised with 10N-NaOH. This solution was then treated with 2.5-N-NaOH (5ml.) and benzoyl chloride (1.5ml.), the mixture shaken for 5min. and the precipitate which formed was filtered and recrystallised to constant activity from 80% aqueous ethanol to give 2-benzamido-1-(4'-benzoyloxyphenyl)-1-benzoyloxypropane. (m.p. 181-182°). On preparation of this tribenzoyl derivative, which appears to be a new compound for analysis, by benzoylation of a further sample of p-hydroxynorephedrine hydrochloride a second benzoylated compound was recovered by fractional crystallisation. This on further recrystallisation from 50% aqueous ethanol had a m.p. of 162-163° and is probably the dibenzoyl derivative, namely 2-benzamido-1-(4'-benzoyloxyphenyl) propan-1-ol. Analysis figures of the suspected di- and tribenzoyl derivatives were respectively: Found C, 73.9; H, 5.9; N, 3.7%. $C_{23}H_{21}NO_4$ requires C, 73.6; H, 5.6; N, 3.7%. Found C, 75.3; H, 5.5; N, 3.0%. $C_{30}H_{25}O_5N$ requires C, 75.1; H, 5.2; N, 2.9%.

(i) 4-Hydroxyphenyl Acetone. The ketone (0.5g.) was dissolved in a portion of acid-hydrolysed urine (see amphetamine), by the addition of sufficient 10N-NaOH. The urine was adjusted to pH4 with conc. HCl and extracted with ether (3x100ml.). The ether extracts were evaporated and the residue was dissolved in ethanol (2.5ml.) and heated on a water bath for 15 min. with a solution containing semicarbazide hydrochloride (0.5g.) and anhydrous sodium acetate (0.7g.) in water (2.5ml.). The semicarbazone of 4-hydroxyphenyl acetone was filtered off and recrystallised from ethanol, m.p. 213°.

(j) Phenylalanine. (±)-Phenylalanine (1.0g.) was dissolved in a portion of urine, solution being encouraged by gentle warming. The phenylalanine that crystallised out on standing overnight was filtered off and washed with water. The crystals were dissolved in a solution of sodium bicarbonate (3g.) in water (25ml.) and then treated with benzoyl chloride (1.5ml.). The precipitate was filtered off and recrystallised from 20% aqueous ethanol to give crystals of N-benzoyl (±)-phenylalanine (m.p. 184°).

(k) 2-Methylamino-1-Phenylpropane. (±)-2-Methylamino -1-phenylpropane hydrochloride (methamphetamine) (1.245g.) was dissolved in a portion of urine. The urine was adjusted to pH13 with 10N-NaOH and extracted with ether (2x100ml.). The ether, after drying with anhydrous Na_2SO_4, was evaporated to 15ml. and then treated with dry HCl. The crystals of methamphetamine hydrochloride that separated were filtered off and recrystallised from a mixture of diethyl ether and ethanol (m.p. 132°).

(l) Phenylacetic Acid. The acid (1.0g.) was dissolved by warming in portions of urine or acid hydrolysed urine (see amphetamine). The urine was adjusted to pH2 with conc. HCl and extracted with ether (3x100ml.). The ether extracts were evaporated to dryness and the residue recrystallised from hot water, m.p. 76°.

(m) Mandelic Acid. This compound was only sought in the unhydrolysed urine. (±)-Mandelic acid (1.0g.) was dissolved in a portion of urine by the addition of a little 10N-NaOH. The urine was acidified to pH1 with 10N-HCl and extracted with ether (3x100ml.). The ether extracts were evaporated to dryness and the residue of impure mandelic acid was converted to its S-benzylthiouronium salt which was recrystallised from hot water, m.p. 166°.

(n) Phenyl Lactic Acid. (±)-Phenyl lactic acid (1.0g.) was added to urine and worked up as for phenylacetic acid. It was recrystallised from benzene, m.p. 94°.

(o) Phenylpyruvic Acid. Sodium phenylpyruvate (1.253g.) was dissolved in a sample of urine. The urine was adjusted to pH1 with conc. HCl and extracted with ether (3x100ml.). The ether extracts were evaporated and the residue was dissolved in alcohol (10ml.) and treated with 2,4-dinitrophenylhydrazine (1.0g.) dissolved in a mixture of conc. H_2SO_4 (2.0ml.) and alcohol (15ml.). The reaction mixture was diluted with 2N-H_2SO_4 when an oil precipitated which solidified on standing. This was recrystallised from aqueous ethanol to give the 2,4-dinitrophenylhydrazone of phenylpyruvic acid

m.p. 191°.

(p) Phenylpropionic Acid. The acid (1.0g.) was dissolved in portions of urine and acid hydrolysed urine and worked up as for phenylacetic acid. It was recrystallised from petroleum (b.p. 40-60°) at -10°.(m.p. 47°).

(q) Cinnamic Acid. Worked up as for phenyl acetic acid. Recrystallised from aqueous ethanol, m.p. 132°.

(r) 4-Hydroxy Benzoic Acid. Worked up as for phenylacetic acid. The acid was recrystallised from xylene-ethanol, m.p. 213°.

CHROMATOGRAPHY.

The R_F values of the compounds relevant to this work are given in table 1 using descending chromatography on Whatman No.1 paper.

The following solvents were used for chromatography:

A. Butanol-2% (w/v) aqueous citric acid (1:1, by vol). In this case the paper had been previously dipped in 5% aqueous sodium dihydrogen citrate and then dried (Curry and Powell, 1954).

B. Butanol saturated with 1.5N-ammonia-ammonium carbonate buffer(Fewster and Hall,1951).

C. 3-Methylbutanol/1,1-dimethylpropanol/water/formic acid (5:5:10:2, by vol.) (Alleva, 1963).

D. Butanol/toluene/acetic acid/water (2:2:1:1, by vol.).

Chromatograms were run for about 35 cm. from the origin.

The following methods were used to detect compounds on the chromatograms.

U. V. Light. All of the substances appeared as dark quenching spots when paper chromatograms were viewed by U. V. light. (254mμ; Chromatolite, Hanovia).

Diazotised p-Nitroaniline. p-Nitroaniline (0.25g.) was dissolved by gentle heating in 25ml. of N-HCl and the solution diluted with ethanol to 50ml. To 10ml. of this stock solution was added 0.1g. of sodium nitrite. Immediately after the nitrite had dissolved the chromatogram was sprayed with the solution. After 5 mins. the chromatogram was resprayed with a 0.5N solution of NaOH in ethanol. Amines and phenols appeared as coloured spots on the chromatogram, but were not permanent (Wichstrom and Salversen, 1952).

Hippuric Acid Detection. The chromatograms were sprayed with a 10% solution (w/v) of p-dimethylaminobenzaldehyde in acetic anhydride. On heating the paper at 120° for 2 mins. in an oven, hippuric acid shows as an orange-red spot. (El Masry, Smith, and Williams, 1956).

Benzoic Acid Detection. The chromatogram was sprayed with a 0.04% solution of bromophenol blue in ethanol, adjusted to a purple tint with sodium hydroxide solution. The benzoic acid appeared as a blue spot. (Opienska-Blauth, Saklawska-Szymonowa and Kanski, 1951).

Table 1. R_F Values and colour reactions of Amphetamine and related compounds.

Compounds	R_F Values in solvent A	B	C	D	Colour with diazotised p-nitroaniline.
Amphetamine	0.60	0.88	0.51	0.57	pale pink
N-Methylamphetamine	0.61	0.92	0.57	0.57	none
N-Acetylamphetamine	-	0.98	0.98	0.98	none
4-Hydroxyamphetamine	0.45	0.79	0.32	0.30	purple
4-Methoxyamphetamine	0.58	0.94	0.50	0.52	pale pink
Norephedrine	0.45	0.82	0.39	0.44	pale pink
4-Hydroxynorephedrine	0.27	0.64	0.17	0.08	purple
2-Amino-1-(3',4'-dihydroxyphenyl)propane	0.22	-	-	0.08	pale pink (slowly)
2-Amino-1-(4'-hydroxy-3'-methoxyphenyl)propane	0.32	-	-	0.29	purple
2-Amino-1-(3',4'-dimethoxyphenyl)propane	0.42	-	-	0.42	pale pink
2-Amino-1-(3',4'-dihydroxyphenyl)propanol	0.16	-	-	0.04	green
Benzoic acid	0.97	0.43	0.93	-	none
Hippuric acid	0.85	0.30	0.82	-	none
Benzoylglucuronide	-	0-0.2	0.60	-	none
4-Hydroxybenzoic acid	-	0-0.1	0.63	-	-

Benzoylglucuronide Detection. The chromatogram was sprayed with napthoresorcinol in acetone (1% w/v) to which 10% phosphoric acid (4:1 v/v) had been added just before use. On heating at 120° the glucuronide showed up as a dark blue spot. (Bridges, Kibby, and Williams, 1965).

BASIC METABOLITES OF AMPHETAMINE

Urine (5ml.) from rats dosed with (±)-(^{14}C) amphetamine sulphate (10mg/kg; 5μc) from that excreted over the first day after dosing was adjusted to pH5 with 2N-acetic acid and incubated with 5ml. of a β-glu-curonidase preparation (Ketodase; Warner-Chilcott) at 37° for 24 hrs., using phenolphthalein glucuronide as a control. The solution was adjusted to pH6 with N-ammonium hydroxide and the urine, to which had been added amphetamine, norephedrine and their 4-hydroxy derivatives, (0.5mg. of each), was passed (4ml/hr.) through a column of IRC-50 resin (standard grade), acid form, of dimensions 11x1 cm. (recycled according to Hirs, Moore and Stein, 1953). The column was washed with water (20ml.) and then eluted with 2N-HCl (40ml.). The eluate was freeze-dried and the residue extracted with ethanol (2.0ml.) and portions (0.05-0.2ml.) of the latter chromatographed on Whatman No. 1 paper using solvent D.

Metabolites were detected on the chromatograms by radio-chromatogram scanning and the peaks compared with standard R_F values and the position of the amphetamine derivatives which had been added to the urine.

CHAPTER 3

THE URINARY METABOLITES OF AMPHETAMINE IN SEVERAL MAMMALIAN SPECIES

In this chapter a study of the metabolism of amphetamine in several mammalian species, including man, is described.

In the introduction the work of previous workers has been outlined, in particular the finding that amphetamine is metabolised "in vivo" in the rat largely by hydroxylation of the aromatic ring, whereas this is a minor "in vivo" route for the rabbit, deamination probably being more important. This deamination to phenyl acetone was demonstrated "in vitro", but whether this was an "in vivo" metabolite, or whether it was further metabolised was not determined. However, at about the same time, El Masry, Smith, and Williams, (1956), were investigating the fate of phenyl acetone in the rabbit and indicated that it could be metabolised to (+)-benzyl methyl carbinol glucuronide and hippuric acid. Recently hippuric acid has been shown to be a metabolite in the rat (Alleva, 1963).

The excretion of unchanged amphetamine in man has been studied by several workers but no other excretory products have been detected.

Bearing the above points in mind the work in this section was undertaken to confirm the previous findings and add further to the knowledge of the urinary metabolites of amphetamine with particular regard to the differences between species.

CHOICE OF ANALYTICAL METHOD

The same methods of analysis of metabolites were

Table 2. The elimination of ^{14}C after ^{14}C-Amphetamine sulphate in various mammals.

SPECIES	RAT			MOUSE	
Optical isomer	(+)	(-)	(±)	(+)	(-)
Dose of drug mg./kg. and route of administration	10 (p.o.)	10 (p.o.)	10 (p.o.)	10 (p.o.)	10 (p.o.)
Dose ^{14}C, µC.	2	10	10	0.6	1.3
No. of animals	3	3	2	3	3
^{14}C output	% of Dose (averages with ranges in parentheses).				
In urine on day 1	78.7 (72.0–90.0)	68.5 (60.2–75.9)	80.8 (79.4–82.2)	77.9 (74.9–82.9)	77.1 (74.1–78.7)
2	5.9 (4.3– 7.6)	11.2 (6.1–14.4)	3.8 (2.1– 5.4)	6.4 (3.9–10.2)	3.0 (2.0– 3.7)
3	3.3 (2.3– 4.6)	1.8 (0.9– 3.5)	1.1 (0.7– 1.4)	2.2 (1.0– 3.0)	2.2 (1.2– 4.4)
Total	87.9	81.5	85.7	86.5	82.3
In faeces on day 1	2.0 (1.0– 2.8)	3.2 (2.8– 3.6)	3.3 (3.3– 3.3)	0.7 (0.2– 1.4)	3.8 (0.5– 7.3)
2	0.8 (0.6– 1.0)	1.5 (1.4– 1.8)	1.0 (0 – 2.0)	-	-
3	0.2 (0.1– 0.3)	0.3 (–)	0.4 (0.1– 0.8)	-	-
Total	3.0	5.0	4.7	0.7	3.8
Total excretion	90.9	86.5	90.4	87.2	86.1

Table 3. The elimination of ^{14}C after ^{14}C-Amphetamine sulphate in various mammals.

Species	Rabbit	Greyhound	Guinea Pig		Rhesus Monkey	
Optical isomer	(±)	(±)	(+)	(-)	(+)	(-)
Dose of drug mg./kg. and route of administration	10 (p.o.)	5 (i.p.)	5 (i.p.)	5 (i.p.)	0.7 (p.o.)	0.7 (p.o.)
Dose ^{14}C, μC	15.0	20.0	4.5	6.0	5.0	10.0
No. of animals	3	3	3	3	2	2
^{14}C output		% of Dose (averages with ranges in parentheses)				
In urine on day 1	72.3 (54.4-81.6)	75.3 (54.2-88.7)	85.5 (83.6-86.5)	82.5 (80.3-83.9)	41.7, 73.4	47.6, 68.9
2	7.2 (1.9-13.9)	2.4 (2.2- 2.8)	1.7 (1.3- 2.2)	2.9 (0.9- 4.0)	13.1, 5.0	9.1, 8.1
3	7.3 (1.5-13.2)	0.4 (0.46- 1.0)	-	-	2.5, 2.0	2.4, 5.3
Total	86.8	78.1	87.2	85.4	57.3, 80.4	59.1, 82.3
In faeces on day 1	4.9 (1.2-10.5)	0	-	-	-	-
2	1.8 (0.5- 3.7)	0	-	-	-	-
3	1.6 (1.4- 1.8)	0	-	-	-	-
Total	8.3	0	-	-	-	-
	95.1	78.1			57.3, 80.4	59.1, 82.3

Table 4. The metabolites of ^{14}C-Amphetamine in the urine of various species.

Dosage and No. of animals as in Table 2. Values are expressed as a % of dose (averages, with ranges in parentheses) and are for the first 24 hrs. in each case except the rat, where they are for the first 48 hrs. after dosing.

SPECIES	RAT			MOUSE	
Optical isomer	(+)	(-)	(±)	(+)	(-)
Metabolites sought					
Amphetamine, free	12.2 (8.8-14.7)	14.6 (9.8-19.2)	10.8 (10.6-11.0)	—	—
total	13.7 (10.1-17.3)	16.7 (13.2-20.5)	12.8 (12.6-13.0)	32.7 (31.4-35.0)	28.0 (21.4-34.9)
4-Hydroxyamphetamine, free	3.4 (2.1- 5.8)	4.7 (3.7- 5.9)	7.3 (3.4-11.2)	3.1 (1.2- 6.6)	6.2 (0 -12.3)
total	47.7 (44.0-49.6)	63.2 (58.2-66.9)	59.7 (57.7-61.7)	14.0 (10.3-19.0)	16.7 (10.0-21.2)
Benzoic acid, free	0	0	0	0	0
Benzoyl glucuronide	0	0	0	8.5 (6.1-10.9)	11.9 (8.6-16.0)
Hippuric acid	2.2 (1.1- 3.0)	2.4 (1.7- 2.8)	3.3 (2.5- 3.6)	22.7 (17.5-26.1)	20.3 (16.6-23.2)
Benzoic acid, total	2.2 (1.1- 3.0)	2.4 (1.7- 2.8)	3.3 (2.5- 3.6)	31.2 (23.6-37.0)	32.2 (27.7-37.0)
Benzyl methyl ketone	0	0	0	0	0
Benzyl methyl carbinol	0	0	0	0	0
Sum of metabolites	63.6	82.3	75.8	77.9	76.9
Total ^{14}C in urine	84.6	79.7	84.6	77.9	77.1

Table 5. The metabolites of ^{14}C-Amphetamine in the urine of various species.

Dosage and No. of animals as in Table 3. Values are expressed as a % of the dose (averages, with ranges in parentheses) and are for the first 24 hrs. in each case.

Species	Rabbit	Greyhound	Guinea Pig		Rhesus Monkey	
Optical isomer	(±)	(±)	(+)	(-)	(+)	(-)
Metabolites sought						
Amphetamine, free	-	-	-	-	-	-
total	3.5 (2.2- 4.8)	29.9 (19.9-38.4)	22 (18-25)	15 (14-16)	3.8, 31.1	23.4, 33.5
4-Hydroxyamphetamine, free	-	-	-	-	0, 0	0, 5.5
total	6.2 (5.0- 7.0)	5.9 (5.1- 7.1)	0	1 (0.5-2)	0, 11.4	1, 6
Benzoic acid, free	-	-	12 (9-16)	40 (18-68)	5.0, 18.3	13.8, 19.3
Benzoyl glucuronide	-	-	29 (23-39)	10 (0-30)	13.5, 12.6	0, 0
Hippuric acid	10.4 (1.8-18.3)	19.5 (16.5-23.6)	21 (14-30)	18 (0-37)	19.5, 0	6.1, 7.5
Benzoic acid, total	22.9 (14.5-35.8)	27.8 (20.3-31.8)	62 (55-67)	68 (67-68)	38.0, 30.9	19.9, 26.8
Benzyl methyl ketone	21.5 (17.5-25.4)	1.4 (0- 2.5)	0	0	0, 0	0, 0
Benzyl methyl carbinol	7.5 (6.5- 7.9)	0.7 (0- 2.0)	0	0	0, 0	0, 0
Sum of metabolites	61.6	65.7	84	84	41.8, 73.4	43.3, 65.8
Total ^{14}C in urine	72.3	75.3	86	83	41.7, 73.4	47.6, 68.9

Table 6. The elimination of ^{14}C after ^{14}C-Amphetamine sulphate in man.

The subjects (RLS, LGD and SF) took 5 mg. of ^{14}C-amphetamine sulphate in water orally. The dose of ^{14}C was 6 μC for the (±)-isomer, and 2 μC for the (+) and (-)-isomers.

