THE METABOLISM OF AMPHETAMINES IN VARIOUS SPECIES

by

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ABSTRACT

Using ^{14}C labelled material, the metabolism of the sympathomimetic drug methamphetamine ((±)-2-methylamino-1-phenyl-[1-^{14}C]propane) has been investigated in the rat, guinea pig and man.

Administered radioactivity was eliminated rapidly from all 3 species, between 50 and 80% of the dose appearing on the first day. In the rat and man this was found almost exclusively in the urine; in the guinea pig, at a dose of 45 mg/kg about 18% was in the faeces with 69% in the urine but with a dose of 10 mg/kg the excreted radioactivity was almost all in the urine.

There are marked differences in the metabolism of methamphetamine between the three species examined. The rat metabolized the compound principally by N-demethylation and ring hydroxylation, while the guinea pig utilized side chain breakdown. In both species, at a dose of 45 mg/kg, sidechain hydroxylation was an important metabolic option, and it would seem that in the guinea pig the metabolism of methamphetamine varies with dose, sidechain hydroxylation being of significance at a high dose but deamination giving a precursor of benzylmethyl ketone being an important pathway at a lower dose (10mg/kg). The guinea pig produced an unknown metabolite at a dose of 45 mg/kg but not at the lower dose.

The biliary excretion of amphetamine and methamphetamine in the rat was shown to occur, and the principal biliary compounds were the O-glucuronides of the ring hydroxylated metabolites. It was concluded that an appreciable enterohepatic circulation of the biliary metabolites of both drugs occurred in the rat.

In man, the metabolism of methamphetamine was very different from that of amphetamine, ring hydroxylation being the major metabolic route and side chain breakdown being very small. Sidechain hydroxylation of both amphetamine and methamphetamine was shown to a minor extent.

The results of the metabolic studies have been employed in the construction of an hypothesis to explain the development of tolerance to methamphetamine.

FOREWORD

The work described in this thesis was carried out between October 1969 and January 1972 in the Biochemistry Department of St. Mary's Hospital Medical School. I wish to thank Professor R.T. Williams, F.R.S., for giving me the opportunity to work in his department, and for the great interest he has shown throughout this study. His vast knowledge and experience have been drawn upon extensively, and I am most grateful.

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Finally, and most importantly, I must thank my wife, Jill, for her patience, encouragement and kindness, which has meant so much to me during my work for my first degree and at St. Mary's. I trust that she, too, finds in the result something of my gratitude to her.

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CHAPTER ONE

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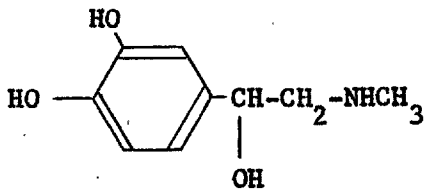
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The Amphetamines - an historical perspective

Around the year 1900, there was great interest in the world of physiology in the workings of the autonomic nervous system, eventually leading to the identification of the endogenous transmitters, noradrenaline in the sympathetic and acetylcholine in the parasympathetic system. However, long before the conclusive identification of the transmitters, the pharmacological importance of these compounds had been realised and exploited.

Oliver and Schaefer (1895) demonstrated the pressor effects of suprarenal extracts, and Abel and Crawford (1897) isolated the active principle and termed it epinephrine (adrenaline). After some controversy, Jowett (1904) was able to confirm that the elemental formula was $C_9H_{13}O_3N$, and proposed the structure to be:



This structure was confirmed by synthesis in the work of Stolz (1904) and Dakin (1905). The potential medicinal value of this and related compounds must soon have been realised, for the literature quickly contained many synthetic methods for these materials.

It will be noted that the carbon bearing the alcoholic hydroxyl group is an asymmetric centre, and it was found that the synthetic adrenaline (which was racemic) had a lesser physiological activity than the natural suprarenal extracts, known to be laevo rotatory. Cushny (1908,1909) showed that natural (-)-adrenaline had twice the pressor activity of synthetic (±)-adrenaline, and that the (+)-form had only one-twelfth of the activity of the (-)-isomer. The finding that one optical isomer has a greater biological activity than another has often been repeated in work using optically active compounds of this type.

As a result of the great number of structural variations on the theme of adrenaline carried out since 1905, in 1910 Barger and Dale were able to publish their classical paper relating chemical structure to the biological activity of this type of amine. They coined the term sympathomimetic, meaning "an action simulating that of true sympathetic nervous systems", and made general conclusions about the structure-activity relationships of sympathomimetic amines. The structure for optimal activity has a benzene ring, with a two carbon sidechain, with a terminal primary or secondary amino group. Activity is enhanced by two phenolic hydroxyl groups at 3' and 4' on the ring, and by an alcoholic hydroxyl group on the first carbon of the sidechain.

From this time onwards until the present day, structural analogues of adrenaline have been investigated for potentially useful pharmacological activity. Ephedrine (2-methylamino-1-phenylpropan-1-ol), an alkaloid from Ephedra species plants isolated by Nagai (1887), was found by Chen and Schmidt (1924) to have a weak but prolonged sympathomimetic action, and to be active orally. Ephedrine has two configurations, each of which can exist in a pair of optical isomers, and the most active form was (-)-ephedrine. The oral activity of this phenylisopropylamine led to investigation of other related compounds, and amphetamine (2-amino-1-phenylpropane), first synthesised by Edeleanu (1887) as a chemical exercise, was found to be a pressor agent by Piness *et al* (1930). Methamphetamine (2-methylamino-1-phenylpropane), which was synthesised by Schmidt (1914) as part of investigations into the structure of ephedrine, was also found to be a potent sympathomimetic by Hauschild (1938).

The activity of these drugs when given orally is due to their resistance to monoamine oxidase, the enzyme which breaks down phenylethylamines (Blaschko *et al*, 1937).

The work described in this thesis is principally concerned with methamphetamine, and in addition some work has been performed on amphetamine. This chapter, then, is a review of the physiology of the sympathetic nerve ending, the mode of action and pharmacology of methamphetamine and the metabolism of the amphetamines in general.

The Physiology of the Adrenergic Nerve Ending

Methamphetamine is a sympathomimetic amine, and as such has its action at the adrenergic nerve ending. To understand its mode of action it is first necessary to consider the physiology and biochemistry of the adrenergic terminal in some detail.

The Concept of the Chemical Neurotransmitter

After the isolation and synthesis of adrenaline in the decade 1895-1905, many workers, notably Lewandowsky (1898) and Langley (1901) observed the close similarity between the effects of sympathetic nerve stimulation and the physiological actions of injected adrenaline. On this basis, Elliot (1905) postulated that, when sympathetic nerves were stimulated, adrenaline was liberated from some precursor in the nerve and excited the effector organ.

In a similar way, Dixon (1907) showed that the alkaloid muscarine had very similar actions to those resulting from stimulation of the vagus nerve, and suggested that parasympathetic stimulation caused release of a muscarine-like substance which acted as a chemical transmitter of the impulse to the effector cell Loewi (1921), in a classical experiment, prepared two isolated frogs hearts and perfused them in series. Stimulation of the parasympathetic nerve supply to the first heart slowed its rate of beating, and it was observed that the second heart slowed also. This was shown to be due to the release of a chemical substance from the first heart which was being washed out and acting on the second heart also. This substance was identified as acetylcholine by Loewi and Navratil (1926). Loewi (1921) also stimulated the sympathetic nerves to the first heart, thereby increasing its rate of beating, and found that the second heart accelerated also. However, although this experiment validated the concept of chemicals being released by nerve stimulation to mediate the effects of that stimulation, the identity of the accelerator substance released by sympathetic stimulation remained obscure for three more decades.

Identity of the Sympathetic Transmitter

Loewi (1921) had shown that both parasympathetic and sympathetic nerves released compounds on stimulation which had the same effects on the organ as direct nerve stimulation. Cannon and Uridil (1921) found an adrenaline-like pressor amine in the liver, and in further work over the next 15 years, reviewed by Cannon and Rosenbleuth (1937), this amine was shown to be the chemical mediator of sympathetic nerves, which they termed "sympathin". This sympathin, although adrenaline-like, was not adrenaline itself as the two materials exhibited marked pharmacological differences as well as similarities.

Barger and Dale (1910), many years previously, had pointed out that primary sympathomimetic amines give effects closer to those of sympathetic stimulation than do adrenaline and other secondary amines. Many suggestions were made that the sympathetic transmitter was demethylated adrenaline, or noradrenaline (see Bacq,1934; Greer et al.,1938) but no definite evidence was forthcoming until, using much improved analytical techniques, von Euler (1946) was able to show that the sympathomimetic substance in highly purified extracts of sympathetic nerves was in fact noradrenaline. The first conclusive evidence of noradrenaline release from nerves was by Peart (1949) and despite some objections (see Tainter and Ludena,1950) it was soon widely accepted that the principal neurotransmitter in mammals was noradrenaline (von Euler,1959).

There is a very large body of evidence, pharmacological, biochemical and histological, that noradrenaline is the chemical transmitter in certain of the fibre tracts of the CNS and that these noradrenaline-containing neurones are functionally similar to the peripheral fibres (see Iversen, 1967).

Biosynthesis of Noradrenaline

In 1938, Holtz and Ludtke discovered the enzyme L-DOPA decarboxylase, which produces dopamine from DOPA. Subsequent to this, Blaschko

(1939) proposed the reaction sequence shown in Fig.1.1 to be the biosynthetic pathway from phenylalanine to noradrenaline and adrenaline, but the verification of this route occupied the next 20 years. It is now well established that adrenal medullary tissue is able to convert radioactive tyrosine to adrenaline by this pathway (see, for example, Demis et al.,1955; Udenfriend and Wyngaarden, 1956) but demonstration of noradrenaline synthesis is technically more difficult. In 1958, Goodall and Kirshner showed that homogenates of sympathetic nerves could convert labelled tyrosine or DOPA into dopamine and noradrenaline and Spector et al.,(1963) similarly demonstrated noradrenaline synthesis in isolated perfused guinea pig heart.