	Recovery of ^{14}C in urine expressed as a percentage of the dose								
Isomer	(±)			(+)			(-)		
Subject	RLS	LGD	SF	RLS	LGD	SF	RLS	LGD	SF
^{14}C output									
Day 1	72.8	61.3	64.3	72.5	62.8	59.0	63.3	53.4	58.2
2	17.3	23.8	17.5	14.4	18.9	20.3	16.8	28.9	22.7
3		6.5	7.6		4.7	4.1	7.1	7.5	8.0
4		1.9			2.4				
Total ^{14}C recovered	90.1	93.5	89.4	86.9	88.8	83.4	87.2	89.8	88.9

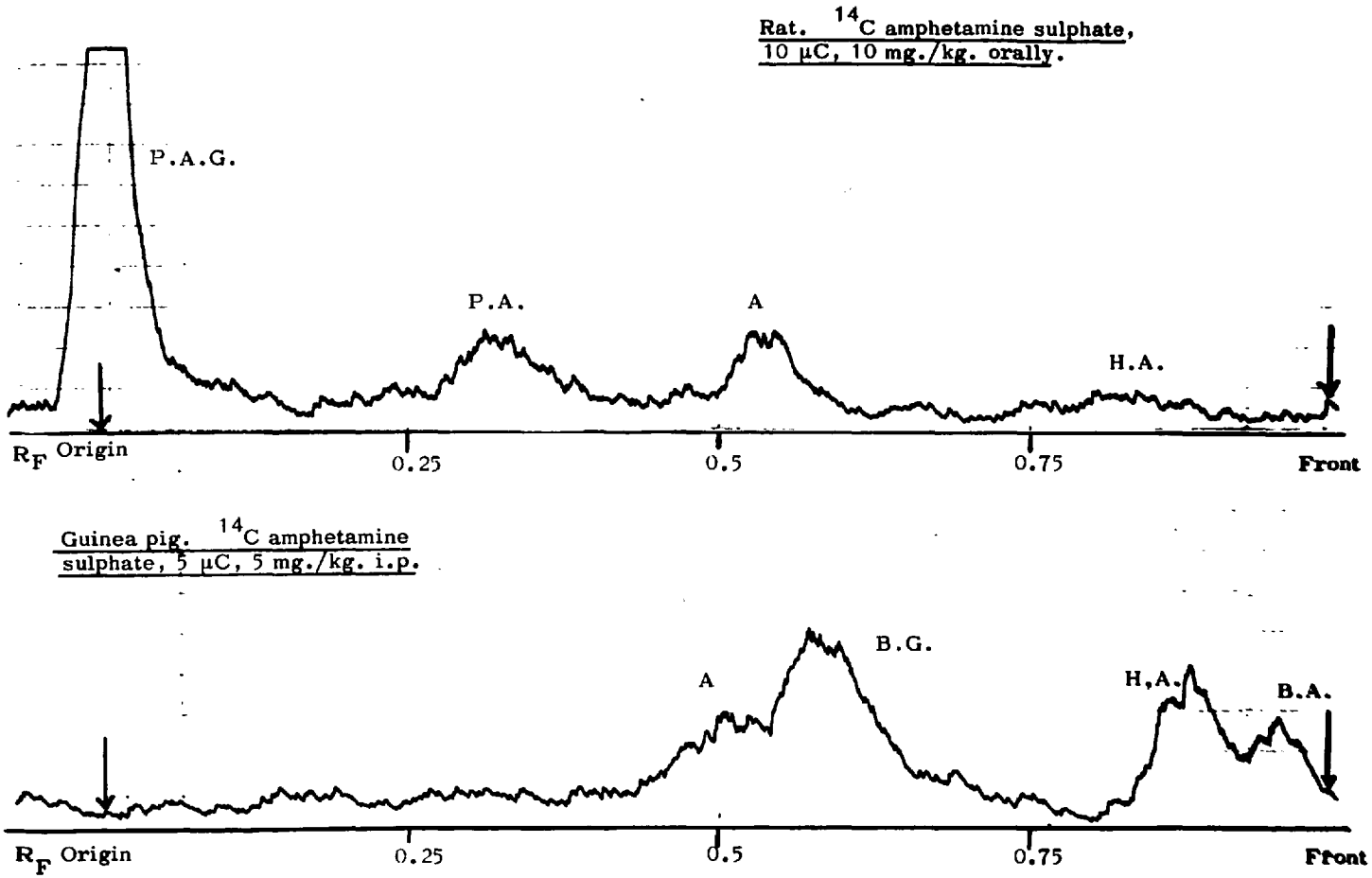
Table 7. The metabolites of ^{14}C-Amphetamine in the urine of man.

The dosages are as in Table 6. Values are expressed as a percentage of the dose and are for the first 24 hrs. in each case.

Isomer	(±)			(+)			(-)		
Subject	RLS	LGD	SF	RLS	LGD	SF	RLS	LGD	SF
Amphetamine (total)	35.1	22.9	32.5	38.5	25.1	37.1	41.3	22.0	38.2
4-Hydroxyamphetamine (total)	0.5	6.0	1.9	0.7	2.5	0.1	0.3	9.2	2.3
Hippuric acid	18.6	17.1	13.6	-	-	-	-	-	-
Benzoic acid (total)	24.3	21.8	17.4	22.0	22.0	20.3	15.8	12.7	9.3
Benzyl methyl ketone	2.0	1.3	6.8	1.8	1.5	3.4	1.0	1.1	1.7
Benzyl methyl carbinol	0	0	0	0	0	0	0	0	0
Sum of metabolites	61.9	52.0	58.6	63.0	51.1	60.9	58.4	45.0	51.5
Total ^{14}C in urine	72.8	61.3	64.3	72.5	62.8	59.0	63.3	53.4	58.2

Fig. 1.

Radiochromatograms of 0-24 hr. Rat and Guinea Pig urine. Chromatographed in solvent C.



P.A.G. = p-OH Amphetamine Glucuronide; P.A. = p-OH Amphetamine; A. = Amphetamine; B.G. = Benzoyl Glucuronide; H.A. = Hippuric Acid; B.A. = Benzoic Acid.

not always used from one species to another. The factors which governed their choice were (a) amount of radio activity which could be given to the animal, and (b) ease of analysis. At the beginning the only suffiently accurate method available was isotope dilution which was used for determinations in the rat, rabbit, and dog. At this point a windowless 2π Packard radiochromatogram scanner became available and therefore subsequent work on small animals was carried out with this. However, the work in man still had to be performed by isotope dilution procedures since only small amounts of radio activity could be given, resulting in a urine with insufficient activity for radiochromatogram scanning.

RESULTS

RAT

In this species well over 70% of the administered ^{14}C with each isomer was recovered in the first day, almost exclusively in the urine (Table II).

The most important urinary metabolite in the first two days was 4-hydroxyamphetamine (See Fig. 1 and Table IV) which came to almost 50% of the dose for the (+) isomer, over 60% with the (-) isomer and a percentage for the racemate which was almost mid-way between the (+) and (-) values. Hydrolysis of the urine with a β - glucuronidase preparation and subsequent chromatography showed that 4-hydroxyamphetamine was excreted largely as a glucuronide conjugate, which had an R_F value in solvent systems B and C of 0.05. Further evidence that the

4-hydroxy metabolite was a glucuronide was obtained by isotope dilution, the value for the percentage of 4-hydroxyamphetamine being the same after β -glucuronidase hydrolysis (See enzyme hydrolysis of benzyl methyl carbinol conjugate in Chapter 2), as after acid hydrolysis for total 4-hydroxyamphetamine.

Benzoic acid is a minor metabolite, accounting for only 2-3% of the dose and it is excreted largely as hippuric acid.

Total amphetamine excretion for both isomers was in the range of 10-20%, the free amphetamine being approximately 2% less than the total value.

In order to examine the rat urine for minor basic metabolites, particularly because such metabolites are active pharmacologically, an ion exchange resin (See Methods) was used to remove the acidic metabolites from β -glucuronidase treated urine, in a separate experiment with rats fed (∓)-amphetamine. After chromatography of the processed urine in system D three new minor peaks became apparent at R_F 0.1, 0.44, 0.98 the first two having exactly the same R_F values as authentic samples of 4-hydroxynorephedrine, and norephedrine which had been added to the urine. Since separation of 4-hydroxynorephedrine and norephedrine from large quantities of 4-hydroxyamphetamine and amphetamine by other chromatography systems was not very reliable, the presence of norephedrine and 4-hydroxynorephedrine in the hydrolysed urine was confirmed by isotope dilution and quantitated accurately at the same time. Norephedrine was found to the extent of 0.3% of the dose in two

separate experiments and 4-hydroxynorephedrine to the extent of 0.3% of the dose in one experiment, insufficient 4-hydroxy compound being available for a second determination. The peak at R_F 0.98 had an R_F of 0.98 in system B too, and amounted to between 1 and2% of the dose in the three animals examined. The R_F values made it probable that this substance was N-acetyl amphetamine.

The following substances were looked for using isotope dilution, in rat urine; p-hydroxybenzoic acid, phenylacetone, benzyl methyl carbinol, phenyl alanine, and phenyl acetic acid, but without success.

MOUSE

Again well over 70% of the administered ^{14}C is excreted in the urine in the first 24 hours.(Table II).

Unlike the rat the major urinary metabolite is not 4-hydroxyamphetamine, although the precentage is higher than any other species studied (ca. 15%) except the rat. The major metabolite is benzoic acid (30%), excreted largely as hippuric acid and benzoyl glucuronide, and about 30% of the amphetamine is excreted unchanged. (Table IV).

Like the rat there was no evidence of phenyl acetone, even after acid hydrolysis.

GUINEA PIG

Urinary excretion of ^{14}C in the urine is high (80% of the dose).(Table III).

The major metabolite is at the other extreme to the rat being benzoic acid and its conjugates (60-70% of the dose).(Table V). No 4-hydroxyamphetamine could be detected in the urine of animals fed with the (+) isomer, and very little in the urine of animals fed with the (-) isomer (0.5-2%). Of the parent drug amphetamine, about 15% of the (-) isomer is excreted unchanged and about 22% of the (+) isomer is excreted unchanged too. It may be that the (-) isomer is more readily metabolised than the (+) isomer.

Again like the rat and mouse there is not evidence for phenyl acetone on hydrolysis of urine.

GREYHOUND

Urinary excretion of ^{14}C in the first day's urine was within the range 70-80% like the rat, mouse. and guinea pig.(Table III).

About 30% of the dose is excreted as free amphetamine, the main metabolite being benzoic acid (25%, largely as hippuric acid). Minor metabolites are 4-hydroxy-amphetamine(6%) and a small quantity of a substance which yields phenyl acetone on acid hydrolysis.(Table V).

RABBIT

In the rabbit the metabolism differs quite markedly from any of the other species studied, however the ^{14}C excreted in the urine in the first 24 hours was in the same range as the other animals, (ca. 70%)(Table III).

Little unchanged amphetamine is excreted (3%)

and 4-hydroxylation is quite low, 6%, being excreted as 4-hydroxyamphetamine. The main metabolite is benzoic acid (and conjugates, 25%). A metabolite so far undetected in any other species is conjugated benzyl methyl carbinol (ca. 7%), which appears to be conjugated with glucuronic acid, since virtually all the carbinol which is liberated from the conjugated form by acid hydrolysis can be liberated equally well by β -glucuronidase incubation (See Methods). The other metabolite which is excreted in quite high percentage (ca. 20%), is a substance, which will be called X, from which phenyl acetone is liberated on acid hydrolysis. (See Table V and Fig. 2). Metabolite X is the subject of a later chapter in this thesis.

Rabbit urine was also examined for p-hydroxybenzyl methyl ketone, phenylpropionic acid, phenyl pyruvic acid, phenyllactic acid, cinnamic acid, phenyl acetic acid, mandelic acid, and p-hydroxybenzoic acid using the isotope dilution procedures, but without success.

RHESUS MONKEY

One of the two monkeys used gave ^{14}C recoveries on a par with the other species examined ie. about 70% of the dose in the first day's urine. On the other hand the other monkey appeared to excrete far less than average in the first 24 hours. However the total ^{14}C recovery was much less than usual (only 60%) so it is quite probable that not all the urine excreted by this monkey was collected. Hence the first days ^{14}C excretion for the monkey is probably similar to the other species studied. (Table III).

The main urinary metabolites appear to be benzoic acid and its conjugates, less (ca. 20%) being produced from the (-) than the (+) isomer (ca. 35%). 4-Hydroxy-amphetamine is a minor metabolite and one of the monkeys produced no 4-hydroxyamphetamine from the (+) isomer. The unchanged parent drug is also an important urinary excretion product.(Table V).

No precursor of benzyl methyl ketone (X) could be detected in the urine.

MAN

Table VI shows the urinary excretion of ^{14}C after administration of the various optical forms of amphetamine to three male subjects. A large proportion of the activity (ca. 90%) is excreted via the urine, and the major part appears in the first day's urine (ca. 60%), there being little difference between the rate of excretion of the isomers. However it does seem that the rate of excretion of activity is slower in man than all the other species studied, except the Rhesus monkey.

The main urinary excretory product (Table VII) is amphetamine. However there is no clear cut difference between isomers, but there is an individual difference, R.L.S. and S.F. excrete between 30-40% unchanged, whereas G.D. only excretes over 20%. This individual difference also extends to the excretion of 4-hydroxyamphetamine but in this case G.D. excretes more than the other subjects. It would appear that there is an isomeric difference between the excretion of 4-hydroxyamphetamine, more being excreted after

dosing with the (-) than the (+) isomer. Less benzoic acid (conjugated largely as hippuric acid) is produced from the (-) (ca. 10%) than from the (+) isomer (ca.20%). Human urine also contains a substance which yields phenyl acetone on acid hydrolysis (1-3%). Again an individual difference was observed, S.F. producing more than the other two subjects.

An isotope dilution for N-methyl amphetamine gave a negative result.

DISCUSSION

The results show an obvious species difference in the metabolism of amphetamine.

Man, monkey and dog appear to metabolise the drug in approximately the same manner. A significant portion of ^{14}C in the first day's urine comprises the unchanged drug and an almost equal quantity of benzoic acid (ca. 20-30% for each). A much smaller quantity is ring hydroxylated to 4-hydroxyamphetamine (1-10%) and an even smaller quantity of the substance which gives phenyl acetone on acid hydrolysis was found in the urine of man and dog.

The four other species differ quite markedly from each other, both qualitatively and quantitatively and will be considered separately.

In the rat the major metabolite is 4-hydroxyamphetamine

conjugated with glucuronic acid (60% of the dose). Unchanged amphetamine (11%) and what appears to be N-acetyl amphetamine (2%) are also excreted. Side chain degradation to benzoic acid is almost negligible (2%) and norephedrine and 4-hydroxynorephedrine, although present are only found in extremely small quantities (<1%). Other ring and side chain hydroxylated and methoxylated products which had been sought because of reports of their formation "in vitro" or because their biosynthesis might be possible by analogy with phenyl amine metabolism were not detected using the experimental system described. This was disappointing, because "in vitro" work of Axelrod, Daly, and Inscoe (1963) had indicated that the formation of 3,4-dihydroxyamphetamine and 3-methoxy, 4-hydroxyamphetamine were distinct possibilities. However Porter and Titus in the same year showed that these two compounds, which are metabolites of α-methyl DOPA in the rat are deaminated to give phenyl acetone derivatives.

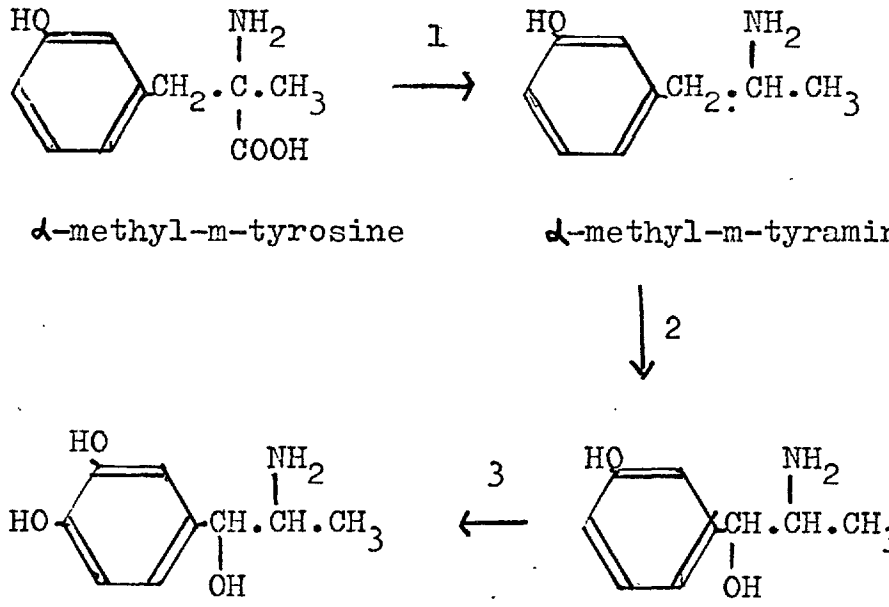
There have been several recent papers on the β-hydroxylation of amphetamine and its 4-hydroxy derivative. Iversen, Glowinski and Axelrod (1966), demonstrated the presence of α-methyl octopamine (4-hydroxynorephedrine) in the hearts of mice after the administration of dl-α-methyl tyramine (4-hydroxyamphetamine). The degree of β-hydroxylation was decreased after immunosympathectomy indicating a probable connection between the β-hydroxylase enzyme and the sympathetic nervous system. Fischer, Horst, and Kopin (1965) infused ^{3}H-α-methyl tyramine into an isolated perfused cat spleen. On stimulation of the splenic nerve, α-methyl octopamine, the β-hydroxylated derivative of the compound was released. This suggests that the latter compound can act as a

false transmitter in the sympathetic nervous system. That amphetamine itself could be β-hydroxylated was shown by Thoenen, Huerlimann, Gey and Haefly (1966). They gave ^{3}H-(±)-amphetamine to a cat and 2 hours later isolated the spleen. On stimulation of the sympathetic nerve p-hydroxynorephedrine and norephedrine were released.

From this literature it seems apparent that the sympathetic nerves are responsible for β-hydroxylation and hence the small percentage of β-hydroxylated amphetamine and 4-hydroxyamphetamine found in the rat is to be expected. Although Goldstein and Anagnoste (1965) found no norephedrine after administration of amphetamine to rats, Thoenen, Huerlimann, Gey, and Haefly were able to demonstrate its presence in the spleen of the cat.

The pharmacological implications of β-hydroxylation of amphetamine and its 4-hydroxy metabolite are of interest. It has been demonstrated by Fischer, Horst and Kopin (1965) that octopamine, α-methyl octopamine-phenylethanolamine and norephedrine can be taken up and released by the sympathetic nerves in the cat spleen. On the other hand the non-β-hydroxylated amines tyramine, α-methyl tyramine, phenylethylamine and amphetamine were not released as false transmitters. This correlates well with the finding that β-hydroxylated amines are retained in the adrenergic nerve storage particles in the rat heart whereas the non-β-hydroxylated amines are not retained (Mussacchio, Kopin, and Weise, 1965). The situation with the β-hydroxylated derivatives of amphetamine and its 4-hydroxy metabolite is therefore

analogous to that of α-methyl metatyrosine which is metabolised to metaraminol and α-methyl noradrenaline (Anden, 1964; Maitre and Staehlin, 1965).



α-methyl-m-tyrosine α-methyl-m-tyramine

α-methyl noradrenaline metaraminol

1 = aromatic-L-amino acid decarboxylase
2 = dopamine-β-hydroxylase
3 = liver microsomal hydroxylating enzyme

Metabolism of α-methyl-m-tyrosine to "false transmitters"

Haefly, Thoenen, and Huerlimann (1965) reported that treatment with α-methyl-m-tyrosine considerably reduced the effects of sympathetic nerve stimulation in the cat nictitating membrane and the spleen. The most credible explanation for this is the depletion

of catechol amines by the α-methyl amines is accompanied by an almost stoichiometric replacement by metaraminol and α-methyl noradrenaline. However these are not nearly as active pharmacologically as noradrenaline and consequently when they are released by sympathetic nerve stimulation the adrenergic effect is much less than normal. Also, unlike noradrenaline, the α-methylated amines are not susceptible to amine oxidase within the nerve and they are also taken up very efficiently by the catecholamine uptake process, so that after release they are soon returned to the catecholamine storage sites (Burgen and Iversen, 1965). This is one probable explanation why these compounds are so long acting. Therefore the β-hydroxylated metabolites of amphetamine should be expected to act in a strictly analogous way and it could be predicted that chronic administration of amphetamine to humans should cause a lessening in the efficiency of the sympathetic nervous system with the accumulation of these metabolites. Indeed, it has been shown that courses of 4-hydroxy-amphetamine in man which is known to be β-hydroxylated to the extent of about 5% of the dose (Sjoerdsma and von Studnitz, 1963), causes a transitory hypertension and then hypotension. (Gill, Mason, Bartler, 1967). This is a particularly interesting example of how a knowledge of the metabolism of a drug can lead to the proper use of the drug. Not long ago (Goodman and Gilman, 1955) 4-hydroxyamphetamine was being recommended for the treatment of orthostatic hypotension.

Rat and mouse are closely related rodents and

it might have been expected that the metabolism in these would be closely similar, however, this is not so. There is a high excretion of unchanged amphetamine and benzoic acid (each ca. 30%), both of which are much lower in the rat. Also, although the 4-hydroxy-amphetamine produced is higher than any other species (ca. 15%) it is much lower than the rat.

Of all the species studied the rabbit appears to metabolise amphetamine most completely, only about 4% appearing in the first day's urine and yet 4-hydroxylation is low (6% of the dose). On the other hand the most important route is side chain degradation, this accounting for ca. 50% of the dose excreted as ^{14}C in the first 24 hours. This 50% is divided into ca. 20% for benzoic acid, largely as hippuric acid, 20% for the metabolite X, which yields phenyl acetone on acid hydrolysis, and 8% for benzyl methyl carbinol, excreted largely as glucuronide.

That phenyl acetone itself was produced "in vitro" was shown by Axelrod (1955) using rabbit liver microsomes. It appears not to be excreted as such in the urine but as substance X which yields the ketone on hydrolysis. The further fate of the ketone in the rabbit was studied by Smith, Smithies and Williams, 1954, and El Masry, Smith and Williams, 1956. They found that after oral dosing of rabbits with the ketone an average of 43% was excreted as benzyl methyl carbinol glucuronide, and 19% as hippuric acid. This is the first time that the carbinol has been detected as an amphetamine metabolite, although this was suggested

by Asatoor, Galman, Johnson and Milne, (1965) as a possible pathway.

In the guinea pig, the metabolism of amphetamine appears to be relatively simple, amphetamine being metabolised to benzoic acid and its conjugates (ca. 60% of the dose in 24 hrs.). About 20% of the drug is excreted unchanged and 4-hydroxylation takes place to only a negligible extent.

These results largely agree with the work of Axelrod (1954,1955) who indicated that 4-hydroxylation was a major route in the rat and a minor one in the guinea pig and rabbit. However the excretion of unchanged drug in the present experiments with the guinea pig are much higher (Axelrod 1%, present results 20%), and yet unchanged drug excretion in dog, rat, and rabbit agree well with the present results. His results also suggest that 4-hydroxylation is a major route in the dog (ca. 20%) whereas the present results show a much lower value (6%), but this could perhaps be due to differences between breeds.

More recent work by Alleva (1963) on the metabolism of amphetamine in the rat, using a labelled compound, demonstrated the presence of a new metabolite, hippuric acid, which has been confirmed in the present work. Ellison, Gutzait, and van Loon, (1965,1966) studied the metabolism in man, rat, dog and monkey and their results are in general agreement with the present work, although the 4-hydroxylation by Beagle dogs is about double that found for the Greyhound, again suggesting

a difference between breeds of dog.

A marked difference in the pharmacological action of the d and l-isomers of amphetamine suggested that the examination of the metabolism of the two isomers might be worth investigating. Alles and Wiesgarver (1961) gave a large dose of racemic amphetamine to an addict and on pooling several large volumes of urine found the extract to be laevo rotatory. Beckett and Rowland (1965) reported that after the administration of d or l-amphetamine, less of the d-isomer was found after a comparable collection period. Yet further support to these findings was given by Gunne (1967) using a gas chromatographic analysis method which could resolve the optical isomers of amphetamine. He was able to administer the racemic compound and watch the excretion of the two isomers in the same urine, thus nullifying any objections about the effect of varying pH on the excretion of the isomers if studied separately. Over the first 24 hours the d:l ratio was 0.8. Gunne and Galland (1967) using a similar technique showed that after administration of racemic amphetamine to rats 8% more l-4-hydroxyamphetamine than d-4-hydroxy-amphetamine was excreted in the urine.

This last result is in agreement with the present findings which show that more 4-hydroxyamphetamine is excreted in the urine after dosing a rat with l-amphetamine than with d-amphetamine. In addition there seems to be a similar trend in excretion of the hydroxylated drug in man which has not been observed before, and, moreover this appears to vary from

from individual to individual. A difference between the excretion of the two isomers in the unchanged drug is not so apparent, but this is probably due to slight changes in the average urinary pH, since Asatoor, Galman, Johnson and Milne (1965) have shown that the excretion of amphetamine is much more dependant on urinary pH than the 4-hydroxy metabolite.

Therefore it seems clear that the fate of the two isomers in man and rat is probably not identical. How this relates to their pharmacology is not at present certain, nor is it clear how this differential excretion comes about. There may be a selective uptake of the d-isomer from the blood into the tissues, combined with a stereospecific metabolism of the d-isomer, or a prolonged retention of the d-isomer followed by a slow excretion. A stereospecific excretion seems unlikely because Gunne showed that variations in the urinary pH did not change the d/l ratio.

Stereospecific metabolism of amphetamine has been shown to occur "in vitro" by Goldstein, McKereghan, and Lauber, (1964), only d-amphetamine being β - hydroxylated. "In vivo" experiments by Goldstein and Anagnoste were only able to demonstrate 4-hydroxy-norephedrine production from amphetamine, but the present work in rats, and that of Thoenen, Huerlimann, Gey and Haefly show that production of norephedrine from amphetamine "in vivo" is possible too. Whether β - hydroxylation alone can account for the difference in excretion of the two isomers and the 4-hydroxy metabolites is doubtful, but it is of considerable interest to note that the decarboxylation of L- α -

methylamino acids "in vivo" with the 2S absolute configuration (Cahn, Ingold, and Prelog, 1956) produces (+)-α-methylamines with the 2S configuration (van Rossum, 1963). Therefore d-amphetamine will have the same configuration as the naturally occuring amines and will be more likely to get caught up in normal metabolic sequences, than the l-isomer, and it could be that the configuration results in a better fit on receptors, and this alone could account for its enhanced pharmacological activity over the l-isomer.

In order to ensure that urinary pH was not concerned in the differential metabolism between species, since it has been shown by several workers that an acid urine increases the amount of unchanged amphetamine excreted and an alkaline urine has the opposite effect, the pH of freshly voided urine was determined for each species on several animals. In each mammalian species the urine pH was usually 6 ± 0.5.

It might have been suspected that the differing degree and mode of metabolism from species to species would be reflected in the toxicity of the drug. Suprisingly, accurate published data on the toxicity in a number of species is not easy to acquire, but there is information for two species where there is a marked difference in metabolism, the rat and rabbit. In the rat where about 12% of the amphetamine is excreted unchanged and the rest of the dose is largely 4-hydroxylated (ca. 50-60%) the LD_{50} is 20mg./Kg. (Schulte et al., 1941). On the other hand the rabbit metabolises the drug almost completely (5% in the first day as unchanged amphetamine), only 7% of the

dose is excreted as the 4-hydroxylated metabolite, and the rest of the dose is excreted as deaminated metabolites (ca. 50%). In the rabbit the LD_{50} is 85mg./Kg. (Reifenstein, 1940) and in the present work the LD_{50} in New Zealand White does has been shown to be even higher (105mg./Kg.).

Table VIII

	Pressor Activity (a)	Threshold Stimulant Dose (b)	LD_{50} (c)
Amphetamine Sulphate	100-425	0.3	20
4-Hydroxy-amphetamine HBr.	50-100	80	> 320

(a) Amount equivalent to unit dose of adrenaline (Leffler et al., 1951)
(b) and (c) Doses mg./Kg. sub. cut. data from paper by Schulte et al., 1941

Summary of the pharmacological properties of amphetamine and its 4-hydroxy metabolite

From the table it can be seen that 4-hydroxylation of amphetamine will cause a twohundredfolddrop in the central effect of the drug measured by the threshold stimulant dose, a doubling in the pressor activity

and at least a tenfold decrease in toxicity. Therefore the major metabolic route in the rat decreases the central action of the drug, but not completely and the hypertensive activity is actually increased.

The rabbit however produces major metabolites which have little pharmacological effect. Therefore regarding the metabolism of amphetamine in the light of detoxication it is obvious that the rabbit metabolises the drug much more completely and therefore should have less of the active drug circulating in its system than the rat and it also produces metabolites which have a lesser pharmacological action than the rat. Hence it could have been predicted from the metabolic patterns of the two animals that amphetamine would be more toxic in the rat than the rabbit, which indeed it is.

CHAPTER 4

THE ISOLATION AND CHARACTERIZATION OF THE PRECURSOR OF BENZYL METHYL KETONE

Some twelve years ago Axelrod (1954) showed that rabbit liver homogenates were able to deaminate amphetamine to benzyl methyl ketone (phenyl acetone). However the free ketone has not been detected as a urinary metabolite of the drug. In Chapter III of this thesis it has been shown how phenyl acetone was detected using an isotope dilution technique in the urine after acid hydrolysis. In order to confirm this result the ketone was isolated and characterised in the following way.

ISOLATION OF PHENYL ACETONE

A total of 2.4g. of (±)-amphetamine sulphate was fed orally during 3 days to 8 rabbits at the rate of 100mg./rabbit/day. The urine was hydrolysed as indicated in Chapter II for the estimation of phenyl acetone and then subjected to a steam distillation. The cloudy steam distillate was treated with 2,4-dinitrophenylhydrazine in ethanol which was added dropwise until precipitation was complete. The pale yellow solid was filtered off and recrystallised from ethanol to constant melting point (151°, 60mg.). A mixed melting point determination with an authentic sample of the 2,4-dinitrophenylhydrazone of phenyl acetone also gave a value of 151°, therefore it is most probable that phenyl acetone is produced by acid hydrolysis of the urine, and since it is not present in the unhydrolysed urine it must be there as a precursor which will be called X.

PROPERTIES OF X

Chromatography of urine from a rabbit fed ^{14}C-

Radiochromatograms of 0-24 hr. Rabbit urine. ^{14}C amphetamine sulphate, 15 μC, 10 mg./kg. orally.

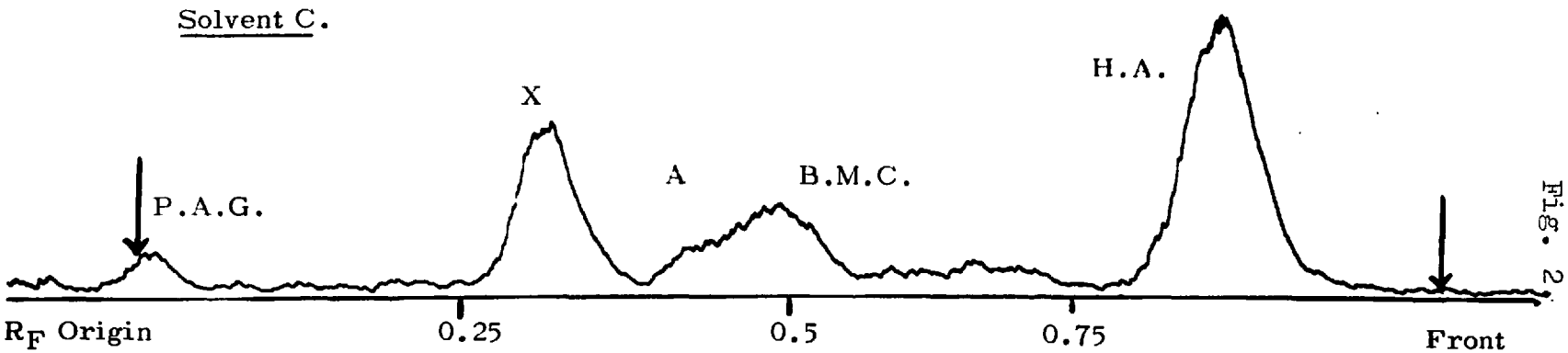
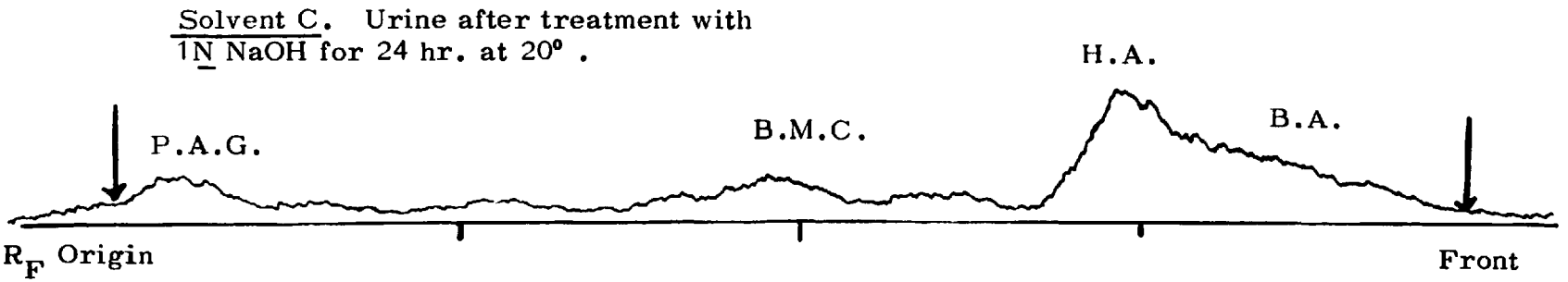


Fig. 2



P.A.G. = p-OH Amphetamine Glucuronide; A = Amphetamine; B.M.C. = Benzyl Methyl Carbinol Glucuronide; H.A. = Hippuric Acid; B.A = Benzoic Acid.

(±)-amphetamine sulphate and subsequent scanning with the radiochromatogram scanner revealed a major peak R_F 0.6 in solvent B and 0.33 in solvent system C which had not been observed on chromatography of the urine from the other animals.(Compare Fig.1 and 2). It was therefore suspected that this might be metabolite X.

In order to confirm that this new peak was indeed X, a portion of the ^{14}C-amphetamine urine was mixed with the first days urine from a rabbit fed 100mg. of amphetamine sulphate and a portion of this urine was streaked onto Whatman 3MM paper and chromatographed using pyridine: pentan-1-ol:water; 7:7:6 by vol. The new metabolite was located with the radiochromatogram scanner (R_F0.67) and eluted with water. The eluate was hydrolysed (See phenyl acetone estimation Chapter II), and the hydrolyzate extracted with ether which was washed with water, dried over sodium sulphate and then removed at reduced pressure. The trace of residual oil was taken up in ethanol and the U.V. spectrum of the extract was shown to be identical with that of an authentic sample of phenyl acetone.

Having confirmed that the new peak was in fact X some of the properties of the compound were examined. On keeping the urine at room temperature overnight in the presence of an equal volume of N-NaOH or N-HCl and then chromatographing and scanning the chromatogram with the radiochromatogram scanner, X had disappeared, and had not been replaced by a new peak. It thus appeared that X was a relatively labile substance and was hydrolysed to give a volatile product, thus giving

further support to the idea that this peak was in fact X, since the hydrolysis product of X, phenyl acetone is a volatile substance. Enzymic hydrolyses were attempted using β-glucuronidase (See method for hydrolysis of benzyl methyl carbinol conjugates in Chapter II) and limpet aryl sulphatase (carried out using sulphatase, Sigma Chemical Co., St. Louis U.S.A.; 0.5ml. 0.2M Sodium acetate buffer, 1mg. enzyme, 0.5ml. urine incubated at 37° for 24 hrs. p-Nitrophenyl sulphate was used as a control), with chromatography and scanning as before. However neither of these enzymes appeared to attack X. The following spray reagents were tried on the area occupied by X on paper chromatograms, but without success.

(i) Ninhydrin (o.2g.) in 100ml. n-butanol, and 2ml. pyridine, followed by heating at 105° for 5 minutes.

(ii) Equal volumes of 0.1M potassium dichromate and glacial acetic acid, followed by 0.1M aqueous silver nitrate. Compounds containing divalent sulphur give a yellow/orange spot on a brown background (Knight and Young, 1958).

(iii)Glucuronide spray. (See benzoyl glucuronide detection in Chapter II)

Hence amino acids and other ninhydrin positive substances, divalent sulphur, and glucuronic acid were unlikely to be present inX. However, these could not be ruled out absolutely at this point in the investigation since it was realised that the concentration

of X on the paper was not far from the limits of detection for spray reagents.