The formation of noradrenaline and dopamine from ^{14}C-tyrosine by slices of brain tissue has also been demonstrated (Masuoka et al.,1961,1963). The presence of all the enzymes required for the synthesis of noradrenaline, and their regional distribution, in human brain, has been shown by Vogel et al. (1969).

These biosynthetic enzymes all exhibit very low substrate specificity but high stereospecificity, only acting on the (-)-isomers of their substrates

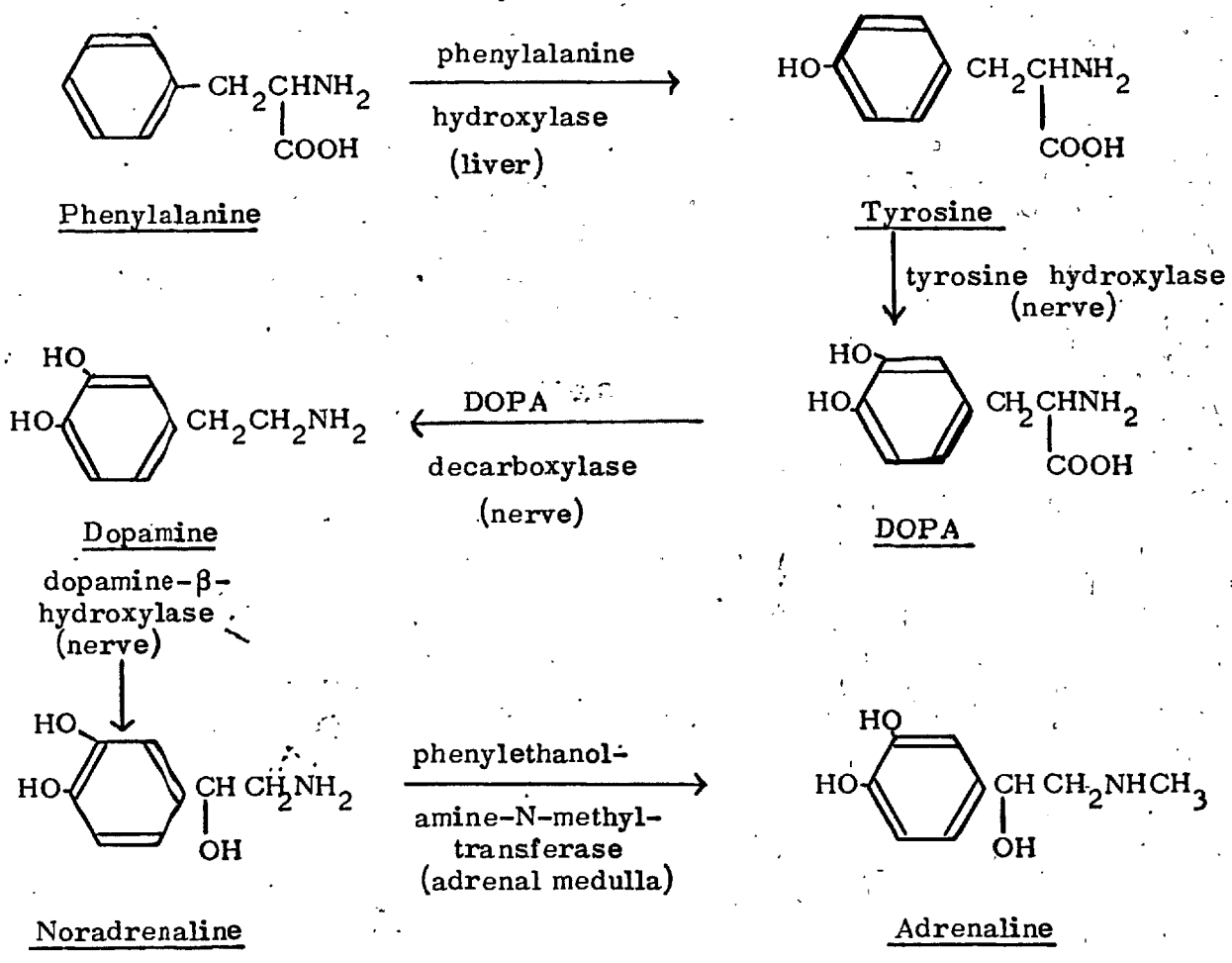
Control of Noradrenaline Synthesis

As will be seen from the biosynthetic pathway described before, there are three possible sites at which control of noradrenaline synthesis may be applied, these being (a) tyrosine hydroxylase, (b) DOPA decarboxylase and (c) dopamine-β-hydroxylase. These enzymes have been partially purified and their K_ms determined using the appropriate substrate.

Tyrosine hydroxylase K_m 1×10^{-5}M (Nagatsu et al.,1964)
DOPA decarboxylase K_m 4×10^{-4}M (Lovenberg et al.,1962)
Dopamine-β-hydroxylase K_m 5.8×10^{-3}M (Creveling et al.,1962)

From these values obtained from "in vitro" studies, it would appear that the rate limiting step in noradrenaline biosynthesis is dopamine-β-hydroxylase. However, "in vivo" studies (performed by perfusing guinea pig

Fig 1.1 The Biosynthesis of Noradrenaline and Adrenaline



hearts with tyrosine, DOPA or dopamine) show that the system may only be saturated with tyrosine, and that the K_m for purified tyrosine hydroxylase lies very close to the K_m for the system tyrosine noradrenaline (Levitt et al.,1965). This suggests that the rate limiting step in noradrenaline formation is tyrosine hydroxylase, the reason for the difference between the prediction based on "in vitro" work and the results of "in vivo" studies probably being due to the dopamine-β-hydroxylase and DOPA decarboxylase activity in nerve tissue being of the order of μmol/g tissue/h and that of tyrosine hydroxylase being mμmol/g tissue/h (Nagatsu et al.,1964).

Various treatments, including sympathetic nerve stimulation (Alousi and Weiner,1966), accelerate the synthesis of noradrenaline but Sedvall and Kopin (1967) have found that when this occurs tissue levels of tyrosine hydroxylase do not alter. Alousi and Weiner (1966) suggested that free intraneuronal noradrenaline is responsible for the regulation of this enzyme, possibly by competing with the reduced pteridine cofactor (Kaufman,1964), for the oxidized enzyme (Ikeda et al.,1966) and evidence for this is provided by findings like those of Weiner and Selvaratnam (1968) who observed that tyramin inhibited tyrosine hydroxylase "in vivo" but not "in vitro" They explained this on the basis of tyramine releasing noradrenaline from its storage granules in the cytoplasm and this released amine inhibiting the enzyme. Wurzburger and Musacchio (1971) have found that bovine adrenal tyrosine hydroxylase is a cytoplasmic enzyme, not granule bound, and as such is available to the free noradrenaline pool for feedback control of its biosynthesis at the rate limiting step.

In the physiological situation, catecholamine synthesis is not influenced by variations in the tissue levels of tyrosine. The K_m of the enzyme is 1×10^{-5}M and the tissue level of tyrosine is about 5×10^{-5}M (Chirigos et al.,1960). In man, the fasting plasma level is about 6.7×10^{-5}M which does not vary in a variety of pathological states (Waalkes and Udenfriend,1957). Spector et al.(1965) have, however, shown that tyrosine hydroxylase inhibitors do lower brain levels of noradrenaline.

Storage of the synthesised Noradrenaline

In 1953, both Blaschko and Welch and Hillarp et al. showed that, in the adrenal medulla, catecholamines are stored in dense granules which are separable from other intracellular components by fractional centrifugation of suitably prepared tissue homogenates. The catecholamines may be released from these granules by treatment with acid or other agents to disrupt the structure of the granule. Similar granules have been obtained from sympathetic nerve terminals (Euler and Hillarp, 1956; Potter and Axelrod, 1962).

The granules are made up of the catecholamine, ATP, a non-enzyme protein (a chromogranin), calcium ions and the final enzyme in the biosynthesis, dopamine-β-hydroxylase. Musacchio et al.(1965) have studies the binding of a number of sympathomimetic amines in the granules, and found that all three hydroxyl groups and the amino-group of noradrenaline are essential for its storage. A number of other amines were tested, the ratio of bound to free being measured, the order being noradrenaline (catechol and β-hydroxyl groups)>dopamine (catechol only)>octopamine (p-hydroxyl and β-hydroxyl). Tyramine (p-hydroxyl only) was not bound. Weiner and Jardetzky (1964) have reported that the β-hydroxyl group is essential to the amine-ATP interaction involved in granule structure.

Release of Noradrenaline

The first unequivocal demonstration of the release of noradrenaline from sympathetic nerves on stimulation was by Peart (1949) and since then many workers have shown this using a variety of techniques. The actual mechanism of noradrenaline release, however, remains very obscure. Stimulation of the nerve causes an action potential to pass down the axon which, when it arrives at the terminal depolarizes the membrane. The catecholamine stored in the granule is then released by a process of exocytosis (reverse pinocytosis) in which the contents of the vesicle are emptied into the synapse, breaking up as this occurs to free the noradrenaline. The process does not derive energy from ATP hydrolysis (Douglas and Poisner, 1966b). In addition to the amine, other components of the granule are released:

Douglas and Poisner (1966a) have reported that ATP and its metabolites are released, and Viveros et al. (1968) (using adrenal medulla) and Gewirtz and Kopin (1970) (using sympathetic nerves) have both shown the release of dopamine-β-hydroxylase. In very recent work, Poisner and Bernstein (1971) have suggested the involvement of microtubular structures seen in the cell wall of the nerve ending in the release of noradrenaline by participating in the exocytosis of the granule.

Action of Noradrenaline

The stimulation of the sympathetic nerve brings about many marked characteristic physiological responses which are evoked by the noradrenaline released by nerve stimulation. This passes across the synapse and acts on receptor areas of the postsynaptic cell membrane by depolarizing them. Ahlquist (1948) has classified the receptors by the effects resulting from their stimulation.

Inactivation of Released Noradrenaline

After the noradrenaline which has been released has depolarized the postsynaptic membrane, it must be inactivated so that the stimulation of the effector cell is terminated. This can occur in two ways:

(i) Metabolic degradation of the amine
(ii) Reuptake of the catecholamine into nerve endings

(i) Metabolic Degradation

There are two principal routes for the enzymic inactivation of noradrenaline, which overlap to some extent. Blaschko et al.(1937) describe an enzyme which destroyed a number of amines including adrenaline and noradrenaline. Richter (1937) termed this enzyme amine oxidase, and found that the amines were degraded to the corresponding aldehyde. Later work showed that the enzyme is located in mitochondria and is widely distributed throughout mammalian tissue, being especially abundant in liver and CNS. Collins et al.(1970) describe at least four different isoenzymes in human brain. The enzyme is now termed monoamine oxidase (MAO).

After deamination, the aldehyde produced is further metabolised either by oxidation to the corresponding acid, reduction to the corresponding alcohol or by further side chain breakdown to a benzoic acid derivative. These acid and alcohol metabolites are excreted both free and as conjugates with glycine, glucuronic acid or sulphuric acid as appropriate (Axelrod,1966).

The alternative pathway for catecholamine inactivation is methylation of one of the phenolic hydroxyl groups by the enzyme catechol-O-methyl transferase (COMT) (Axelrod,1957). The methyl group is donated by the high energy compound, S-adenosylmethionine, and the urinary metabolites are methylated almost exclusively in the meta-position.

As stated, these two pathways of catecholamine inactivation may overlap, methylated metabolites being deaminated and vice versa. Fig.1.2. shows the major routes of noradrenaline metabolism as described by Axelrod (1966).

These pathways provide very efficient inactivation of the adrenergic neurotransmitter, but the principal mode of catecholamine inactivation is reuptake in to the nerve ending.

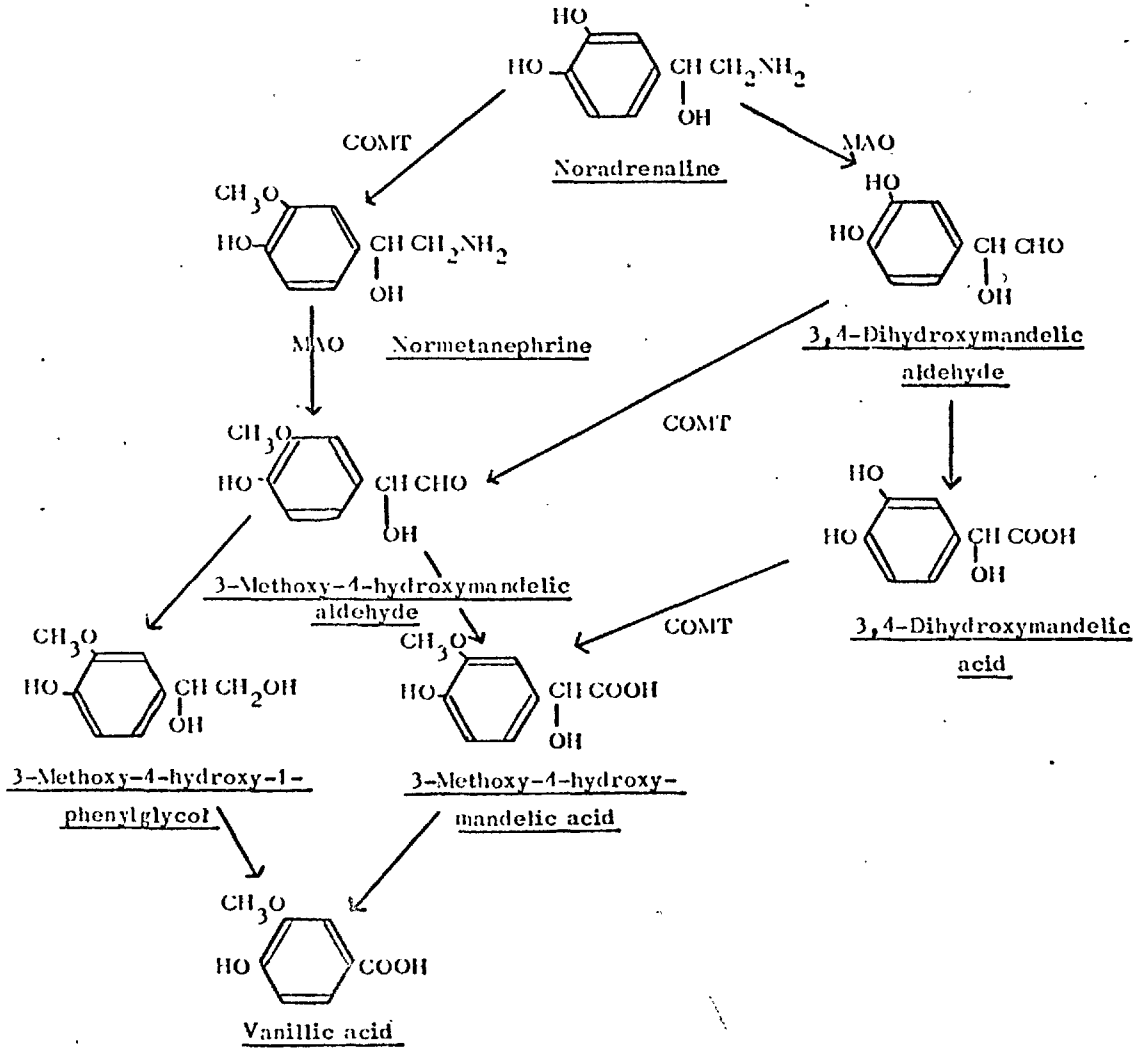
(ii) Uptake of Noradrenaline into the Nerve Endings

In 1932, Burn suggested that exogenous catecholamines could be taken up in storage sites in peripheral tissues, and Raab and Humphreys (1947) demonstrated an increase in the catecholamine content of cat and dog heart after injection of adrenaline and noradrenaline. Axelrod et al.(1959) were the first to show that tissue uptake was an important factor in the disposition of small doses of catecholamine, some 70% of a dose being metabolized quickly, largely by methylation, and 30% taken up into those tissues with extensive sympathetic innervation. Since these experiments, many workers have found that the process of uptake is carried out by most sympathetically innervated tissues in a wide range of species (see Iversen, 1967).

This uptake proceeds against a concentration gradient, the ratio of accumulated noradrenaline to that in the external medium being greater than

Fig 1.2 The Main Pathways of Noradrenaline Breakdown

after Axelrod, 1966



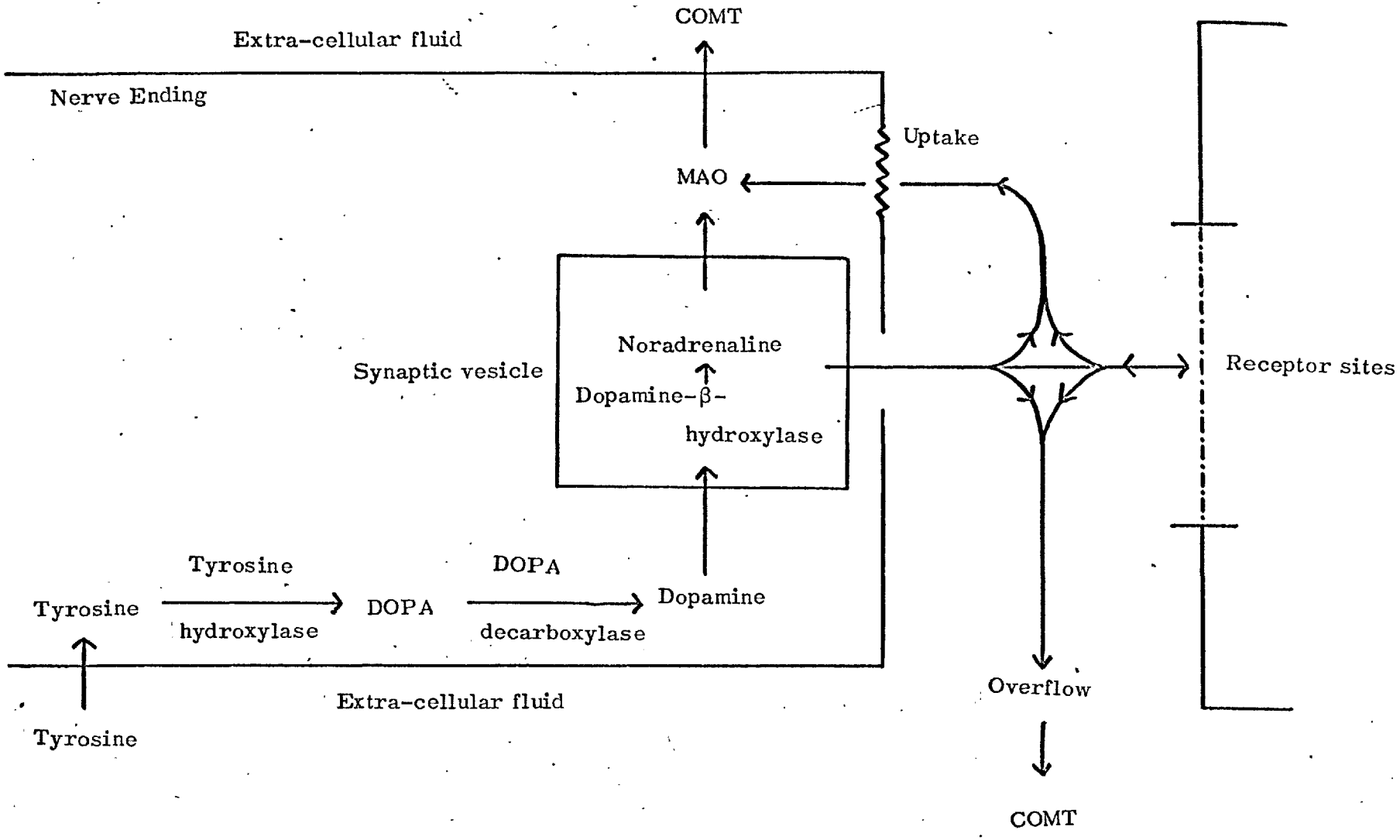
10000:1. Dengler et al.(1962) were the first to suggest that this uptake was mediated by a saturable membrane transport process and applied Michaelis-Menten kinetics to it. They were able to determine V_{max} for the process and the K_m for the uptake site-noradrenaline complex. Iversen (1963) showed that this uptake was stereospecific, K_m for the (-)-antipode being 5 times less than for the (+)-isomer.