ATTEMPTED ISOLATION OF X

In order to find out more about the nature of X, especially since it did not appear to contain any easily identifiable chemical group, a partial isolation of X was attempted.

Three New Zealand White doe rabbits were given orally daily for three days (±)-amphetamine sulphate (25mg./Kg.). The urine (900ml.) was mixed with 100ml. of urine obtained from a rabbit dosed with ^{14}C-(±)-amphetamine sulphate (20μC) so that X could be located during the extraction procedure by radiochromatogram scanning. The urine was then filtered through glass wool and freeze-dried. The residue was extracted with 95% ethanol (4x100ml.) and the bulked extracts filtered and evaporated *in vacuo* to 20ml. The solids which separated, mainly urea, were filtered off and the filtrate used for preparative thin layer chromatography.

Aliquots (0.5ml.) of the filtrate were applied to each of 20 alumina plates (aluminium oxide-G; Merck Ltd.; 20x20cm. 1mm. thick. The plates were activated by heating at 110° for an hour before use) which were then developed with pyridine:pentan-1-ol:water; 7:7:6 (by vol.). The position of X was located on an identical plate (5x20cm.) which had been developed at the same time. The band of R_F 0.7-0.8 which contained X was scraped off the glass plate and eluted with 90% aqueous ethanol. The eluates were bulked, filtered and evaporated to

11ml. *in vacuo*.

An aliquot (2ml.) of the eluate was applied to each of 5 alumina plates as above and developed using solvent C (Chapter II). X was again located on a 5x20cm. plate and the band at R_F 0.2-0.3 which contained the metabolite was scraped off, eluted, bulked and reduced in volume by evaporation *in vacuo* as before, to give 1ml. of brown solution.

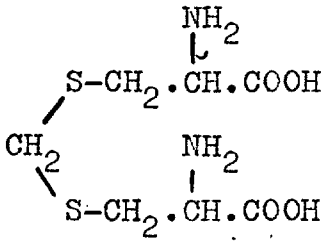
This 1ml. was then chromatographed on alumina plates once again using pyridine:pentan-1-ol:water; eluted after location of X, and the extract reduced to 1ml. which was then freeze-dried to a pale yellow solid (about 20mg.).

THE PROPERTIES OF THE ISOLATED MATERIAL

In order to confirm that the isolated solid did in fact give phenyl acetone on acid hydrolysis 4mg. of the material was hydrolysed in 3ml. of 5N-HCl under reflux at 100° for 2 hrs. The cooled hydrolysate (2.5ml.) was warmed with 2,4-dinitrophenylhydrazine in ethanol (5mg. in 1ml.). The precipitate which formed was filtered and recrystallised from ethanol to give deep yellow platelets of the 2,4-dinitrophenylhydrazone of phenyl acetone, m.p. and mixed m.p. 152 -153°. In ethanol, the crystals showed a light absorption at $\lambda_{max.}$ 360mμ and $\sim$260mμ which are the values for the authentic compound (El Masry, Smith, and Williams, 1956). The other portion of the hydrolysate (0.5ml.) was extracted with ether, which was washed with water, then taken to dryness *in vacuo*. The resulting trace

of oil was dissolved in ethanol and examined for U.V. light absorption which was found at $\lambda_{max.}$ 260mμ and ~285mμ. The authentic ketone shows $\lambda_{max.}$ 260mμ $\epsilon_{max.}$ 249, and ~285mμ, ϵ156 (Kumler, Strait, and Alpen, 1950).

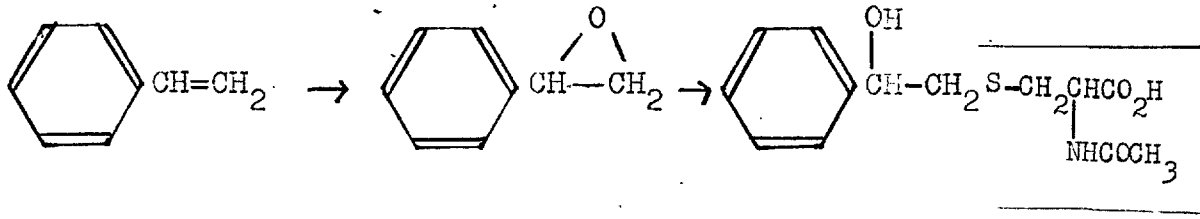
A small sample (0.5mg.) of the isolated solid was subjected to a qualitative elemental analysis using the Lassaigne sodium fusion method. This gave a strong positive test for sulphur. Working on the assumption that phenyl acetone was an intermediate in the synthesis of X a search of the literature was made for sulphur containing compounds which are known to be produced from compounds containing keto groups. A compound, djenkolic acid, has been isolated from the djenkol bean, and was shown by du Vigneaud and Patterson (1936) to be the cysteine thioacetal of formaldehyde.



djenkolic acid

Armstrong and du Vigneaud (1948) showed that the mercaptoles of acetone and benzaldehyde were formed quite readily in acidic solution. Later, Black (1966) in work on addition of L-cysteine to unsaturated lactones and related compounds noted that this amino acid gave a thio-amino acid with styrene oxide in aqueous solution at pH7. Again,

if an epoxide can be considered as a theoretical intermediate in the deamination of amphetamine, premercapturic acids and mercapturic acids of the type found in the metabolism of napthalene (Boyland and Williams, 1965) and of alkyl halides (Barnsley, 1964) are also possibilities. James and White (1967) found that β -hydroxyphenethylmercapturic acid was a product after feeding styrene and styrene oxide to rabbits.



styrene styrene oxide β -hydroxyphenethyl-mercapturic acid

Metabolism of styrene and its oxide in rabbit (James and White, 1967)

The formation of these mercapturic acid conjugates is considered to take place by the initial reaction with glutathione since conjugates of glutathione, cysteinylglycine and cysteine are excreted as metabolites of iodobenzene, napthalene and pyrene (Boyland and Sims, 1964).

Consequently, it was decided to examine a hydrolysed sample of the material for amino acids, particularly sulphur amino acids, since sulphur had been detected in the sodium fusion analysis.

AMINO ACID ANALYSIS OF HYDROLYSATE OF X

The isolated material (0.5mg.) was placed in a 4x30mm. glass tube and dissolved in 0.25ml. of 6N-HCl. Using an oxygen-coal gas flame, the top of the tube was pulled out to form a thin neck. Oxygen-free nitrogen was then bubbled through the solution, via a capillary, for sufficient time to expel all the air in the tube, which was then sealed off at the neck. The tube was heated at 110° for 15 hrs. in order to hydrolyse the compound and any peptides present. The tube was then cooled, broken open, and the solution transferred to a watch glass which was placed in a vaccuum dessicator in order to remove all the volatile acid and water. The solid remaining was redissolved in 0.1ml. water, taken to pH7 with $NaHCO_3$ and left in the air for two hours in order to oxidise any cysteine present to cystine.

The hydrolysate was then examined as follows:

(a) Paper Chromatography. Samples of the hydrolysate were run together with authentic samples of amino acids in two ascending chromatography systems on Whatman No. 1 paper for 19cm. In the first system, butan-1-ol: acetic acid:water; 120:30:50 by vol. two ninhydrin positive spots were present at R_F 0.06, and 0.23 which corresponded to cysteine and glycine. In the second system, phenol:water; 160g.:40g., two ninhydrin positive spots were again apparent at R_F 0.27, and 0.4 which again corresponded to cysteine and glycine.

(b) Amino Acid Autoanalysis. A volume of the hydrolysate equivalent to 200μg. of the isolated substance was acidified with a drop of dil. HCl to pH2-3 and subjected to separation on a resin column using a Locarte autoanalyser. The print out indicated the presence of only three ninhydrin positive substances, which were probably cysteic acid, glycine and possibly arginine, but these were present in such low concentrations $<$ 10μg. that they were probably impurities.

These results therefore suggest that the sulphur was not present as an amino acid.

Although the limpet aryl sulphatase had not hydrolysed X it was realised that sulphatases are relatively specific, so a sulphate ester could not be completely ruled out. When a small sample of X was warmed with 5N-HCl, followed by a few drops of barium chloride solution, a white precipitate formed, suggesting the presence of sulphate.

Therefore if X did contain sulphate, was the rest of the molecule an intermediate between amphetamine and phenyl acetone, or was it phenyl acetone itself? In order to try and answer this question the metabolism of phenyl acetone in the rabbit was examined in more detail to see if X could indeed be produced by feeding phenyl acetone.

METABOLISM OF PHENYL ACETONE IN THE RABBIT

(a) Preparation of 1-phenyl(1-^{14}C)propan-2-one. A female New Zealand White rabbit (3.0Kg.) was given orally ($\pm$)-(^{14}C)-amphetamine sulphate (25μC; 30mg.)

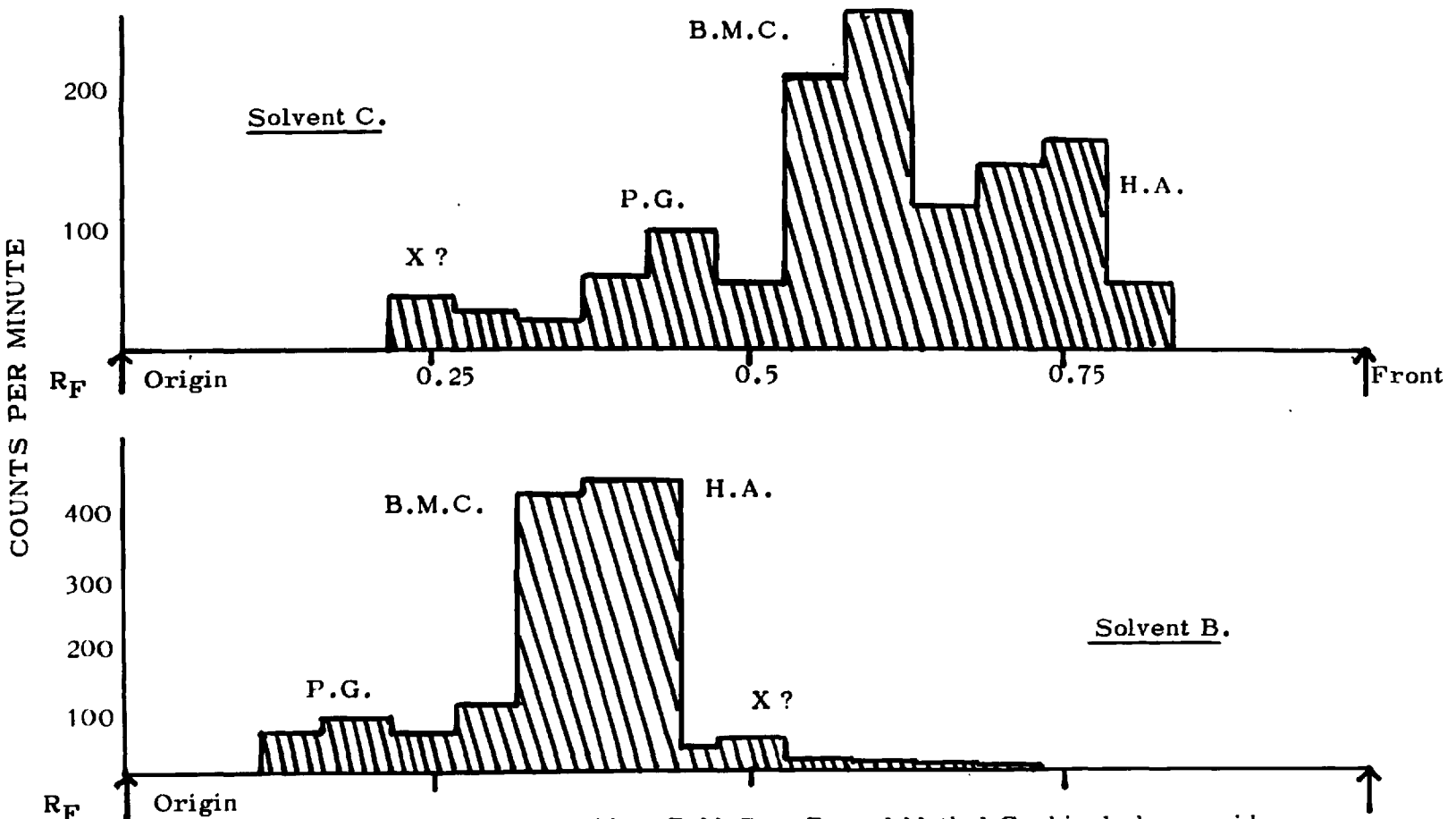
in water. The 24hr. urine (100ml.) was mixed with an equal volume of 10N-HCl and heated under reflux for 2hrs. 1-Phenylpropan-2-one (3ml.) was added to the cooled solution and the mixture extracted with ether (3x50ml.). The extract was washed with 2% $NaHCO_3$ solution (2x50ml.), water (2x50ml.) and finally evaporated at reduced pressure and room temperature. The residual oil was treated with excess sodium bisulphite solution (4.5g. in 17ml. 30% aqueous ethanol). After 30min. the bisulphite addition compound of phenyl acetone (2.6g.) which had separated out was filtered, washed with a little water, followed by ethanol and ether and sucked dry. The bisulphite compound was then decomposed using 2N-NaOH (20ml.) and the ketone which separated was taken up in ether (50ml.). The ether solution was washed with water, dried over anhydrous sodium sulphate and evaporated to a pale yellow liquid (0.76ml.). The 1-phenyl(1-^{14}C)propan-2-one had a specific activity of 2μ C/g.

(b) Feeding 1-phenyl(1-^{14}C)propan-2-one. (^{14}C)Phenyl acetone (1-phenyl(1-^{14}C)propan-2-one) (2g.; 1.1μC) was fed orally to a doe rabbit.(3Kg.) and the urine collected for 24hr. periods over 3 days.

Of the administered ^{14}C, 91% was excreted in the first 24hr. and a further 3% over the next two days.

Samples (0.1ml.) of the first day's urine were chromatographed on Whatman No. 1 paper using either solvent B or solvent C (See Chapter II). After drying, the chromatograms were cut into strips 4cm. wide and

Radiohistograms of 0-24 hr. Rabbit urine. ^{14}C phenylacetone 1 μC, 2 g. orally.



P.G. = Phenolic Glucuronide; B.M.C. = Benzyl Methyl Carbinol glucuronide;
H.A. = Hippuric Acid.

Fig. 3.

then cut into sections 1.5cm. long and the radioactivity of each piece was determined in the scintillation spectrometer using the dioxan scintillator of Bridges, Davies and Williams, (1967).

On plotting the radiohistograms for each solvent system (See Fig. 3) it was apparent that four radioactive peaks were present. Several samples of the urine were chromatographed in both systems concurrently with the ones for which the distribution of radioactivity was examined and sprayed with various colour reagents. The following table summarises the results

R_F solvent B	R_F solvent C	%dose (Solvent C)	Colour Reaction
0.21	0.48	13.6	glucuronide phenol
0.37	0.63	41.4	glucuronide
0.42	0.79	30.0	hippuric acid
0.58	0.26	6.0	----

The first two peaks (0.21, 0.37 and solvent B) gave a positive napthoresorcinol test for glucuronide (See Chapter II), and in addition the first peak, after elution and hydrolysis with dilute HCl gave a positive ferric chloride test for a phenol. Thus the first peak is probably a glucuronide of a phenol derived from phenyl acetone and the second peak the glucuronide of benzyl methyl carbinol, a known metabolite of the ketone. The third peak (R_F 0.42 solvent B) gave a

positive test for hippuric acid (See Chapter II), had the right R_F and in addition is a known metabolite of phenyl acetone (Smith, Smithies and Williams, 1954; El Masry, Smith and Williams, 1956; found an average of 43% was excreted as the glucuronide, and 19% as hippuric acid). These previous results are in general agreement with the present findings. The fourth peak (R_F 0.58 in solvent B, and 0.26 in solvent C) corresponded almost exactly to the R_F values for the metabolite X in the two systems.

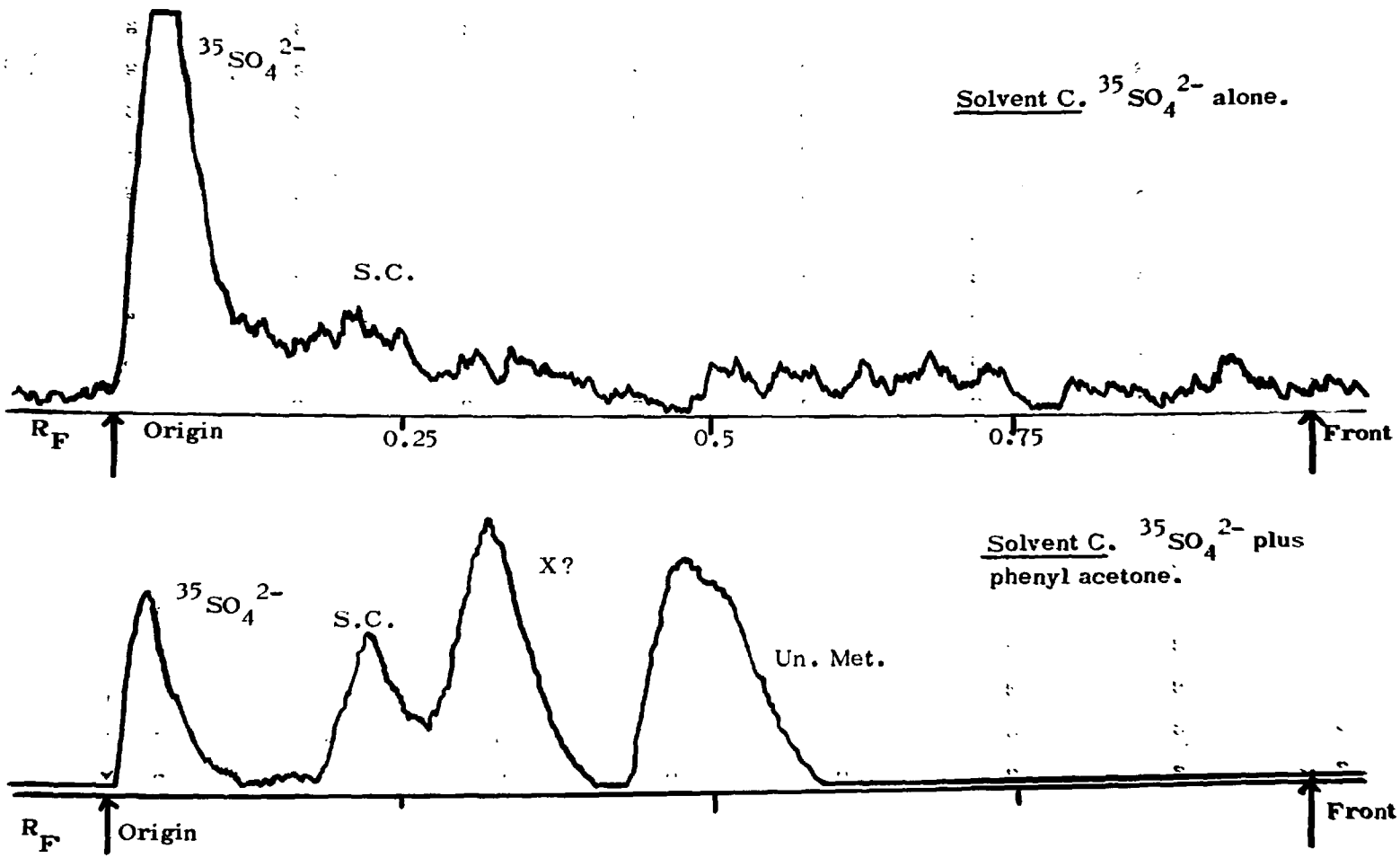
An isotope dilution for phenyl acetone on a sample of urine showed that no free ketone was present in the urine but, after acid hydrolysis (See Chapter II), an isotope dilution showed that 14.9% of the dose was present as the ketone.