After reuptake, the noradrenaline which has entered the adrenergic terminal is found both in the cell sap and in a microsomal fraction which also contains the endogenous catecholamine storage granules, first shown by Campos and Shideman (1962). Fischer and Iversen (1966) found that, in cat spleen, ^{3}H-noradrenaline taken up from the circulation is released preferentially over the endogenous stores, the released amine having a higher specific activity than that stored in the tissues.

These two processes enable the fate of released noradrenaline to be described fully. Noradrenaline in the synaptic cleft can be either taken up again by the nerve terminal or else it finds its way into the circulation and hence to the liver where either or both COMT and MAO contribute to its breakdown. The noradrenaline taken up is either stored in the granules and thus available for release again or is in a free pool in the cell sap. This free pool is open to attack by the nerve mitochondrial MAO, and the products of deamination pass out of the neurone to be excreted, after further metabolism and conjugation in most cases. Reuptake is the primary process of catecholamine inactivation and initially metabolic degradation is of lesser importance in the real physiological state. Fig.1.3 illustrates the physiology and biochemistry of the sympathetic nerve ending as discussed here.

Fig 1.3 Diagrammatic Representation of the Adrenergic Nerve Terminal

from Geffen et al , 1970



The Mode of Action of Methamphetamine

The precise mechanism of action of the amphetamine group of drugs on the adrenergic neurone has been the subject of much investigation in the past fifteen years. Most of the conclusions drawn are based on work performed using amphetamine, but some work has been done on methamphetamine and the modes of action appear to be very closely related, as would be expected. Four main hypotheses have been advanced, all based on the assumption that the effects of noradrenaline within the CNS are excitatory, in the same way as in the peripheral nerves.

(a) Monoamine Oxidase Inhibition

Blaschko (1938) found that the phenylisopropylamines inhibit MAO, the intraneuronal enzyme which is able to inactivate noradrenaline. It has been suggested that this inhibition causes an accumulation of the endogenous transmitter, which causes the stimulatory effect. It is known that the (+)-isomer of amphetamine causes more CNS excitement than the (-)-form (Schulte et al.,1941) and the MAO inhibition shows a small but similar, stereospecificity.

(b) Liberation of Noradrenaline

Moore (1963,1964) found that amphetamine depleted brain noradrenaline levels and that the (+)-antipode was 3-5 times more effective than the (-)-form; this ratio is the same as the ratio of the CNS potency of the two isomers. Glowinski and Axelrod (1965) similarly found that amphetamine was able to displace ^{3}H-noradrenaline from the brain, after it had been given by intraventricular injection.

(c) Direct Action on Noradrenaline Receptors

Van Rossum et al.(1962) found that amphetamine exerted its behavioural effects in animals whose noradrenaline stores had been depleted with reserpine, and postulated that the drug was acting directly on the noradrenaline receptors. However, if repeated doses are given the effects

should stay the same, but they do, in fact, diminish (Burn and Rand,1958). Glowinski and Axelrod (1965) found evidence for the existence of two separate noradrenaline pools within the nerve ending, one amphetamine-sensitive and the other reserpine-sensitive, which would explain the inability of reserpine to remove completely the effects of amphetamine.

(d) Inhibition of Uptake

As has been outlined previously, the noradrenaline uptake process is the prime mode of inactivation at the adrenergic nerve endings and inhibition of this process by drugs may be expected to prolong and potentiate the actions of the adrenergic transmitter. As the noradrenaline-containing neurones in the CNS may be considered functionally identical with those in the periphery then drugs inhibiting uptake will have potent central effects. Among many known inhibitors of the uptake process are a variety of the amphetamine group of drugs, listed in the following table, taken from Iversen (1967).

Drug	Conc. for 50% inhibition of noradrenaline uptake, in moles x 10^{-7}
(+)-amphetamine	1.8
(±)-amphetamine	4.6
(-)-amphetamine	37
(+)-methamphetamine	6.7
(±)-norephedrine	20
(-)-ephedrine	22

Drugs inhibiting the uptake of noradrenaline into neurones may be expected to produce an increase in the O-methylated metabolites of noradrenaline and a decrease in the deaminated products, due to the noradrenaline not being open to attack by the intraneuronal MAO (Rutledge,1970). This is the picture seen after amphetamine, but in this case the decrease in deaminated noradrenaline metabolites is so great that it resembles the picture seen after MAO inhibition. As previously mentioned, the amphetamines have long been known to be potent "in vitro" inhibitors of MAO, and

Iversen et al.(1966), for amphetamine, and Cook and Schanberg (1970) for methamphetamine, have suggested that the inhibition of MAO as well as noradrenaline uptake plays an important role in the mode of action of these drugs at the molecular level.

The amphetamines illustrate the extreme complexity of the actions of drugs on nerve physiology and biochemistry. It is an oversimplification to conclude that, because a compound can be shown to have a specific effect on nerve function, this will explain the pharmacological actions of the drug on nerve fibres. The amphetamines have several distinct actions on adrenergic fibres, and it would be premature at present to decide which, if any, of these actions is of importance in explaining their mode of action.

Pharmacology

Methamphetamine has marked effects on those body functions controlled by the sympathetic nerves, and has a potent action on the CNS and the psyche. Its pharmacology may be conveniently considered under two headings, (i) peripheral and (ii) central actions.

Peripheral Actions

Hauschild (1938,1939) showed that methamphetamine is a potent pressor agent in cats and dogs. With increased dose, the rise in blood pressure is more prolonged, and on repeated administration, tachyphylaxis occurs. The activity of the two isomers is identical. Derouaux (1942) showed that the pressor effect is due to peripheral vasoconstriction, and that methamphetamine reduced the bleeding time in rabbits. Allen (1948) found that methamphetamine increased the systolic blood pressure but left the diastolic unchanged, and Churchill-Davidson and Swan (1952) found that it increased cardiac output.

Novelli and Tainter (1943) noted that methamphetamine caused a persistent hyperventilation, and Graham et al.(1945), using a perfused rabbit lung preparation, showed this to be due to bronchodilation.

Churchill-Davidson et al.(1951)found that methamphetamine increased the secretion of urine due to raised renal blood flow. Methamphetamine is known to increase blood sugar and free fatty acids, and to mobilize stored glycogen, all characteristics of sympathetic stimulation (see review by Ellis, 1956).

Central Actions

Methamphetamine is a very potent CNS stimulant in man and animals, and the ratio of CNS to peripheral actions is higher than for amphetamine.

Hauschild (1938) found that methamphetamine increased both motor activity and facility of maze learning by both mice and rats, and noted that the (+)-isomer was the more potent. He also reported the hyperthermic effect of methamphetamine, which is probably mediated through a central site of action, like amphetamine (McCullough et al.,1970).

Animal experiments provide only a partial indication of CNS actions in man, as these are principally on the psyche. In patients, the effects of methamphetamine vary with the personality and the mental condition. The principal effects of the normal dose are wakefulness, alertness, decreased fatigue and mood elevation with an increase in confidence and concentration. Elation and euphoria are often seen, with increases in motor and speech activity. Performance of simple tasks is improved, although increases in rats are not always associated with increases in quality. Overdosage and/or repeated administration will cause a great variation in these effects (Wiess and Laties, 1962).

Not all subjects respond in the above way, as many experience headache, palpitations, dizziness, agitation, delirium, vasomotor disturbance or confusion (Wiess and Laties,1962).

The appetite is markedly depressed by methamphetamine and weight losses are observed, this being caused by a drop in food intake rather than increased metabolic activity. However, tolerance is developed to this

effect very rapidly, and in man dietary restriction is required for useful anorexia (Goodman and Gilman,1965).

Therapeutic Uses

Methamphetamine has found many therapeutic uses which are best looked at under the two headings used previously.

Peripheral Actions

Methamphetamine was used in sprays for the relief of nasal congestion and bronchial asthma, but this was discontinued in 1955 by law after excessive non-medical use. Methamphetamine can be used for the maintenance of blood pressure in spinal infiltration anaesthesia.

Central Actions

These are the basis of the major therapeutic applications of methamphetamine. It may be used to treat narcolepsy and for the relief of rigidity in Parkinsonism. This disorder is thought to be due to a lesion of the dopaminergic neurones in the basal ganglia of the brain, and it is of interest to note that the (-)-isomer of amphetamine is equipotent with the (+)-form with respect to dopamine uptake inhibition (Coyle and Snyder,1969). This is in contrast to the other central actions of amphetamine which exhibit marked stereospecificity for the (+)-antipode. Methamphetamine has been used as an anorectic, as noted previously, and the dose regime must be carefully constructed to avoid insomnia.

The use of methamphetamine in the treatment of mental disease has been widespread, ranging from attempts at the relief of mood disorders and nervous exhaustion to schizophrenia and depression in manic-depressive psychoses.

Medical opinion now opposes the use of such drugs due to the rapid production of tolerance and the high liability for psychic dependence. The drug has been restricted to hospital use in the U.K. since September 1968

and its use is opposed by all responsible professional opinion (Dunlop,1970).

The central effects are, however, the reason for the main interest in this drug as they give it its very high abuse potential. In the Spanish Civil War and World War 2, the amphetamines were used first by the Germans and then by Allied forces to combat battle fatigue, and by the early 1950's the illegal use of the amphetamines was well established. They were at that time available without prescription, but despite tighter and tighter legal controls, the non-therapeutic use of these drugs is still prevalent throughout the world. A fuller discussion of this problem is in Chapter 6.

The toxic effects of large single doses are extensions of the effects enumerated previously, acute renal failure, hyperthermia giving rise to high fever, cardiovascular collapse and death due to cerebrovascular accident (Zalis and Parmley,1963).

The Metabolism of the Amphetamines

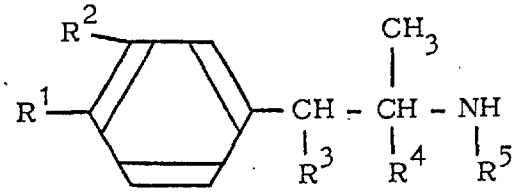
Metabolism is one of the most important factors influencing the pharmacology and toxicology of a foreign compound. Much interspecies variation occurs in the metabolism of drugs and great difficulties are met with in the extrapolation of toxicological data from animals to man.

The importance of the amphetamines drugs has been outlined, and in the last 15-20 years the metabolic fate of a number of such compounds has been investigated by a variety of workers. In only two cases, those of amphetamine and ephedrine, is there sufficient data for interspecies comparisons to be made, but this review also contains material on a number of drugs whose fate has been investigated in one or two species only. Structures of compounds discussed are shown in Table 1.1.

Amphetamine

Of all the drugs in this group, amphetamine is the one whose metabolism is best known, having been investigated by a number of workers in a wide variety of species. In addition, a good deal of "in vitro"

Table 1.1 Structures of Amphetamine Drugs Discussed in the Text



Compound	Substituent R^1	R^2	R^3	R^4	R^5
Amphetamine	H	H	H	H	H
Norephedrine	H	H	OH	H	H
Ephedrine	H	H	OH	H	CH_3
4-Hydroxyamphetamine	OH	H	H	H	H
p-Methoxyamphetamine	CH_3O	H	H	H	H
Fenfluramine	H	CF_3	H	H	C_2H_5
Mephentermine	H	H	H	CH_3	CH_3
Chlorphentermine	Cl	H	H	CH_3	H
Methamphetamine	H	H	H	H	CH_3
Ethylamphetamine	H	H	H	H	C_2H_5
N-3-chlorpropyl-amphetamine	H	H	H	H	$(CH_2)_3Cl$
Prenylamine	H	H	H	H	$(CH_2)_2CH(C_6H_5)_2$

work is recorded in the literature.

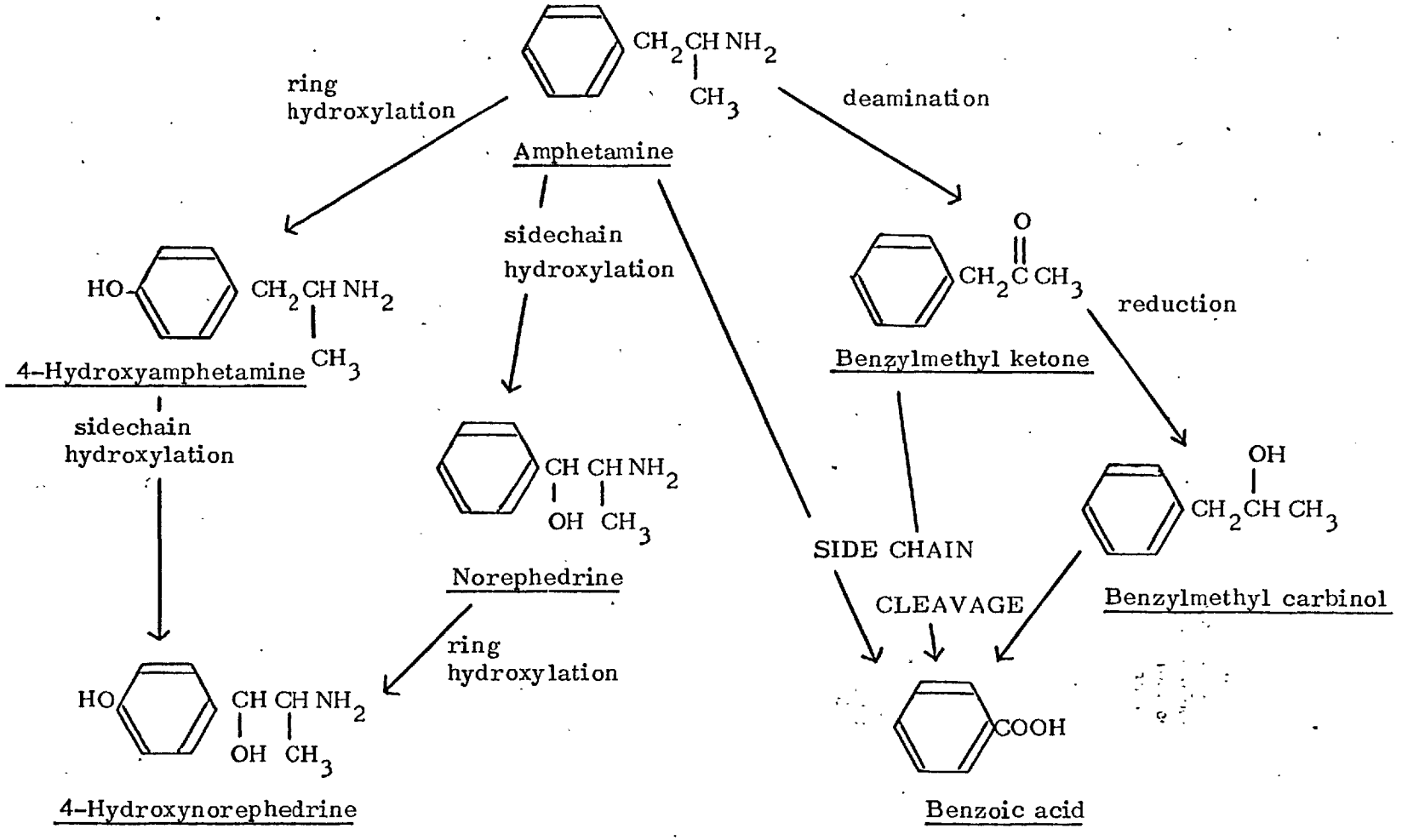
Fig.1.4 shows the principal metabolic pathways of amphetamine as determined "in vivo" and Table 1.2 shows their species distribution. The principal routes are two, namely ring hydroxylation to give 4-hydroxyamphetamine, and degradation of the sidechain to benzoic acid, excreted free and as conjugates. Table 1.2 shows that most species except the rabbit excrete significant amounts (15-30%) of the unchanged drug; that ring hydroxylation is at a low level in most species examined except the rat, and to a lesser extent the mouse; and that all species except the rat excrete considerable amounts of benzoic acid and/or its conjugates.

Amphetamine and 4-hydroxyamphetamine are both known to be substrates for dopamine-β-hydroxylase (Creveling et al.,1962), the terminal enzyme in noradrenaline synthesis, and 4-hydroxynorephedrine has been shown as a urinary metabolite of amphetamine in man (Cavanaugh et al.,1970) and in rat (Dring et al.,1970). Norephedrine is a metabolite of amphetamine in rat (Dring et al.,1970). In addition, both these metabolites have been found in rat heart and brain (Lewander,1971b) and in cat heart and spleen (Thoenen et al.,1966). The reaction is a minor one in all species studied.

The mechanism of sidechain breakdown to benzoic acid is uncertain. Dring et al.(1970) have noted the presence, in the urine of rabbits and certain other species after amphetamine dosage, of an acid- and alkali-labile precursor of benzylmethyl ketone. On the basis of n.m.r. and other data they have proposed the structure of this material to be the O-sulphate of the enol form of benzylmethyl ketone. Benzylmethyl ketone may also be reduced stereospecifically to the corresponding alcohol, which is excreted in some species as a glucuronide conjugate, and is known to be metabolized to benzoic acid by the rabbit (El Masry et al.,1956).

The mode of deamination is at present a topic attracting considerable attention. The reaction was first recorded in rabbit liver microsomes (Axelrod,1955a), and Brodie et al.(1958) postulated that amphetamine was

Fig 1.4 Metabolic Pathways of Amphetamine



All compounds, plus conjugates where appropriate, have been detected in urine after amphetamine dosage. Source and species distribution are shown in Table 1.2

Table 1.2 Species Distribution of Metabolic Pathways of Amphetamine

Data from Dring et al (1970). Figures given are % of dose excreted in that form, and represent total excretion, after hydrolysis where appropriate.

Species	Amphetamine	4-Hydroxy-amphetamine	Sidechain hydroxylation	Benzoic acid + conjugates	Benzylmethyl ketone	Benzylmethyl carbinol
Man	30	2	-	21	3	n.d.
Monkey	27	3	-	30	n.d.	n.d.
Dog	30	6	-	28	1.4	0.7
Rabbit	3	6	-	23	22	7
Rat	12	60	0.6*	3	n.d.	n.d.
Guinea pig	19	n.d.	-	65	n.d.	n.d.
Mouse	30	15	-	32	n.d.	n.d.

*Norephedrine 0.3%, 4-hydroxynorephedrine 0.3%. n.d. = not detected

first being hydroxylated on the carbon bearing the nitrogen, yielding an unstable carbinolamine which spontaneously lost ammonia to give the ketone. Smith and Dring (1970), as an alternative, suggested that there is an initial dehydrogenation to the imine, which would yield the ketone on hydrolysis.

Hucker et al.(1971) have found that the major metabolite of amphetamine in guinea pig liver microsomes is the oxime of benzylmethyl ketone. This ketoxime is known to hydrolyse readily to give the ketone, and this is suggested to be the route of deamination. Parli et al.(1971), using ^{18}O labelling, have found that both benzylmethyl ketone and the ketoxime are formed from amphetamine by rabbit liver microsomes, and have proposed the reaction sequence shown in Fig.1.5.

Dring (1968) has suggested that, in the rat, amphetamine may be N-acetylated to a very small extent, and Truhaut et al.(1969) have reported a transient acetylation in rabbits, but the occurrence of this pathway has not yet been confirmed satisfactorily.