It would therefore appear that X can indeed be formed from phenyl acetone, and the following experiment was devised to find out if inorganic sulphate was combined in some way with the ketone.

EXPERIMENTS WITH RABBITS INJECTED WITH $^{35}SO_4^{2-}$

A doe rabbit was injected intraperitoneally with 250μ C of $^{35}SO_4^{2-}$ (carrier free $^{35}SO_4^{2-}$, Radiochemical Centre, Amersham) in 0.5ml. of water, and the 24hr. urine collected for use as a control. On the second day the rabbit was given the same amount of $^{35}SO_4^{2-}$ as before and an hour later an oral dose of phenyl acetone (2g.). The urine (65ml.) was collected for the next 24 hrs.

Radiochromatograms of 0-24 hr. Rabbit urine after dosing with $^{35}SO_4^{2-}$ i.p., and $^{35}SO_4^{2-}$ immediately before phenylacetone 2 g. orally.



S.C. = Normal SO_4^{2-} conjugate; Un. Met. = Unidentified Metabolite.

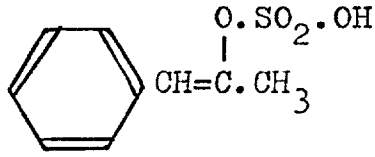
Fig 4.

Samples (0.01ml.) of both the control urine and the urine after dosing with phenyl acetone were spotted onto Whatman No. 1 paper, chromatographed in either solvents B or C and then scanned for radioactivity using the radiochromatogram scanner. Comparison of the two scans (See Fig. 4) showed two peaks, after the administration of phenyl acetone, which were not present in the control urine of R_F 0.57 and 0.7 in solvent B, and 0.31 and 0.48 in solvent C. The smaller of the two peaks of R_F 0.57 in solvent B and 0.31 in solvent C corresponds to metabolite X formed from (^{14}C) amphetamine and ^{14}C-phenyl acetone.

In order to confirm that this smaller peak did indeed contain organically bound sulphate and that sulphate and phenyl acetone were released on acid hydrolysis, urine from the rabbit fed sulphate and the ketone was streaked across Whatman 3MM paper (18x19") and chromatographed in solvent C. After drying the paper a 4cm. strip was removed from the centre of the sheet and scanned to locate the smaller of the two new peaks. This same area was cut from the rest of the sheet and eluted with 95% ethanol. A small portion of this eluate was chromatographed on a thin layer alumina plates (prepared as described earlier in this chapter) when it showed a single radioactive peak when scanned of R_F 0.18 in solvent C and 0.7 in pyridine: pentan-1-ol:water (7:7:6 by vol.). These too are the same R_F values as were found for X. The ethanol eluate was evaporated to dryness, the residue dissolved in water (1ml.) and the solution treated with 10% barium nitrate (1ml.) and 10% Na_2SO_4 (1ml.). The

precipitated $BaSO_4$ was centrifuged and washed twice with water, but was found to contain no radioactivity. The supernatant was mixed with an equal volume of 10N-HCl and heated under reflux for 2hr. The cooled solution was extracted with ether, the ether separated and carefully evaporated. The residual trace of oil was treated with 2,4-dinitrophenylhydrazine (10mg.) in ethanol (1ml.) and 10N-HCl (0.05ml.). The mixture was extracted with $CHCl_3$ (25ml.) and the extract washed with 2N-HCl (2x10ml.). The $CHCl_3$ was removed, the residue taken up in absolute ethanol and the light absorption spectrum determined. This corresponded to the spectrum of the 2,4-dinitrophenylhydrazone of phenyl acetone ($\lambda_{max.}$ 360mμ and ~260mμ). The hydrolysate after ether extraction was treated with barium nitrate and sodium sulphate as before. The precipitated $BaSO_4$ was found to contain ^{35}S and had a specific activity of 2μC/g.

From the foregoing experiments it seemed reasonably certain that X contained phenyl acetone combined in some way with inorganic sulphate. A search of the literature revealed a very interesting fact about the ketone, namely that in ethanolic solution almost 3% exists as the enolic form (Gero, 1954). This suggested that X might conceivably be the sulphate ester of the enolic form of the ketone, i.e.



Histogram of the more prominent ions in the mass spectrum of the diazomethane treated partially pure metabolite X.

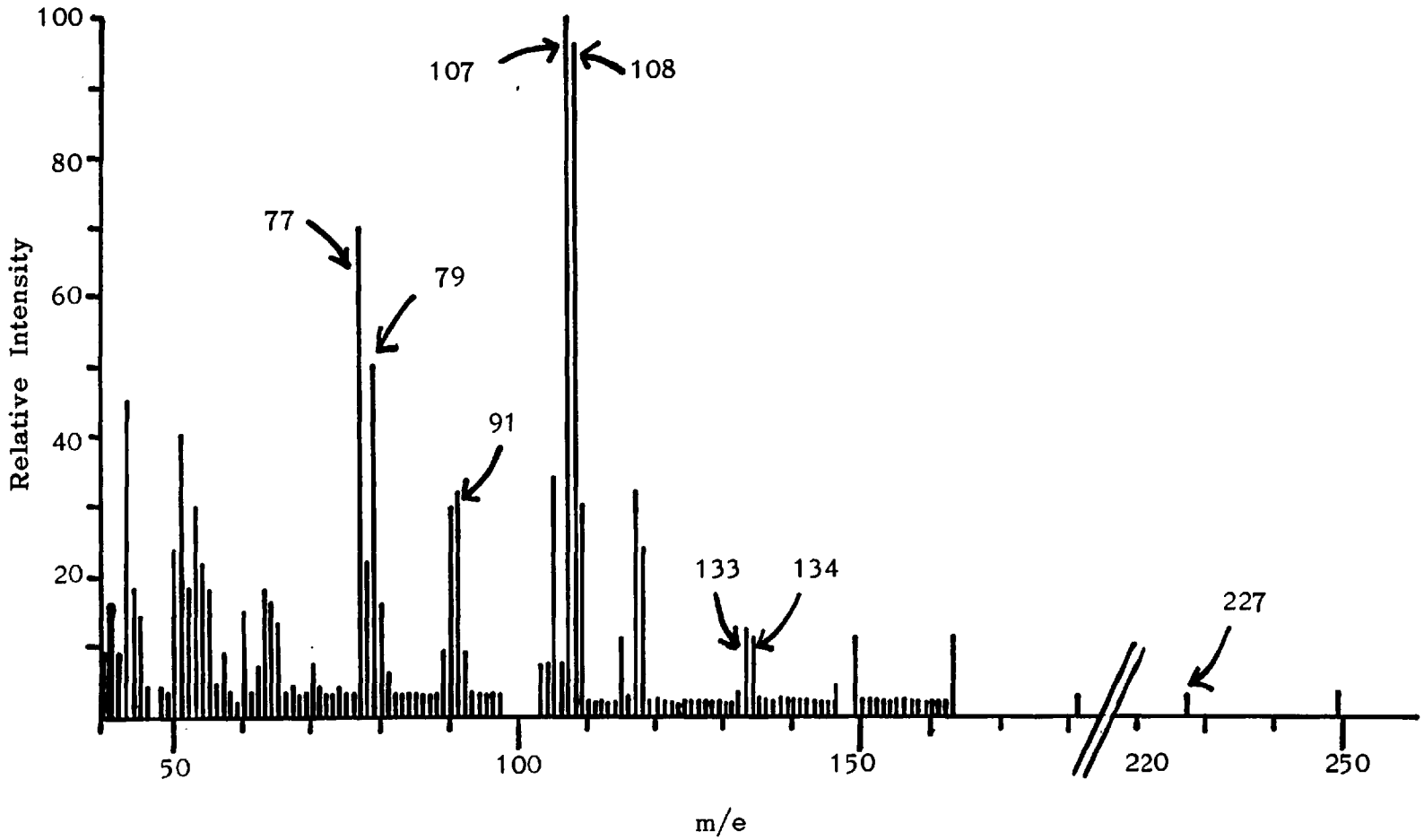


Fig. 5.

Therefore the physical properties, in particular the spectra of impure isolated X were examined.

PHYSICAL PROPERTIES OF THE ISOLATED MATERIAL

Infra-red Spectrum. The isolated material was not pure enough to give a sharp spectrum.

Mass Spectrum. This was determined using an AEI MS 902 mass spectrometer. After insertion of the sample some time elapsed before a spectrum was obtainable, indicating that the substance was not particularly volatile. Examination of the resulting spectrum indicated that considerable pyrolysis must have taken place (insertion probe temp. 270°), since the largest peaks were at m/e 107 and 108.

In order to obtain a greater volatility 1mg. of the sample was methylated in methanol with excess ethereal diazomethane which was removed after 5min. exposure to the reaction mixture by blowing nitrogen through the reaction solution. The spectrum of this material was considerably better, peaks in excess of m/e 200 being obtained (See Fig. 5). In the histogram of the more prominent ions in the mass spectrum the next to highest peak is at m/e 227 which corresponds to M-1 for the methylated enol sulphate, those at 133, and 134 to $C_9H_9O^+$, and 134 to $C_9H_{10}O^+$. Suprisingly, the most abundant ions at 108 and 107 correspond to $C_7H_8O^+$ and $C_7H_7O^+$, probably benzyl alcohol formed by some sort of internal rearrangement within the molecule. The major peaks below m/e 100 are well documented and have been observed on numerous occasions in the mass

Ultra violet absorption spectra of phenyl acetone, β-methyl styrene, and X in ethanol. ϵ for X calculated assuming mol. wt. 214.

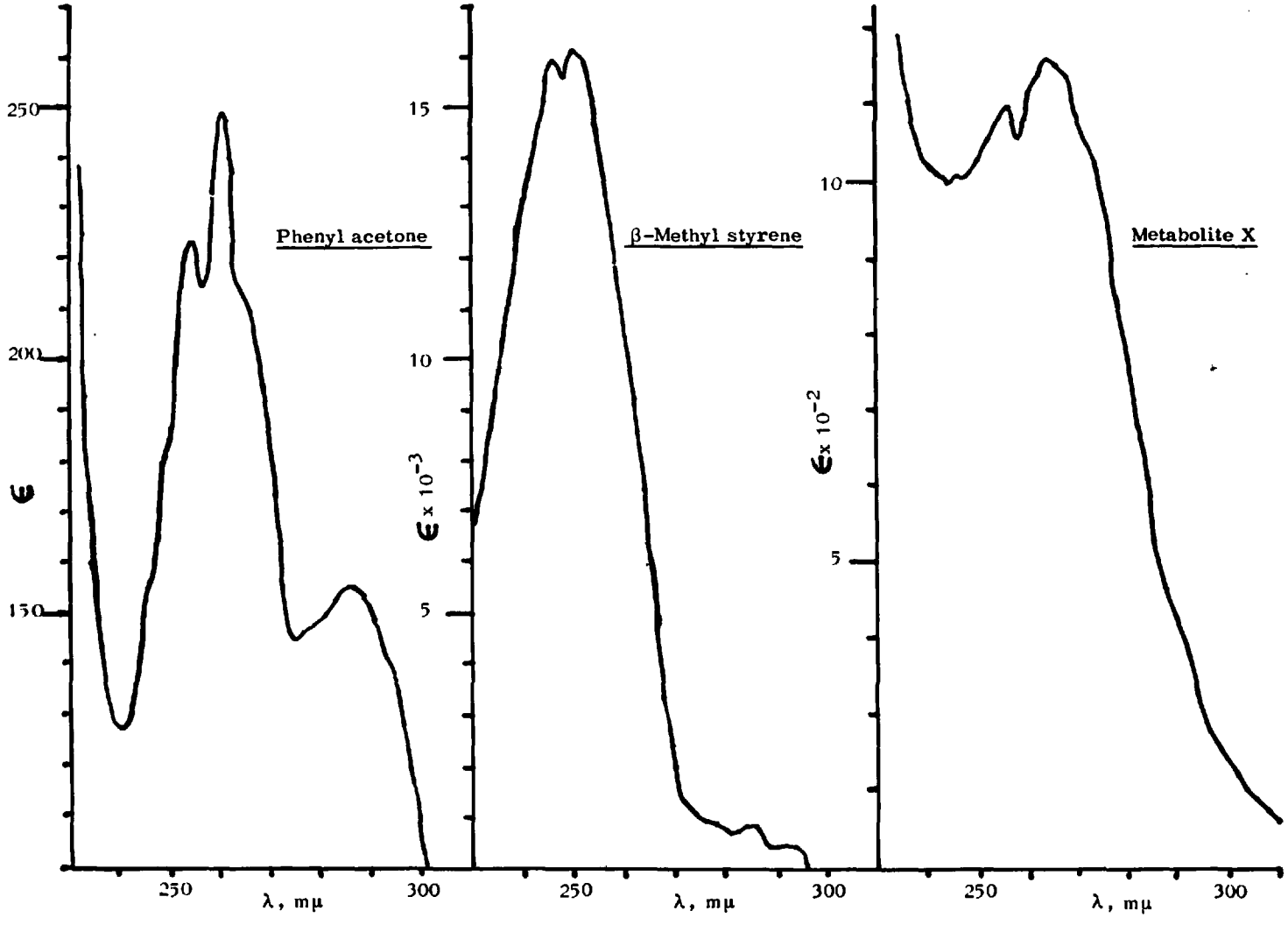


Fig. 9.

spectra of benzyl compounds (eg. benzyl alcohol; Myerson, Rylander, Eliel, and McCollum, 1959. 1-phenylpropane-1,2-diol; Audier, Dupin, Jullien, 1966). Thus m/e 91 is the familiar tropylium ion, m/e 79 the benzenium ion, and m/e 77 the $C_6H_5^+$ ion.

Therefore, although the highest m/e recorded was not that of the postulated methyl ester of the enol sulphate there was one at 227 which corresponds to the molecular ion minus one for this compound. In addition, the other important peaks recorded support the idea that X contains a phenyl acetone nucleus.

U.V. Spectrum. The U.V. spectrum of the isolated material was determined in ethanol and it had a $\lambda_{max.}$ of 264mμ, and an $E^{1\%}_{1cm.}$ of 54.3, or assuming a molecular weight of 214 if indeed it is the enol sulphate of phenyl acetone an ϵ value of 1157. Comparing this with phenyl acetone which has a $\lambda_{max.}$ 260mμ and $\sim$285mμ, $\epsilon_{max.}$ 249 and 156 respectively (Kumler, Strait and Alpen, 1950) it can be seen that X, even though impure has a five-fold increase in absorbancy over phenyl acetone. This in itself suggests the possibility of a conjugated system. However, when the spectrum of β-methyl styrene (1-phenylprop-1-ene) (Gauthier and Gauthier, 1933) was examined in ethanol it was found to have $\lambda_{max.}$ 250mμ and $\epsilon_{max.}$ 16,100 (this is about halfway between values for cis and trans isomers given by Mixer, Heck, Winstein and Young, 1953) and this contains the type of conjugated system postulated for the enol sulphate. However it is known that the sulphate group is strongly electron

N.M.R. Spectrum of Metabolite X in D_2O.

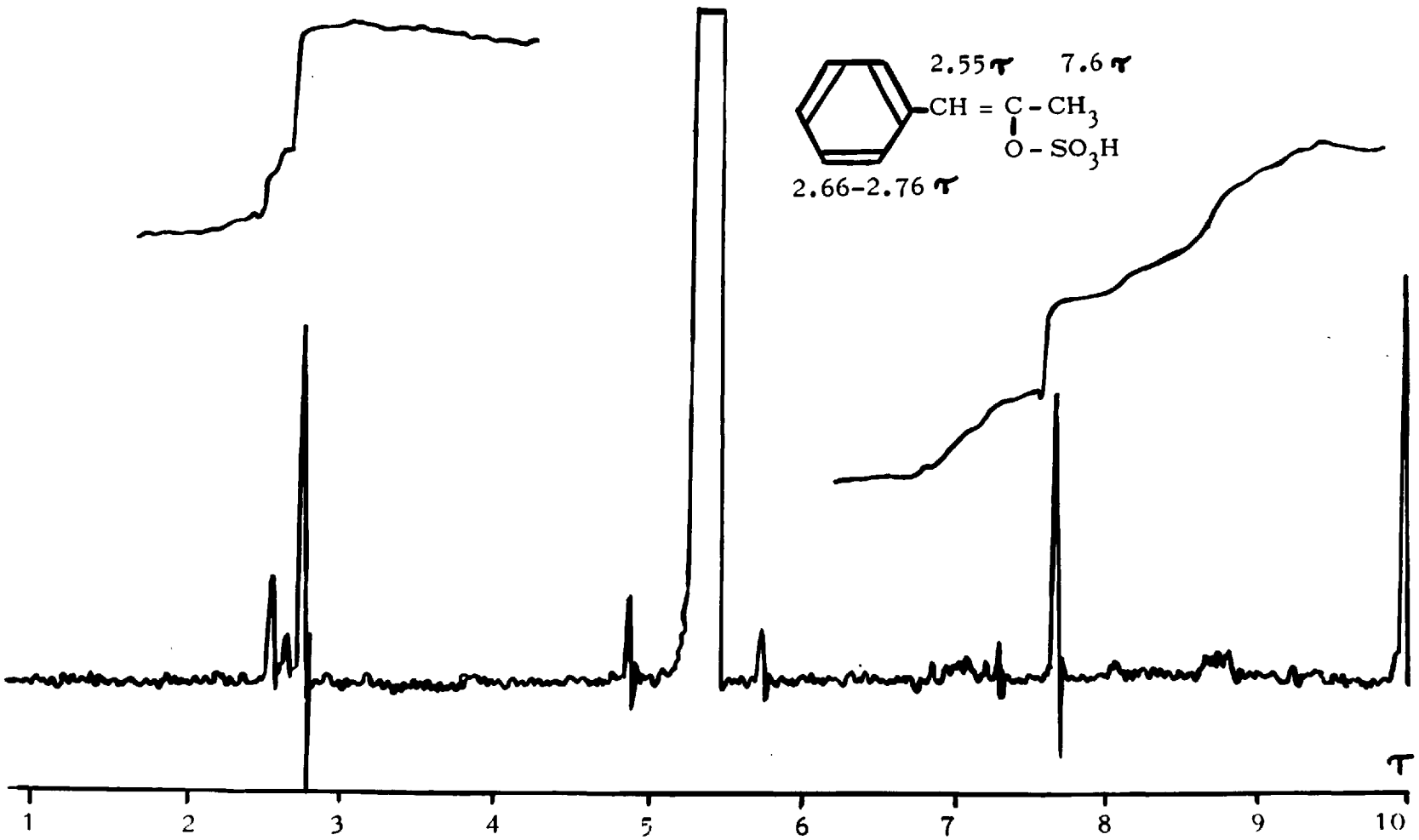
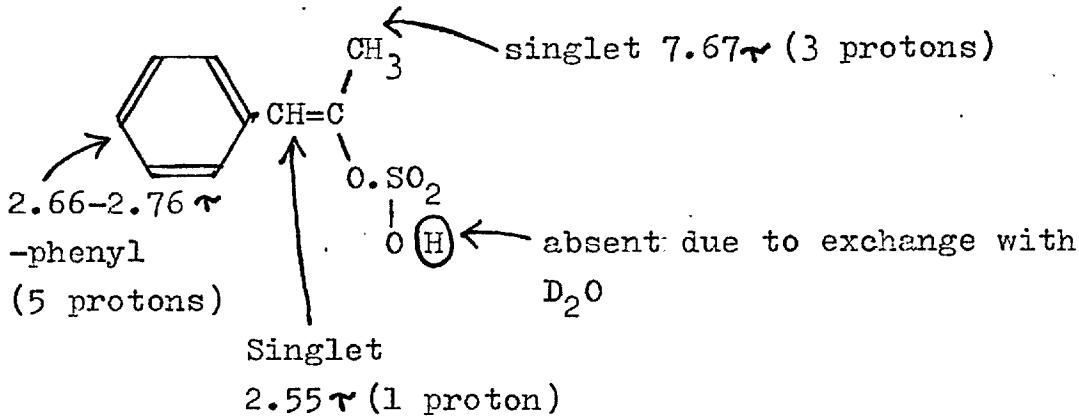


Fig. 7.

withdrawing and it could be that this is decreasing the degree of conjugation if X is indeed the enol sulphate.

N.M.R. Spectrum. Proton magnetic resonance spectra were measured in D_2O using a Perkin-Elmer 60Mc/sec. instrument, chemical shifts being measured downfield from tetramethylsilane (as 3-(tetramethylsilyl) propane-1-sulphuric acid Na salt) as internal standard.

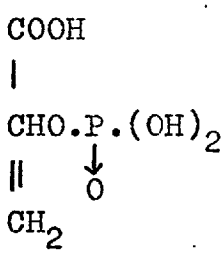
Looking at Fig. 7 it is obvious that the sample is not pure but it would appear to exist as a single cis, or trans isomer. The absence of any olefinic protons in the 3-4 τ region would at first sight appear to eliminate the suggested structure, however, examination of tables indicates that the olefinic proton may appear at very low field indeed (eg. Ph.$\underline{CH}$=C(CO) = 2.2τ). Hence it is possible that the singlet at 2.55τ may be assigned to the olefinic proton. The integration of the spectrum would then agree with the suggested structure as indicated below.



Therefore the n.m.r. spectrum would appear to agree with the suggested structure for metabolite X.

DISCUSSION

The first and probably best known inorganic acid enol ester is phosphoenol pyruvic acid (PEP). In 1934 Meyerhof and Lohman were investigating the enzymatic conversion of D-glyceric acid-3-phosphate to pyruvate and orthophosphate and they observed the formation of an hitherto unknown organic phosphate. They succeeded in isolating the new intermediary compound and established its constitution as that of PEP.



Several workers thereafter attempted the synthesis of PEP, since its isolation from biological material was rather difficult. However yields very rarely exceeded 10%. The most recent method published is that of Ohlmeyer (1957).

Sulphate esters are a common class of conjugate which can be divided into several types.

(a) Aryl sulphates eg. phenyl sulphate

(b) Alkyl sulphate eg. ethyl sulphate

(c) Sulphamates. Sulphate esters of amines eg. phenyl sulphamate.

(d) Steroid sulphates - esters of alcoholic and phenolic steroids.

(e) Carbohydrate sulphates eg. Chondroitin sulphate.

De Meio, Wizerkaniuk and Schreibman separated the sulphate synthesising system in rat liver into two fractions, a sulphate activating and a sulphate transferring enzyme. The active sulphate was soon shown to be an adenylic acid derivative and it was identified in 1957 by Robbins and Lipmann as adenosine-3'-phosphate-5'-phosphosulphate (PAPS). The formation takes place in two steps:

$$ATP + SO_4^{2-} \xrightarrow{\text{ATP-sulphate adenylyl transferase}} APS + PP$$

adenosine-5'-phosphosulphate

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

The transfer of the sulphate is catalysed by a sulphotransferase (sulphokinase) and can be represented:

$$PAPS + ROH \xrightarrow{\text{sulphokinase}} PAP + R.SO_3H$$

Several sulphokinases are known eg. phenol sulphokinase transfers sulphate from PAPS to phenols and is found in the soluble fraction of mammalian liver and it has been shown to be different from steroid sulphokinases of which several exist (Nose, Lipmann, 1958).

It is known that sulphate conjugation occurs in all mammals, although the pig does not form such conjugates readily (Stekol, 1936).

From the above evidence it can be seen that enol esters are already known as intermediary metabolites and that sulphate esters have been studied in some detail. In addition to this Roy (1960) in a study of the synthesis of aryl sulphamates showed that 17-oxo steroids greatly increased their rate of synthesis in rat but not guinea pig liver preparations. He postulated that the data was consistent with the hypothesis that steroid 17-enol sulphates were being formed from PAPS and that the sulphate was subsequently transferred from them to the arylamine. This suggested the occurence of a further group of compounds with a high sulphate potential besides PAPS, namely the 17-enol sulphates. Unfortunately he was not able to synthesise any of the postulated enol sulphates.

Assuming that X is the enol sulphate of phenyl acetone and it is realised that verification is needed

by either synthesis, which so far has proved unsuccessful, or by isolation of the pure compound from biological material, a further question is that why should it occur in the rabbit, but not say the guinea pig which also metabolises amphetamine by side chain degredation? The difference cannot be in the distribution of PAPS since all mammals can perform sulphate conjugations. However it could lie in differences in sulphotransferase activity since Roy implied that guinea pigs were unable to make steroid-17-enol sulphates. Alternatively it could lie in the variability of a tautomerase enzyme. An enzyme catalysing the tautomerisation of the enol form of phenyl pyruvic acid with the keto form has been identified in the organs of several mammals (Knox and Pitt, 1957). Conceivably such a tautomerase may exist for alkyl ketones.

CHAPTER 5

THE AMPHETAMINE - BARBITURATE MIXTURE

Multiple prescribing appears to be a common practice in medicine nowadays, particularly in psychiatry. Of all the drug mixtures which are used probably the best studied is the amphetamine-barbiturate mixture, and it has been shown in the introduction to this thesis that the mixture does indeed appear to have some pharmacological justification for its use. However no work appears to have been done on the biochemical basis of this interaction, if indeed there is one, and the work in this chapter is a preliminary attempt at such an investigation.

In the first part of the chapter the influence of phenobarbitone on the blood level of amphetamine in the rabbit is described and in the second part on the urinary excretion of amphetamine and its metabolites in the rat.

THE EFFECT OF PHENOBARBITONE ON THE BLOOD LEVELS OF INJECTED DEXAMPHETAMINE IN THE RABBIT

MATERIALS AND METHODS

New Zealand White does weighing 2.5-3.5 Kg. maintained on diet No. 41 (Associated Millers) and water ad lib. were used in these experiments.

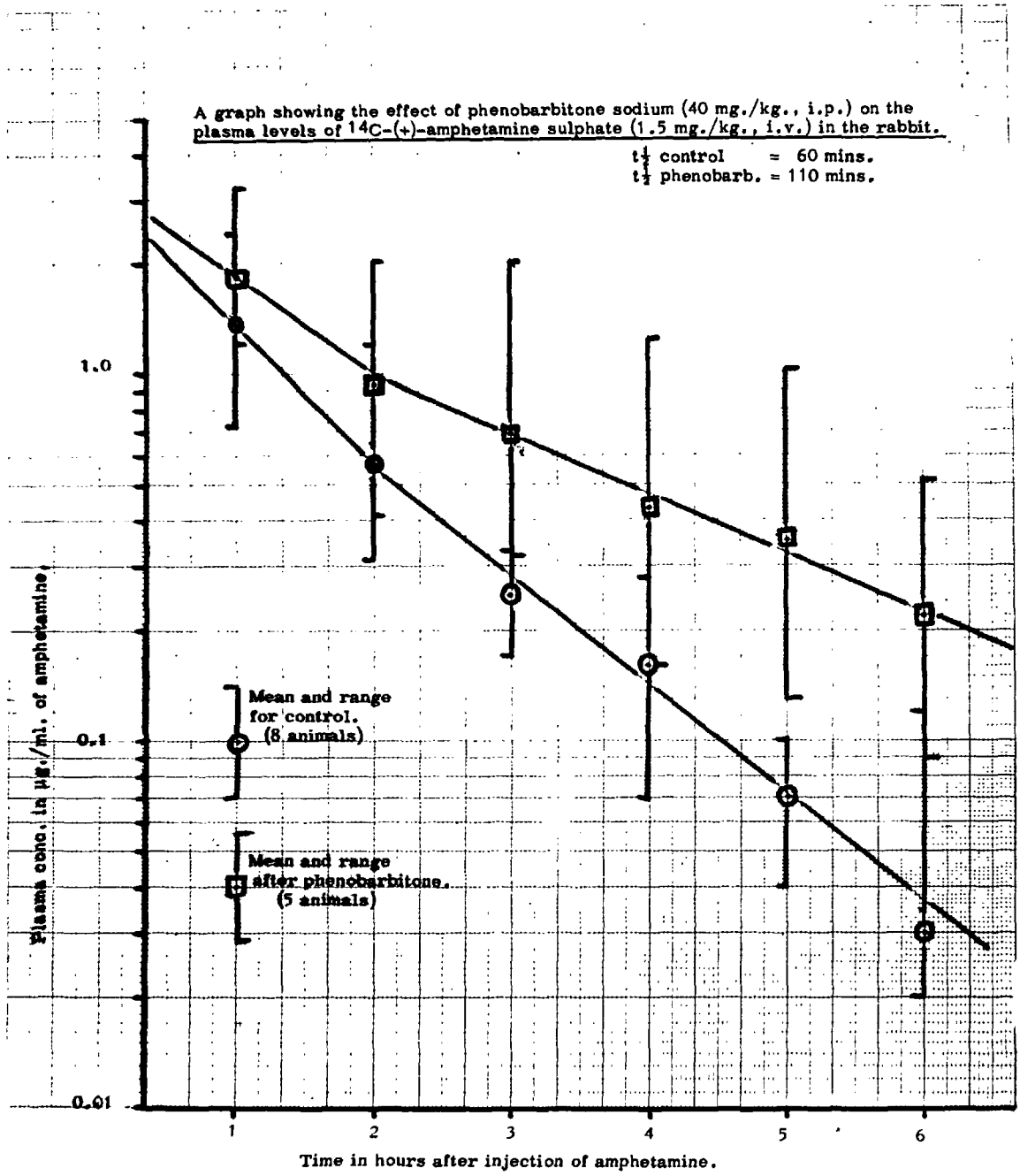
(+)-^{14}C-Amphetamine sulphate (1.5mg./Kg., 7μC) was injected as a solution in normal saline into the marginal ear vein, and blood samples were then taken for the following six hours, at hourly intervals.

In experiments involving phenobarbitone, the barbiturate (40mg./Kg.) was administered intraperitoneally as a solution of the sodium salt 10 mins. prior to the administration of the amphetamine.

Estimation of amphetamine in the plasma. The blood samples (ca. 3ml.) were centrifuged at 2,000 r.p.m. for 10mins. and the plasma removed. The plasma samples (ca. 2ml.) were acidified to pH2 with a few drops of 2N-HCl and extracted with ether (2x5ml.). The ether layers were separated and discarded, and the washed plasma samples adjusted to pH12 with 2N-NaOH and extracted with ether (2x5ml.). The ether layers were separated, bulked, and washed with saturated sodium bicarbonate solution (5ml.). Aliquots of the ether were transferred to scintillation counter glass vials containing 1 drop of 2N-HCl and the ether allowed to evaporate at room temperature. The samples were then counted in the Packard Tri-Carb Liquid scintillator as described in Chapter III.

That this method was specific for amphetamine was shown by chromatographing the processed ether extracts of plasma on Whatmans No. 1 paper in solvents A,B and C. In each case after cutting the chromatogram into small pieces and estimating the radioactivity on each piece (See chromatography of the metabolites of ^{14}C-phenyl acetone in Chapter IV) only one radioactive spot was found R_F 0.60, 0.88 and 0.51 in solvent A, B and C respectively. These R_F values correspond to amphetamine.

Fig. 8.


A graph showing the effect of phenobarbitone sodium (40 mg./kg., i.p.) on the plasma levels of ^{14}C-(+)-amphetamine sulphate (1.5 mg./kg., i.v.) in the rabbit.
t½ control = 60 mins.
t½ phenobarb. = 110 mins.
1.0
0.1
0.01
Plasma conc. in µg./ml. of amphetamine.
Mean and range for control. (8 animals)
Mean and range after phenobarbitone. (5 animals)
1
2
3
4
5
6
Time in hours after injection of amphetamine.

RESULTS

(a) Effect of amphetamine and the amphetamine-barbiturate combination on the behaviour of the rabbits.

Following the injection of (+)-amphetamine alone the rabbits remained calm during the entire experimental period, even when blood samples were taken. The only obvious responses to the drug were pupil dilation and vasoconstriction which lasted for up to 90mins.

On the other hand following treatment with amphetamine and the barbiturate the rabbits became hyperactive and inquisitive. The pupil dilation and vasoconstriction were more pronounced and prolonged than that seen with amphetamine alone. In addition the animals urinated and defaecated throughout the entire experimental period and became highly excited when blood samples were taken.

(b) Effect of phenobarbitone on the plasma levels and plasma half-life of amphetamine. A graph showing the effect of phenobarbitone on plasma levels of amphetamine is shown in Fig. 8.

The disappearance of amphetamine from the plasma seems to follow an exponential curve since the semi-logarithmic plot relating plasma level to time is linear. Also, both curves appear to be biphasic. This is not quite as apparent here as it was in the case of one ot two animals where several determinations were made between 0-2 hrs. In these it was quite obvious that there was a definite change in rate of plasma amphetamine dissappearance at between 2 and 3 hrs. after dosing.

Although it would appear that the level of amphetamine is increased and the rate of disappearance decreased between 0-2 hrs. there are insufficient points to give an accurate measure of this. However between 2-6 hrs. this difference is even more obvious, the plasma half life of amphetamine for the control ($t^{\frac{1}{2}}$ control) being 60 mins. and the plasma half life for the phenobarbitone treated animals being 112 min.

Therefore treatment with phenobarbitone would appear to decrease the rate of disappearance of amphetamine from the plasma with a resulting increase in amphetamine levels over the controls.

THE EFFECT OF PHENOBARBITONE ON THE URINARY EXCRETION OF AMPHETAMINE AND ITS METABOLITES IN THE RAT

METHOD

Female Wistar Albino rats (200 ± 20g.) were dosed orally with $(\pm)$-^{14}C-amphetamine sulphate (10mg./Kg., 4μC). The urine was collected from each rat at 2, 4, 6, 8.5 and 24 hrs. after dosing, subjected to chromatography and the resulting chromatograms scanned with the radio chromatogram scanner in order to quantitate each radio active area (See Chapter II).

In the experiments using phenobarbitone, each animal was injected with phenobarbitone sodium (60mg./Kg., i.p.) 30 mins before dosing with amphetamine.

Histogram showing cumulative excretion of amphetamine, 4-hydroxyamphetamine, and hippuric acid in rats after dosing with ^{14}C-(±)-amphetamine sulphate (10 mg./kg., 4 μC, p.o.) and after pretreatment with phenobarbitone sodium (60 mg./kg., i.p.) 30 mins. before dosing with amphetamine. Each value is the mean for 3 animals.