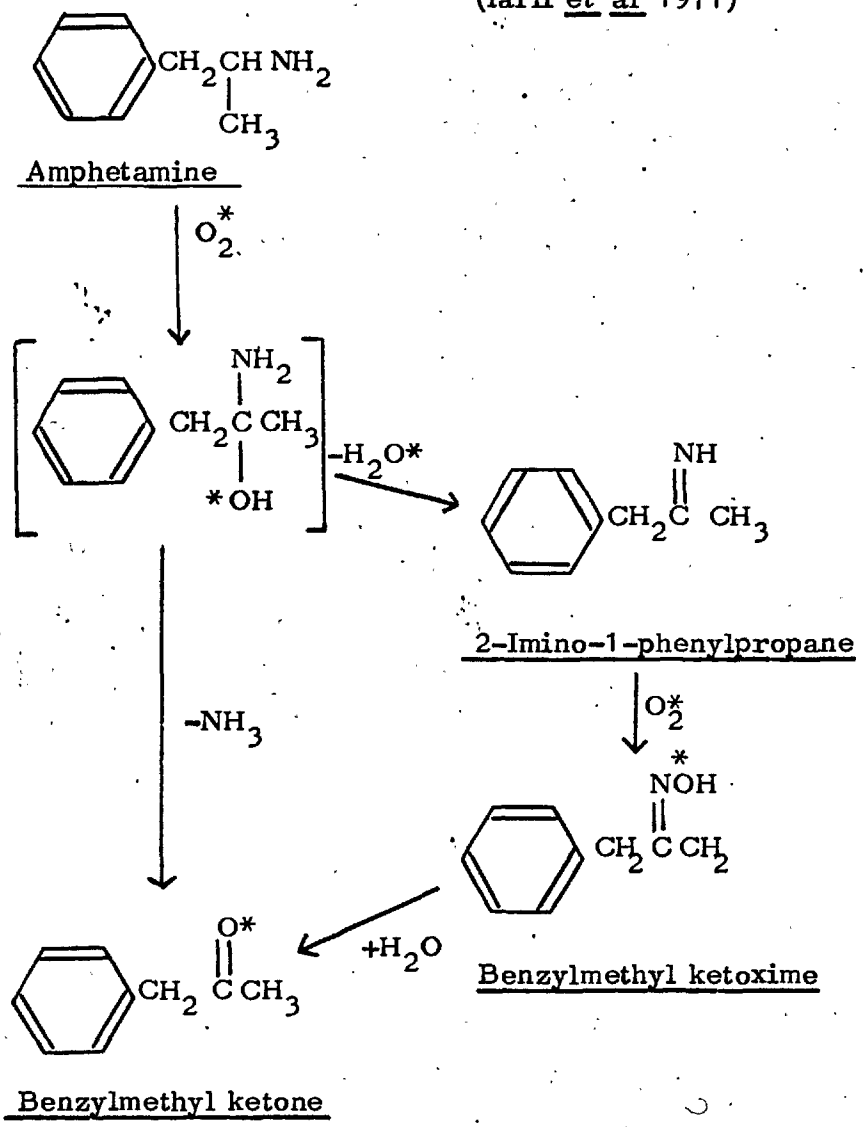
Aromatic Hydroxylation of Amphetamine

The enzyme system which carries out the aromatic hydroxylation of amphetamine has several characteristics notably different from other drug hydroxylation mechanisms. Daly et al.(1967) and Dingell and Bass (1969) have both found it impossible to obtain rat liver microsomes to hydroxylate amphetamine, although in the isolated perfused liver the reaction proceeds as in the whole animal. Creaven et al.(1970) have investigated the hydroxylation and found that while it is inhibited by the usual inhibitors of drug metabolism like SKF-525A, it is not induced by pretreatment with phenobarbitone or benzo[a]pyrene; failure to induce with 3-methylcholanthrene has also been reported (Groppetti and Costa, 1969). It is uniquely inhibited by ethanol treatment "in vivo" (Creaven and Barbee,1969) which has no effect on acetanilide or biphenyl hydroxylation.

Fig. 1.5 Microsomal Oxidation of (+)-Amphetamine

Proposed reaction sequence in rabbit liver microsomes

(Parli et al 1971)



* Indicates ^{18}O labelling

Ephedrine

The fate of ephedrine, an alkaloid from Ephedra spp. plants has been investigated in a range of species and Fig.1.6 shows the metabolic routes "in vivo", and Table 1.3 their species distribution. The main metabolic reactions are N-demethylation, the extent of which is species dependent, ring hydroxylation, which follows the same species pattern as for amphetamine and side chain degradation, which is less important than for amphetamine. The rabbit is the only species in which the unchanged drug and/or its demethylated metabolite, norephedrine, are not the major metabolites. The β-hydroxyl group in the side chain renders the molecule much more water soluble than the corresponding deoxy compound, (Vree et al.,1969) and it has been suggested that the possession of this group is the reason for the relative stability of these drugs to metabolism. It is interesting to note that Nagase et al.(1967) found that only the (-)-isomer was metabolised by the rat, the (+)-form being resistant. Bralet et al.(1968) have investigated the fate of (±)-ephedrine in the rat, and have found that the racemic compound is biotransformed in a very similar way to the (−)-antipode.

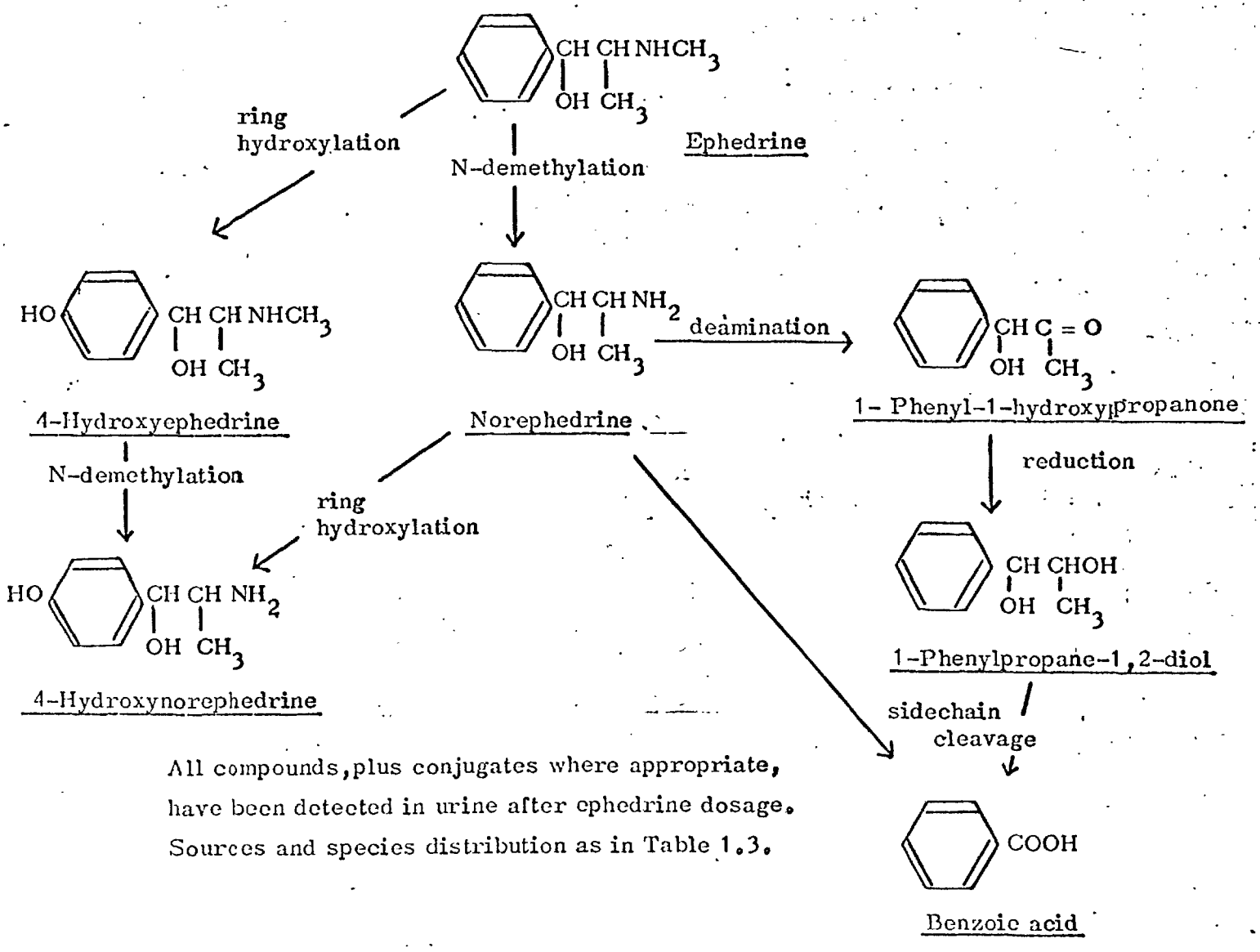
Using a ^{14}C label, the fate of (-)-ephedrine in rabbit liver microsomes has been investigated (Baba et al.,1969), the metabolites being shown in Fig.1.7. The occurrence of the ketol and diol metabolites is of interest, as these are urinary metabolites of ephedrine in the rabbit and pony, and show that deamination is a significant metabolic pathway for this drug as well as for amphetamine, at least in the se two species.

Ring Substituted Amphetamines

A wide variety of ring substituted amphetamines has been synthesised and tested pharmacologically, but the metabolism of very few has been investigated.

The β-hydroxylation of 4-hydroxyamphetamine to 4-hydroxynorephedrine has been observed in 2 species; in rat (Lewander,1971a) without quantification, and in man, Sjoerdsma and von Studnitz (1963) found 4-9% of a dose hydroxylated in the sidechain.

Fig. 1.6 Metabolic Pathways of (-)-Ephedrine



All compounds, plus conjugates where appropriate, have been detected in urine after ephedrine dosage. Sources and species distribution as in Table 1.3.

Table 1.3 Species Distribution of Metabolic Pathways of (-)-Ephedrine

Sources as below. Figures given are % of dose excreted in that form, and represents total excretion, after hydrolysis where appropriate.