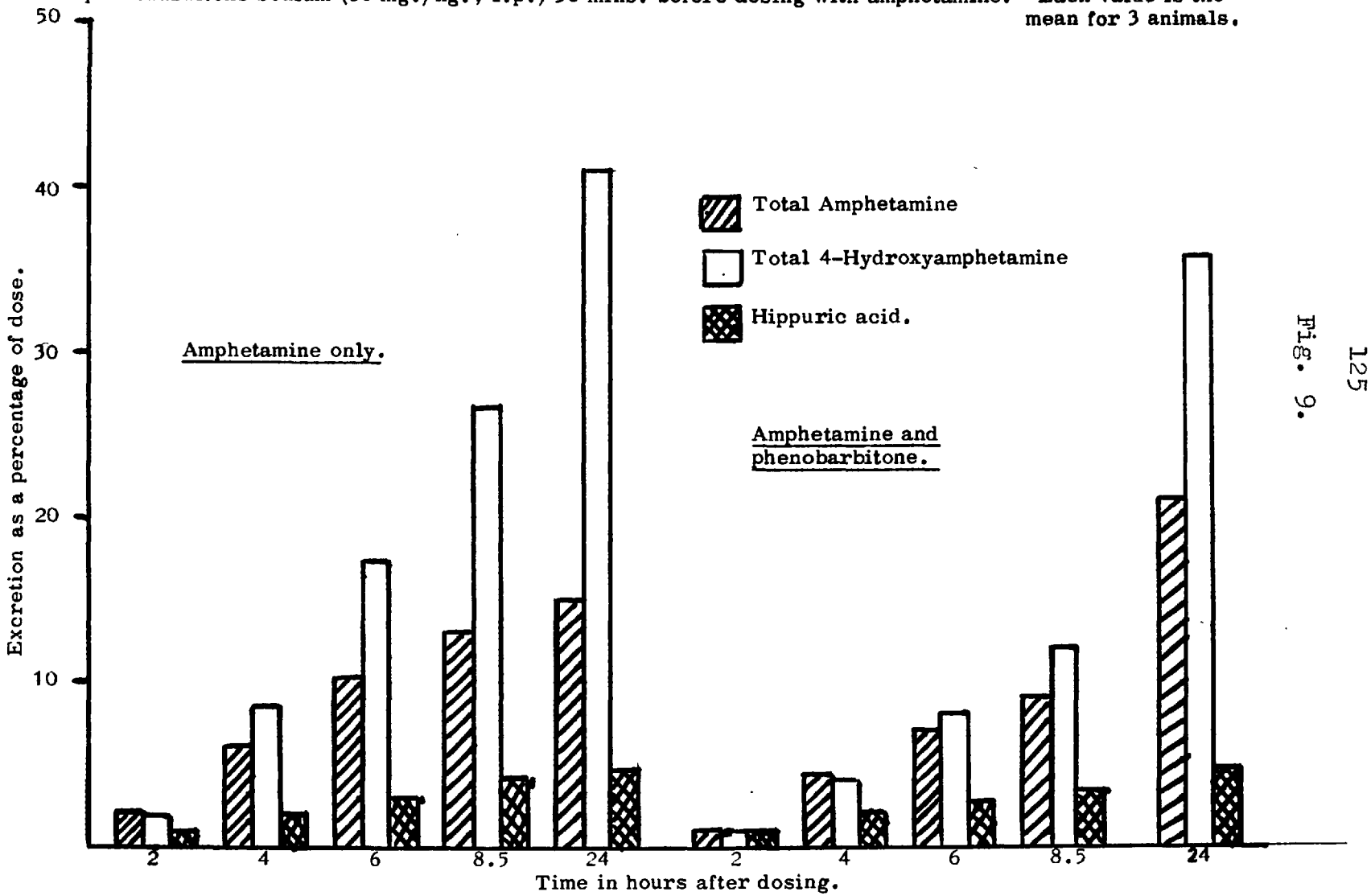


Fig. 9.

RESULTS

In the histogram comparing the cumulative excretion of amphetamine, 4-hydroxyamphetamine and hippuric acid at different times after dosing with amphetamine or the amphetamine-barbiturate combination (Fig. 9) it can be seen that in the barbiturate treated animals the rate of excretion of amphetamine in the first 8½ hrs. has been decreased, but not significantly so. However over the first day the total amphetamine excretion is actually increased over the controls. Again the rate of excretion of 4-hydroxyamphetamine in the phenobarbitone treated animals is definitely depressed 12%:27% at 8½ hrs. for treated and control animals respectively. The total amount of 4-hydroxyamphetamine excreted over the first 24 hrs. in the treated animals is also depressed compared with the controls, and it may be significant that this depression is almost equivalent to the increase in amphetamine excretion. The quantity and rate of excretion of hippuric acid in both control and treated animals appears to be similar, but since the total excreted is only a very small percentage of the dose, significant changes would be difficult to see.

DISCUSSION

The plasma half life determined in the rabbit is of the same order as that found in the rat by Maickel, Cox, Segal and Miller (1966) after i.p. administration of (+)-(H^3)- amphetamine. They also found that the plasma levels declined in a bi-phasic exponential curve (half life 30min. during 0-120 min. and 176 min. during 2-24 hrs.). Since amphetamine is not so completely metabolised in the rat as the rabbit a longer half life

in the second phase would be expected.

The mutual potentiation of amphetamine by barbiturates may find an explanation, at least in part, by the findings of these two experiments. Phenobarbitone would appear to prolong and increase the blood level of injected amphetamine in the rabbit, and secondly it would appear to prevent the metabolism to some degree of amphetamine given orally to rats since the amount of free drug excreted in the first day was increased and the amount of the 4-hydroxy metabolite decreased by a similar amount.

The mechanism by which phenobarbitone prolongs the plasma half life is not known but it could theoretically influence one or more of the sites of loss of the drug, namely tissue distribution, metabolism, or excretion. Excretion is probably not an important factor in the rabbit since very little unchanged amine is excreted in the urine (See Chapter III). However looking at the depression of the metabolism, especially in the first eight hours, of amphetamine in the rat, it is probable that this depression is the factor which causes the increase in the blood level of amphetamine and hence the enhanced pharmacological effect of these compounds.

It has already been noted in Chapter I that various workers have shown that drugs can competitively inhibit each others metabolism. Kato, Chiesara, and Vasanelli (1964) showed that phenobarbitone was such an inhibitor, but only a weak one. At the same

time Rubin, Tephly and Mannering (1964a,b) thought that this inhibition might involve competitition at the liver microsomal level for a "hydroxylase" enzyme.

Other drugs are also known which increase the pharmacological effects of amphetamine in a similar way, namely imipramine and chlordiazepoxide. Carlton (1961) showed a potentiation of the behavioural effects of amphetamine by imipramine, and Lapin and Shchelkunov (1963) showed that it prolonged the stereotyped behaviour induced by amphetamine. In addition Stein (1964) found that imipramine increases the facilitating effect of amphetamine on self stimulation of the brain for sub-threshold reinforcement. Another parameter by which the pharmacological effect of amphetamine can be measured is its hyperthermic effect. Valzelli, Consolo and Morpurgo (1966) showed that imipramine and desipramine (DMI) prolonged the hyperthermia induced by amphetamine in rats and they increase also the levels of brain and liver amphetamine. Sulser, Owens and Dingell (1966) found essentially the same, namely a correlation between the lengthened psychomotor stimulation and enhanced and lengthened brain levels of amphetamine induced by pretreatment of rats with DMI. Both groups suggested that these observations could be explained by the inhibition of the metabolism of amphetamine by DMI.

This observation was followed up by Consolo, Dolfini, Garattini and Valzelli who showed that DMI (2.5-5mg./Kg.) increases the urinary excretion of amphetamine given i.p. at doses of 7.5-15.0mg./Kg. and decreases the excretion of p-hydroxyamphetamine almost

to zero. They suggest that DMI impairs the hydroxylation of amphetamine in liver, thereby increasing the blood and brain level of the amphetamine. Compounds which cannot be 4-hydroxylated eg. 4-chloromethylamphetamine on the other hand does not have its activity enhanced by DMI, indicating again that DMI may enhance amphetamine activity by inhibiting its 4-hydroxylation.

The lesser decrease in hydroxylation of amphetamine by phenobarbitone as compared with DMI could have been predicted since Kato, Chiesara, and Vassanelli showed that imipramine was a much stronger inhibitor of metabolism by liver microsomes than phenobarbitone for a number of drugs.

Rushton and Steinberg (1966) found a similar enhancement of activity in rats with a (+)- amphetamine -chlordiazepoxide mixture (1.18mg./Kg. and 25mg./Kg. respectively), and in man with a mixture of chlordiazepoxide, 20mg. and (+)-amphetamine, 5mg. (Besser and Steinberg, 1967). Although no work appears to have been done on chlordiazepoxide as a metabolic competitor, and its metabolism is not completely understood (Koechlin, Schwartz, Krol and Oberhansli, 1965) it is known that the phenyl ring in the drug can be 4-hydroxylated in the rat (personal communication, Dr. R. Long). It could therefore again be quite possible that chlordiazepoxide might be working by slowing down the metabolism of amphetamine by competition for the hydroxylase enzymes at the microsomal level.

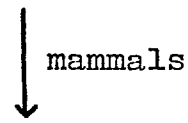
The results with imipramine in particular would seem to lend support to the idea that barbiturates enhance the effect of amphetamine by suppression of the oxidative metabolism of the amine probably at the liver microsome level.

CHAPTER 6

GENERAL DISCUSSION

A study of the species difference in the metabolism of a chemical substance, be it an endogenous substance, or a non-endogenous one like a drug, can have a variety of uses. The examination of the fate of a compound from one species to another can often be of use in designing compounds which are selectively toxic; eg. malathion, a contact organophosphorus insecticide, is rapidly metabolised in mice, rats, and dogs principally by hydrolysis of the ethyl ester bonds, whereas in insects there is oxidation to malaoxon which is 1000 times more active than malathion as an anticholinesterase (Kruger and O'Brien, 1959).

Malathion



de-esterification

Another reason for the study of metabolism differences and one which has received much attention of late (Cafruny, Cosmides, Rall, Schroeder, Weiner, International Symposium of Comparative Pharmacology, 1967) is the attempted correlation of the metabolism of drugs in various animal species with the metabolism in man. If this were to become possible, the screening of compounds for possible therapeutic use would become much simplified. The activity of a compound is

dependant upon either conversion to a more active metabolite or the speed of inactivation of the compound if it is active itself. How these two factors operate can be seen in the following two examples:

(a) There is a 50 fold difference in the duration of action and rate of metabolism of barbiturates in various animal species and man. It is interesting to note however that each species recovers from the effect of the drugs at the same plasma level. This appears to be a frequent observation (Quinn, Axelrod and Brodie, 1958), i.e. that a similar blood level produces the same effect in several species. It would seem that the difference in sensitivity of target sites postulated by the pharmacologist could be replaced by the theory that the differential rates and modes of metabolism of drugs would more readily explain species differences in the pharmacology of a drug.

(b) The therapeutic possibilities of such a drug as imipramine would have been missed if it had only been investigated in rabbits or mice since these animals fail to convert the drug in appreciable amounts to the active metabolite desipramine. (Dingell, Sulser and Gillette, 1964).

From these two examples it is apparent that the use of an animal which metabolises drugs in the same way as man would be extremely desirable. At one time it was thought that such an animal would be found amongst the primates, perhaps one of the apes. Unfortunately Brodie (1964) has dampened some of the initial

enthusiasm in this direction by suggesting that the correlation between man and monkey in some of his studies is not particularly good. It is probably desirable, for this reason, to investigate the metabolism of new drugs in man at an early stage in the drugs development so that an animal may be selected, using the data obtained, that is most suitable metabolically for prolonged toxicity studies. This idea was discussed recently by a W.H.O. committee (1966).

It was with these ideas in mind that the study of the species differences in the metabolism of amphetamine was undertaken, particularly since the work of Axelrod (1954) had indicated that a marked difference was to be found. The degree and extent of these differences has already been described in Chapter III and therefore the general picture which evolved will be compared with known species differences in the metabolism of other drugs.

Chemical reactions which take place within the organism can be broadly classified into oxidations, reductions, hydrolyses and syntheses, as indeed can all chemical reactions. A drug can undergo any one or a combination of these reactions, and is frequently metabolised in two phases. In the first phase (phase I) all the reactions except syntheses can occur, the second phase (phase II) is largely synthetic. (Williams, 1959). Probably one of the most common reactions is that of oxidation, and as will be seen shortly most of the major reactions undergone by

amphetamine itself can be considered to be oxidative.

CURRENT THEORIES ON THE OXIDATIVE METABOLISM OF DRUGS

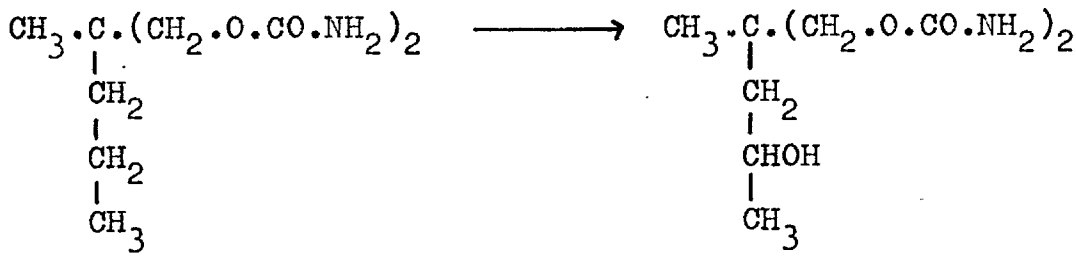
Oxidations include not only conversion of methyl groups, alcohols and aldehydes to carboxyllic acids but also oxidative O, and N dealkylation, oxidative deamination, aromatic hydroxylation, alicyclic ring oxidation and oxidation of sulphur and nitrogen. The following are a few examples of the general type of oxidative reaction possible.

EXAMPLES OF DRUG OXIDATIVE METABOLISM

1. Oxidation of alcohols.

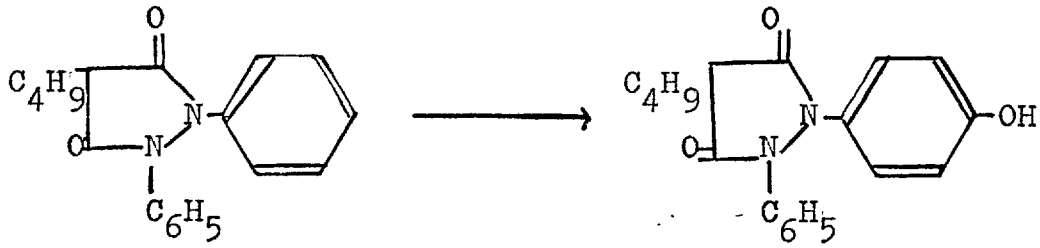
Chloral hydrate — Trichloracetic acid

2. Alkyl chain oxidation.



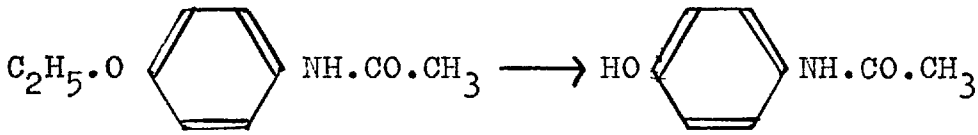
Meprobamate — Hydroxy meprobamate

3. Aromatic ring oxidation.



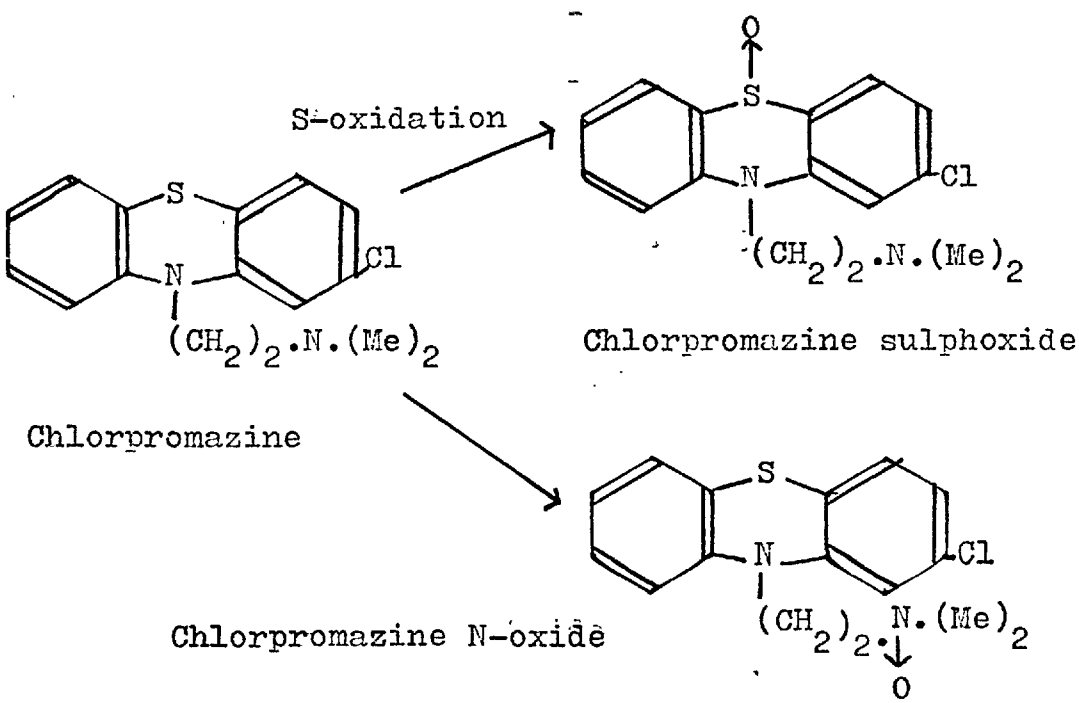
Phenylbutazone — Hydroxyphenylbutazone

4. Dealkylation.



Phenacetin — p-Acetamidophenol

5. S and N oxidation.



Chlorpromazine — Chlorpromazine sulphoxide

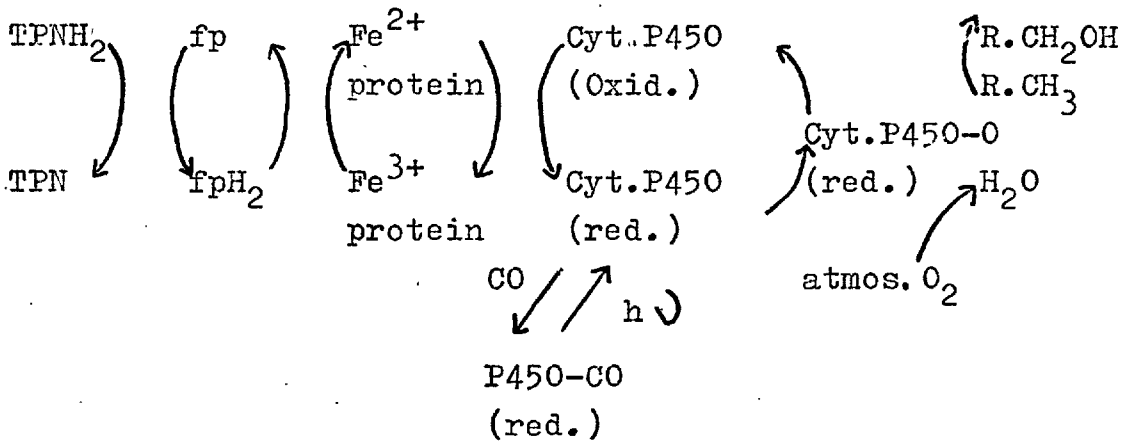
Chlorpromazine N-oxide

In recent years it has been found that the enzymes associated with these oxidative reactions, known as "mixed function oxidases", are associated with the endoplasmic reticulum of a number of tissues, particularly the liver. When cells are subjected to the usual preparative procedures such as homogenisation and centrifugation this reticulum is broken up into small vesicles, known as microsomes, and therefore enzymes present on the endoplasmic reticulum are termed microsomal enzymes.