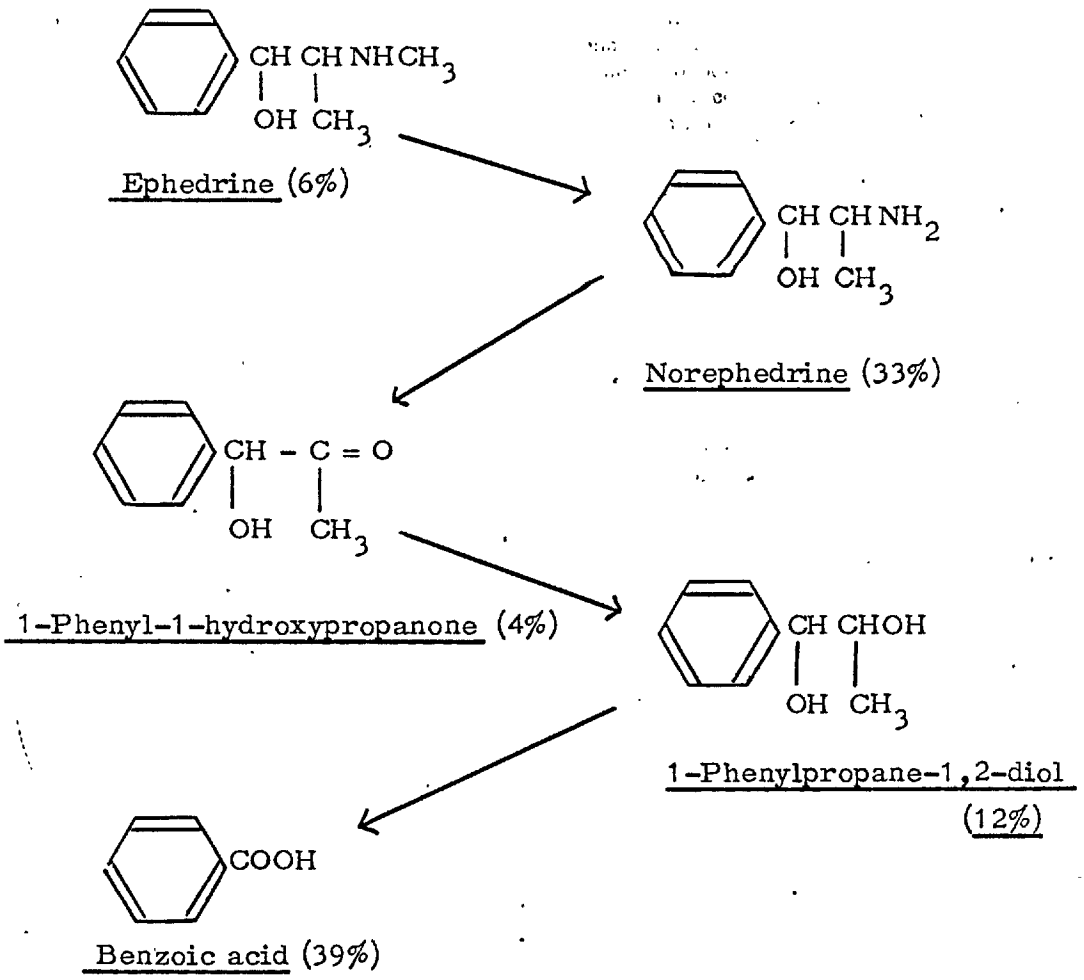
Species	Ephedrine	Norephedrine	4-Hydroxy-ephedrine	4-Hydroxy-norephedrine	1-Phenyl-1-hydroxy-propanone	1-Phenyl-propane-1,2-diol	Benzoic acid + conjugates	4-Hydroxy-benzoic acid + conjugates
Man (a)	80	10	-	-	-	-	-	-
Pony (b)	n.d.	29	3		-	19	-	-
Dog (c)	6	58	0.3	1	-	-	-	-
Rabbit (c,f)	0.5	2	1	-	1	29	43	19
Guinea pig (c)	2	39	1		-	-	-	-
Rat (c,d,e)	38	5	2	12	-	-	3	-

n.d. = not detected

Sources (a) Wilkinson and Beckett (1968), (b) Nicholson (1970, 1971), (c) Axelrod (1953), (d) Nagase et al, (1967), (e) Bralet et al (1968) using (±)-ephedrine, (f) Matsuda et al, (1971).

Fig 1.7 Metabolism of (-)-Ephedrine by Rabbit Liver Microsomes

(from Baba et al 1969)



Figures given are % of ^{14}C in that form after incubation

The hallucinogen p-methoxyamphetamine is extensively metabolised to unknown compounds by man, only 3-12% of an oral dose being excreted unchanged (Schweitzer et al.,1971).

Ho et al.(1971) have investigated the fate of 2,5-dimethoxy-4-methylamphetamine, the hallucinogen STP or DOM, in rats after i.p. injection. In this species the principal urinary metabolites are the acid and alcohol resulting from the oxidation of the 4-methyl group (see Fig.1.8) both free and as conjugates, and there is also a significant faecal excretion, the metabolites in the faeces also being shown in Fig.1.8.

The metabolism of the widely used anorectic fenfluramine has been partially investigated in man and dog. Beckett and Brookes (1967) were able to show N-deethylation in man, but the unchanged drug and its metabolite accounted for at most 20% of the dose. Bruce and Maynard (1968) showed in addition to the drug and desethylfenfluramine, m-trifluoromethylhippuric acid as the major urinary metabolite in man. They were able to account for virtually all of the dose as these three compounds, and the great accent on sidechain breakdown in man resembles the trend seen with amphetamine, with ring hydroxylation being interfered with by the bulky substituent on the aromatic ring. The same authors, in a non-quantitative study in a dog, found in addition to the three compounds found in man a fourth metabolite, m-trifluoromethylbenzoic acid.

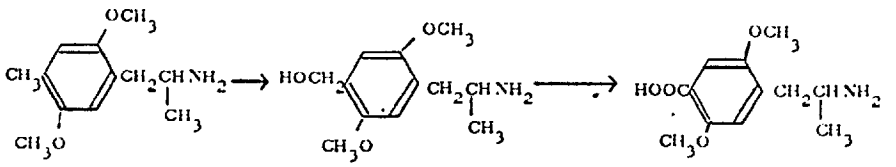
Sidechain Methylated Amphetamines

The addition of a second α-methyl group to the sidechain of amphetamine gives the nucleus of the phentermine compounds, some of which have been used clinically as anorectics. Of these the fate of p-chlorophentermine (chlorphentermine) and N-methylphentermine (mephentermine) have been investigated.

Walkenstein et al.(1955) report that, after a dose of [^{14}C]-mephentermine to dogs, rats and rabbits, no unchanged drug appears in the urine. Two metabolites, phentermine and p-hydroxyphentermine, were found, and their

Fig 1.8 Metabolites of STP in Rat Urine and Faeces

(after Ho et al., 1971)



1-(2,5-dimethoxy-4-methyl phenyl)-2-aminopropane (STP, DOM) I

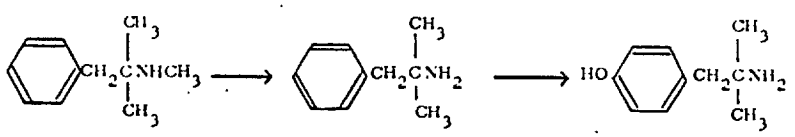
1-(2,5-dimethoxy-4-hydroxy methylphenyl)-2-aminopropane II

1-(2,5-dimethoxy-4-carboxyphenyl)-2-amino-propane III

	% of dose as that metabolite in Urine	Faeces
I	5.0	tr
II free	16.8	1.3
sulphate	11.8	0.3
glucuronide	2.3	-
III	17.4	16.8
total metabolites	53.3	18.4
total ^{14}C excreted	62.7	20.2

Fig 1.9 Metabolites of Mephentermine in Urine of Various Species

from Walkenstein et al., 1955



Mephentermine I

Phentermine II

4-Hydroxyphentermine III

% of dose as that metabolite in urine

	I	II	III
Dog	0	16	28
Rat	0	13	32
Rabbit	0	22	5 + 1 unknown

structures and the quantitative data are shown in Fig.1.9. In all three species, only 50% of the dose is excreted in the urine, with 30% in the faeces.

Dubnick et al.(1968) have investigated the metabolism of chlorphentermine in rats and mice. Both species excrete nearly all the drug in 24h, in the rat principally as the unchanged material but in the mouse some 15% as a novel conjugate in addition to the unchanged drug. The conjugate is not N-acetyl or N-glucuronosidochlorphentermine. The excretion of the unchanged drug in the rat is probably due to the chlorine in the aromatic ring blocking ring hydroxylation, the prime mode of metabolism of these compounds in the rat.

N-Alkylated Amphetamines

The N-alkylation of the amphetamine nucleus is a commonly used molecular modification to alter the pharmacology of the drug (see Boissier et al.,1970) and the metabolism of four such compounds has been investigated "in vivo".

Beckett and Rowland (1965a) and Gunne (1967) have both found that, in man, N-methylamphetamine (methamphetamine) is excreted unchanged and is demethylated to amphetamine. In 24h, some 23-45% of a dose is excreted as methamphetamine and 2-4% as amphetamine. Axelrod (1954) showed that, in the dog, methamphetamine is demethylated to amphetamine which is then ring hydroxylated to 4-hydroxyamphetamine, excreted free and as its glucuronide conjugate.

Beckett et al.(1969) report that N-ethylamphetamine is excreted by man both unchanged and as its deethylated metabolite amphetamine after oral dosage. In 24h, with normal urine pH, 13-24% of the dose is recovered unchanged and 4-10% as amphetamine.

In the same paper, the authors have correlated data for the excretion and metabolism of the enantiomers of amphetamine, methylamphetamine and ethylamphetamine in man and conclude that while there are only small

differences in the elimination and metabolism of the isomers of amphetamine (see also Gunne,1967) these differences become more marked on N-alkyl substitution. N-alkylation increases the extent of metabolism of the (+) but not the (-)-isomer, and the larger the alkyl group, the greater the relative metabolism of the metabolically susceptible (+)-form.