It now seems likely that these so called "drug -metabolising enzymes" handle normal body constituents as well as drugs and that species variations in the metabolism of drugs are merely a reflection of a species variation in metabolism of normal body constituents. Triphosphopyridine nucleotide (TPNH)- dependant enzymes in liver microsomes have been shown to metabolise steroid hormones (King, Mason, Morrison, 1965), fatty acids (Stoffel, 1961), tyramine (Lemberger, Kuntzman, Conney and Burns, 1965) and various indoles (Jepson, Zaltzman and Udenfriend, 1962). The biosynthesis of cholesterol requires TPNH-dependant enzymes in liver microsomes.

The "mixed function oxidases" require reduced TPN and atmospheric oxygen (Brodie, Gillette and La Du, 1958). Since all the reactions are of the same type it would seem a reasonable assumption that a common intermediate is involved somewhere. The most attractive proposal so far is that of the same writers who describe all the oxidative reactions in terms of

a hydroxylation, a common step in all the oxidative reactions being the production of an intermediate hydroxyl donor, which in conjugation with a group of non-specific enzymes oxidises the substrates. Species difference studies and particularly differences in sensitivity to enzyme inhibitors and inducers have shown that different enzymes exist for the final hydroxylating steps (Creaven, Parke and Williams, 1965) although the production of the "active" oxygen is a step common to all the oxidations. The system which produces this active oxygen has been shown to contain a flavoprotein, a non-haem iron protein (probably $TPNH_2$-cytochrome c dehydrogenase) and a CO-binding haemoprotein known as cytochrome P450 (Omura, Sanders, Estabrook, Cooper and Rosenthal, 1966). This system is thought to work as follows.



Gillette (1963) proposed the following scheme for the various classes of drug oxidation, assuming that the common mechanism is hydroxylation.

Aromatic Hydroxylation:

$$CH_3.CO.NH.C_6H_5 \xrightarrow{(OH)} CH_3.CO.NH.C_6H_4.OH$$

Aliphatic Hydroxylation:

$$R.CH_3 \xrightarrow{(OH)} R.CH_2.OH$$

N-dealkylation:

$$R.NH.CH_3 \xrightarrow{(OH)} (R.NH.CH_2.OH) \longrightarrow R.NH_2 + HCHO$$

O-dealkylation:

$$R.O.CH_3 \xrightarrow{(OH)} (R.O.CH_2.OH) \longrightarrow R.OH + CHO$$

Deamination:

$$R.CH.(NH_2).CH_3 \xrightarrow{(OH)} (R.C(OH)(NH_2).CH_3) \longrightarrow R.CO.CH_3 + NH_3$$

Sulphoxidation:

$$R.S.R' \xrightarrow{(OH)} (R.S^+(OH).R') \longrightarrow R.SO.R' + H^+$$

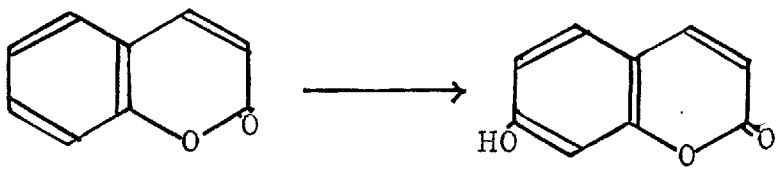
In Chapter 5 of this thesis it was shown how phenobarbitone raised the blood level of amphetamine and increased its half life in the rabbit and also how it inhibited the metabolism of the drug in the rat as seen by the amount of free amphetamine in the urine and the decrease in 4-hydroxylation. These observations can perhaps be explained at the enzymic level using the current theory of oxidative metabolism as outlined above. The metabolism of both phenobarbitone and amphetamine is oxidative and it is not inconceivable that these drugs inhibit the metabolism of each other competitively at the microsomal level, either at the relatively non-specific hydroxylase level

or through a competition for "active oxygen".

SPECIES DIFFERENCES IN OXIDATIVE METABOLISM

The large body of work in the literature, where several species have been examined, is largely confined to hydroxylation of aromatic ring systems, most probably because these are the most easily analysed. Species differences in aromatic hydroxylation could occur in at least three ways, (a) hydroxylation may occur in one species and not at all in another, (b) hydroxylation may be orientated, and this orientation may vary with species, (c) hydroxylation in one position could vary quantitatively with species (Williams, 1967).

The first category of hydroxylation, where hydroxylation may occur in one species, and not at all in another, is well illustrated by the 7-hydroxylation of coumarin.

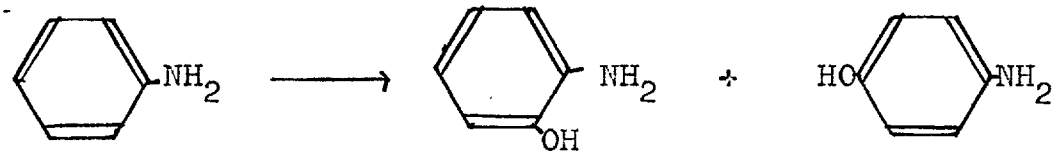


Coumarin 7-hydroxycoumarin

"In vitro" liver preparations from rats and mice cannot hydroxylate in this position at all, whereas the reaction does occur with preparations made from human, pigeon, cat, rabbit, guinea pig and coypu liver.

(Creaven and Williams, 1965). These "in vitro" results agree well with "in vivo" results where coumarin is 7-hydroxylated to the extent of 12% of the dose in rabbit and only 0.4% in the rat. (Kaighen and Williams, 1961).

An example of the second type of hydroxylation, where the hydroxylation is orientated, is shown especially well by aniline. In some species hydroxylation is directed para, and in others largely ortho.



Aniline O-aminophenol p-aminophenol

In this case a sufficiently large number of animals has been examined to classify the animals according to order and species.

Hydroxylation of ^{14}C-aniline by various animal species (After Williams, 1967)

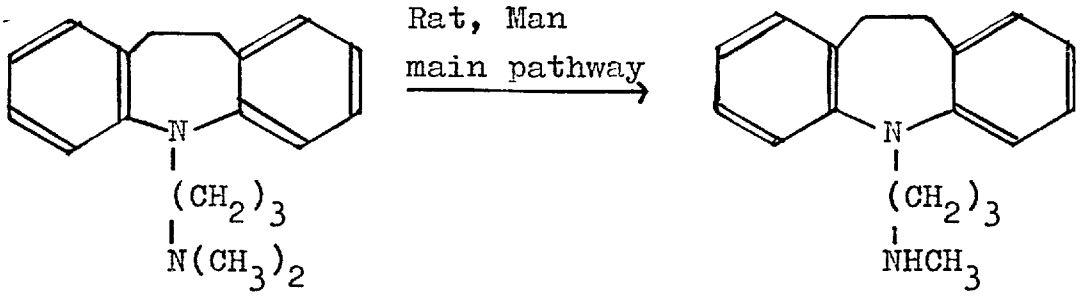
Class	Order	Species	Para/ortho ratio in urine
Aves	Galliformes	Hen	4
Mammalia	Carnivora	Dog	< 1
		Cat	< 1
		Ferret	1
	Rodentia	Rat	6
		Mouse	3
		Golden hamster	10
		Guinea pig	11
		Gerbil	15
	Lagomorpha	Rabbit	6

Notable are the carnivora with a low p/o ratio and the rodentia with a relatively high ratio. Since these hydroxylation figures are not complicated by other pathways of metabolism it would appear that the aniline O-hydroxylase is either more active or occurs in greater amounts in the carnivora than the rodentia. That the ratio changes with time has been shown in the dog, and this suggests that two different enzymes are involved in the hydroxylation (Parke, 1960).

An example of the last category of hydroxylation is imipramine which can be either hydroxylated or demethylated. Herrman and Pulver (1960) showed that

6-10% of a dose of the drug is metabolised in man, the remainder being excreted unchanged, and that in the rabbit 40-50% of the dose was metabolised. Later work by Dingell, Sulser and Gillette (1964) using liver microsome preparations delineated the relative importance of the two pathways, rat and human liver microsomes converting imipramine to desmethylimipramine, whereas the rabbit metabolises imipramine to the 2-hydroxy compound (Leybold and Staudinger, 1962). Incidentally, these findings also go some way towards explaining why imipramine has potent antireserpine and presumably antidepressant activity in rats, where the major metabolite is the desmethyl compound and yet in rabbits has little effect, presumably because the compound is metabolised not by demethylation but probably by 2-hydroxylation.

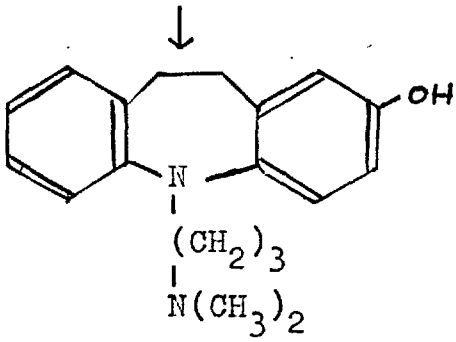
Major pathways of imipramine metabolism (After Dingell, Sulser, and Gillette, 1964)



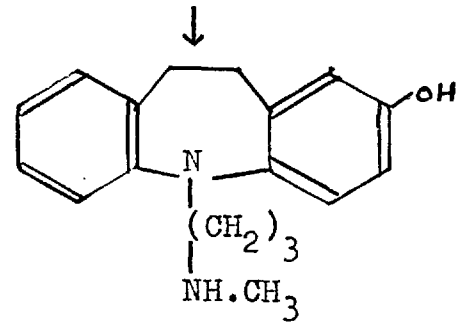
imipramine

desmethylimipramine

Rabbit
main pathway?



2-hydroxyimipramine



2-hydroxydesmethylimipramine

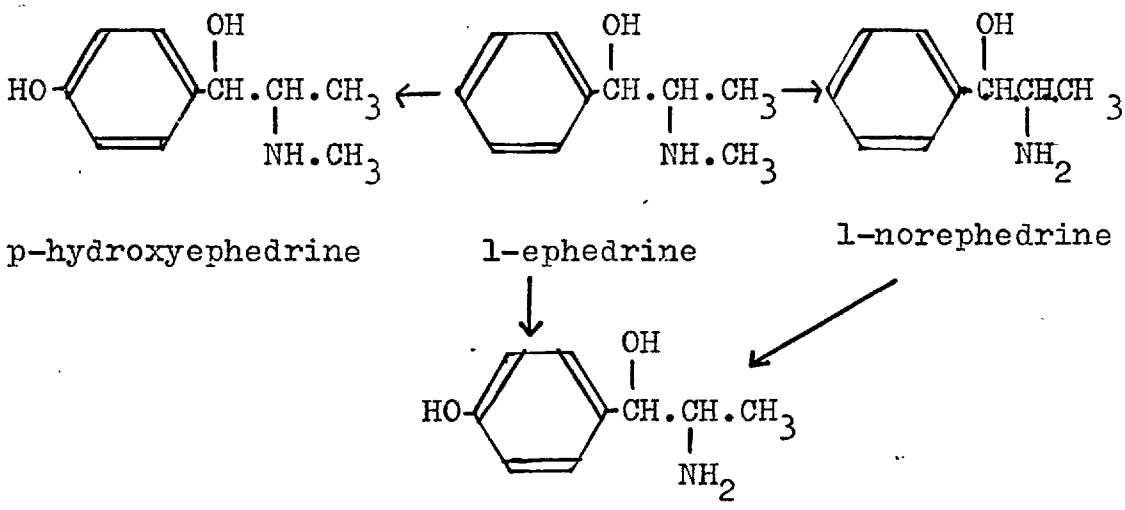
glucuronide conjugate

glucuronide conjugate

From these few detailed examples, there would seem to be several microsomal aromatic hydroxylases the activities of which are dependant not only on species but also the nature of the compound to be metabolised. The picture is made even more complex in compounds like imipramine where there is more than one reaction competing for a compound. With amphetamine too, there are at least two primary reactions competing for the drug and as has been seen in Chapter 3, 4-hydroxylation is the overriding one in the rat, and side chain degradation in the guinea pig with the other species studied having varying degrees of aromatic hydroxylation and side chain degradation. At present insufficient numbers of compounds have been studied in sufficient species to enable prediction from one species to another to become a practical possibility. Again, many of those compounds studied are widely divorced from each other in chemical characteristics and size,

so that probably greater advances could be made in the field of prediction if closely related compounds were studied. Thus the study of the species differences in the metabolism of ephedrine would be a step in this direction since it is a close relation of amphetamine. Axelrod (1953) showed that the routes of metabolism in different species did indeed bear a relationship to amphetamine metabolism, particularly as regards ring hydroxylation. He showed that ephedrine can be either directly hydroxylated or demethylated, then hydroxylated.

Suggested scheme for the metabolism of l-ephedrine (After Axelrod, 1953)



The extent to which ephedrine and norephedrine are hydroxylated is shown in the next table.

Fate of ephedrine in a number of species (After Axelrod, 1953)

	Ephedrine	Norephedrine	4-Hydroxylated ephedrine and norephedrine
Dog	6.5	57.8	1.5
Guinea pig	2.0	38.5	0.9
Rat	32.0	7.5	12.8
Rabbit	0.1	1.8	1.9

50mg./Kg. i.p. to each animal. Results as % of dose.

It can be seen that ephedrine follows the same pattern of 4-hydroxylation as does amphetamine, rat being the greatest, guinea pig being the least. The nature of the unidentified rabbit metabolites might well be predicted from experience with amphetamine.

If species differences in drug metabolism are considered merely to be a reflection of the amount and type of enzymes present in that organism, then the existence of such large differences should not be at all suprising especially since the actual outward appearance of an animal is dependant on cellular structure of which the enzymes are an integral part. Again, it is a well known principle in nature that economy of effort is of the utmost importance. Therefore it would seem unlikely that microsomal enzymes are merely for the metabolism of foreign compounds. There is mounting evidence that this is indeed the

case and it is now known that there are great similarities between "drug" and steroid hydroxylases in liver microsomes (Kuntz, Jacobson, Schneidman, Conney, 1964). This suggests that differences in patterns of drug hydroxylation in different species may reflect differences in the liver microsomal hydroxylation of steroid hormones. Kuntzman, Welch and Conney (1966) have shown that the pattern of progesterone metabolites depends on the species studied. Much of the work in basic biochemistry has been done in only one species frequently of a low order, like the yeast, and it would seem that work with foreign compounds is again providing a biochemical tool and is this time underlining the relative importance of endogenous metabolites.

INDIVIDUAL VARIATIONS IN AMPHETAMINE METABOLISM IN MAN

Of the three subjects studied in detail it seems possible that the variation in hydroxylation may be subject to genetic control, since very similar results for each subject were obtained over a nine month period, each estimation being performed at approximately 3 month intervals. Also there was almost a ten fold difference between R.L.S. and L.G.D. and therefore this could be another example of "polymorphism" (Price Evans, 1967).

The study of the variable ability of individuals to metabolise drugs is becoming both a useful tool for the human geneticist and also the physician. The latter recognised many years ago the variability in

response from patient to patient to certain drugs. This phenomenon of polymorphism is observable in the response of a population to drugs, and these pharmacogenetic polymorphisms can be divided into polymorphisms of drug metabolism with (a) all phenotypes common and (b) one phenotype rare. The first group is exemplified by isoniazid, the rate of metabolism of which is genetically determined. (Evans, Manley and McKusick, 1960). The second by the inability of a small group of patients to hydroxylate diphenylhydantoin, and consequently, on normal dosage to suffer ataxia, dizziness and, sometimes, coma. That it was a very specific genetic effect was shown by the fact that such patients had no difficulty in hydroxylating phenobarbitone.

If indeed further research does reveal that the metabolism to 4-hydroxyamphetamine is indeed subject to genetic control it would be of even greater interest to see if there is any relationship between hydroxylating ability (or inability to metabolise the hydroxy compound further) of an individual and his propensity to become habituated to the drug.

Another important clinical observation, which may be connected with the ability to hydroxylate amphetamine is that certain people who take excessive amounts of amphetamine exihibit symptoms indistinguishable from schizophrenia (Connel, 1958). Recently it has been shown that 4-methoxy amphetamine is the most potent behaviour disrupting compound known after

Lysergic acid diethylamide (Smythies, Johnston, Bradley, Morin and Clark, 1967). Other psychogenic methoxylated amphetamine derivatives are known too (Shulgin, and Sargent, 1967). One of the most widely accepted explanations for schizophrenia and one which was put foreward by Harley-Mason, Osmond, and Smythies in 1952 suggested that the disease might be caused by an abnormality of O-methylation of noradrenaline, to give a psychotoxic metabolite such as dimethoxyphenyl ethanolamine. Indeed such a methylated amine was found in the urine of schizophrenic patients in 1962 by Friedhoff and Van Winkle, not as it happens the ethanol amine derivative, but 3,4-dimethoxyphenylethylamine. However the methylated amine is not found in all patients so whether in fact faulty trans methylation is indeed the sole cause of this mental disease is not at all certain at present (Parry, Hansen and MacIntyre, 1964). It would be interesting to speculate upon the fate of amphetamine in these psychotic patients and if indeed a derangement in O-methylation does occur then the O-methylated metabolites of amphetamine would be more readily detectable due to the greater stability of the α-methylated side chain in man than almost any other species.

GENERAL CONCLUSIONS

The metabolism of amphetamine varies markedly from species to species and within a species it varies with the optical isomer and indeed from one individual to another. This variation takes the form of differing ratios of ring hydroxylation to side chain degradation with the rat at one extreme metabolising the drug

largely by ring hydroxylation and the guinea pig at the other metabolising the drug by side chain degradation. In addition the rabbit is peculiar amongst the species studied in that it alone has benzyl methyl carbinol as a metabolite and excretes another metabolite, thought to be the enol sulphate of phenyl acetone, in much greater quantities than any other species.

Barbiturate pretreatment of rabbits dosed with amphetamine caused an increased blood level of the amine, and a similar pretreatment of rats dosed with the amine increased the amount of unchanged amphetamine in the urine and decreased the amount of the 4-hydroxy metabolite. A possible biochemical basis for the enhancement of the pharmacological effect of amphetamine by barbiturates may therefore be an increased blood level of the amine which has been caused by an inhibition of its metabolism by the barbiturate.

This present study further underlines the great interspecies difference in the oxidative metabolism of drugs and also the difficulty of relating the metabolism of one drug to that of another in the same species. However the compounds which have been previously studied possessed greatly divergent chemical characters and it may be that examination of the metabolism of more closely related compounds would be more rewarding.

The finding of a marked individual variation, particularly as regards hydroxylation, in man, makes a more detailed study in a larger group of people

seem worthy of further investigation, particularly since it is felt that it may give an insight into two related problems of today's society, namely mental disease and drug dependence.

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