Long (1970), in investigating the metabolism of N-3-chloropropylamphetamine (Pondinil, Roche) in a range of species, found that it was almost completely metabolized in man, rat and the guinea pig, and that in dog and rabbit only 17% of the dose was unchanged. The extent of 4-hydroxylation as compared with amphetamine was increased in all species except the rat, where it was already high, and the rabbit. The results are:

	Unchanged Drug	4-Hydroxylated Metabolites
Man	<1	37
Dog	17	22
Rat	<1	58
Rabbit	18	6
Guinea pig	3	15

With this N-3-chloropropyl substituent, the extent of ring hydroxylation in man, dog and guinea pig is markedly increased over amphetamine itself, and the total amount of metabolism, as reflected in the excretion of unchanged drug, is increased in all species except the rabbit.

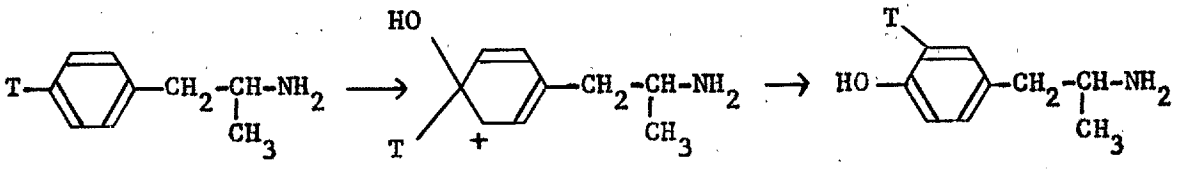
Palm and Grobecker (1968) and Palm et al.(1968) report the formation of amphetamine, norephedrine and their 4-hydroxy analogues as metabolites of prenylamine (N- 3'-phenylpropyl-(2')1,1-diphenylpropyl amine) in man and rat. The rat also produces traces of 4-hydroxyprenylamine, both free and conjugated, in the urine. Neither report quantifies the metabolites or excretion but it appears that extent of metabolism along these routes is small in both species.

From these papers, then, it is apparent that N-substitution has a

profound effect on the metabolism of amphetamines. As would be expected, all species examined are able to N-dealkylate, giving amphetamine, which is a metabolic option obviously not open to other substituted amphetamines. In addition, the hydroxylation of N-3-chloropropylamphetamine as compared with amphetamine appears to be enhanced in man. The amount recovered as amine metabolites (unchanged and as amphetamine) after methamphetamine and ethylamphetamine decreases with size of alkyl group, so that N-alkylation appears to emphasise other metabolic pathways such as hydroxylation and sidechain cleavage.

N.I.H. Shift

The hydroxylation of aromatic compounds is a topic which has received a great deal of attention over the last 15 years, and studies using specific deuterium and tritium labelling have revealed what is apparently a fundamental feature of these hydroxylation reactions. These experiments show that as a frequent consequence of hydroxylation there occurs an intramolecular migration of the atom or group displaced by the new hydroxyl function to an adjacent position on the aromatic ring. This phenomenon has been termed the "N.I.H. Shift" by Guroff et al.(1967) and is in marked contrast to the classical ideas of hydroxylation as a direct replacement of a ring substituent by a hydroxyl group. Among a number of reactions exhibiting the N.I.H. Shift, Daly et al.(1967) found that 4-tritioamphetamin was converted to 3-tritio-4-hydroxyamphetamine.80-90% retention of tritium by rats,both "in vitro" and "in vivo", was observed.



The diagram shows the suggested intermediate.

Similar migrations have been observed in the hydroxylations of phenylalanine and tyrosine.

Besides giving a great deal of insight into the mechanism of the enzymic hydroxylation of amphetamine itself, the almost general occurrence of this reaction will prove useful in predicting metabolites of ring substituted amphetamines, notably the halogenated compounds now being used in pharmacological investigations.

Interactions between Amphetamine and Other Drugs

Drugs may interact with one another in a number of ways, and one of the most important of these is at the metabolic level.

Amphetamine-barbiturate mixtures have been used for many years in psychiatry for the treatment of anxiety and depressive neuroses. These combinations usually contain dexamphetamine sulphate and amylobarbitone, the best known being Smith, Kline and French's "Drinamyl", also known as "purple hearts". In the past, there have been four mixtures using methamphetamine with a barbiturate, but these have now been discontinued. There has been a great deal of official discouragement of the prescribing of these mixtures, but large quantities are still dispensed (Dr. P.S. Sever, personal communication).

Although amphetamine and the barbiturates have very different pharmacological effects in man and animals, when given together they can be "mutually corrective", the side effects antagonizing each other, leaving the principal effects unchanged (Myerson,1939). Although meaning that the mixture is valuable in psychiatry, the problem of dependence and the abuse potential of both agents is increased. As well as amphetamine habituation, the possibility of barbiturate dependence (Eddy et al.,1965) is introduced.

When single doses of amphetamine and a barbiturate are given together, the drugs antagonize each others metabolism. Klissiunis et al.(1965) gave amphetamine and pentobarbitone together and found that the blood level of pentobarbitone was elevated compared with controls. Similarly, Dring (1968) gave amphetamine and phenobarbitone together and found that the blood level of amphetamine was elevated and that its metabolism was impaired.

However, it is known that the prolonged administration of barbiturates causes a non-specific induction of the activity of the drug metabolising enzymes of the liver (see Conney,1967) and it may be that repeated dosage with an amphetamine-barbiturate mixture will reverse the antagonistic effect of the drugs on each others metabolism and enhance the breakdown of both agents.

A number of workers have observed that various antidepressant drugs potentiate the effects of amphetamine on animal behaviour, and in several cases this has been shown to be due to the metabolism of amphetamine being inhibited by the antidepressant, thus prolonging the life of unchanged amphetamine in the body. In the rat, the only species examined so far, the hydroxylation of amphetamine is suppressed by these drugs. Among the compounds shown to act in this way are desmethylimipramine (Dingell and Bass,1969), iprindole and chlorpromazine (Lemberger et al.,1970a,b). The butyrophenone antidepressant haloperidol, however, has very little effect on amphetamine metabolism (Lemberger et al.,1970b).

Creaven and Barbee (1970) report that ethanol markedly inhibits amphetamine hydroxylation in the rat; other studies show that this is due to ethanol itself, not its metabolite, acetaldehyde (Creaven et al.,1970).

As it is well-known that drug abusers and sick patients use a wide variety of drugs, not just one, any metabolic interactions are of great significance. From the drug abuse point of view, these interactions are of profound importance as some of the compounds interacting with amphetamine in this way have potent CNS activity, and hence a high abuse potential.

Scope of the present work

It will be seen from the review of the literature that very little is known about the metabolism of methamphetamine, N-demethylation having been shown in man and ring hydroxylation and N-demethylation in the dog.

Accordingly, a detailed investigation of the fate of this compound in two laboratory animal species and man has been carried out. Only the racemic compound has been investigated.

In addition the biliary excretion of amphetamine and methamphetamine has been investigated in the rat, and following a major project from this laboratory (Dring et al.,1970), the metabolism of amphetamine in man has been re-examined, with reference to the sidechain hydroxylation of this compound.

CHAPTER TWO

MATERIALS AND METHODS

Chapter Two Materials and Methods

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Compounds

(±)-2-Methylamino-1-phenylpropane (methamphetamine) hydrochloride, m.p. 132-134°, was bought from K and K Labs., Plainview, N.Y., U.S.A. (±)-2-Amino-1-phenylpropane (amphetamine) sulphate, m.p. 302° (decomp.), (±)-2-amino-1-(4'-hydroxyphenyl) propane (Paredrine) hydrochloride, m.p. 190-192° and 1-(4'-methoxyphenyl) propan-2-one were the gifts of Smith, Kline and French Laboratories Philadelphia, U.S.A. (±)-2-Methylamino-1-(4'-hydroxyphenyl) propane (Pholedrine) sulphate, m.p. 296°, was the gift of Knoll A.G., Ludwigshafen, Germany. (±)-2-Amino-1-phenylpropane-1-ol (norephedrine) hydrochloride, m.p. 191-194°, was purchased from Sigma Chemical Co., St. Louis, Missouri, U.S.A. (±)-2-Methylamino-1-(4'-hydroxyphenyl) propan-1-ol (4-hydroxyephedrine) hydrochloride, m.p. 188-189°, was purchased from R.N. Emanuel Ltd., Wembley, U.K. (±)-2-Amino-1-(4'-hydroxyphenyl) propan-1-ol (4-hydroxynorephedrine) hydrochloride, m.p. 190-192°, was a sample prepared by Dring et al.(1970).

An authentic sample of anti-benzylmethyl ketoxime, m.p.63°, was the gift of Mr. J. Gorrod, Chelsea College, London, S.W.3. A mixture of syn- and anti-benzylmethyl ketoxime was prepared according to Hey (1930). Other compounds were purchased and purified as appropriate.

(±)-[1-^{14}C]Amphetamine Sulphate (see Blackburn and Burghard, 1965) of specific radioactivity 16.4 μCi/mg was a gift of Smith,Kline and French Laboratories.

(±)-2-Methylamino-1-phenyl-[1-^{14}C]propane

[Carboxy-^{14}C]Benzoic acid

This material (m.p. 121°; 3.6 μCi/mg) was prepared from Ba[^{14}C]CO_3 (Radiochemical Centre, Amersham, Bucks.) and phenylmagnesium bromide according to Dauben et al.,(1947).

[Carbonyl-^{14}C]Benzoyl chloride

The above acid (2.12g) was converted to the acid chloride by boiling under reflux with thionyl chloride, as described by Calvin et al.(1949). (Yield 2.08g).

[Carbonyl-^{14}C]Benzamide

This material was prepared by adding the acid chloride dropwise to ammonia solution (sp.gr.0.88; 10 ml) cooled in ice. The amide which separated was filtered off and recrystallized from water (yield 1.42g; m.p.129°).

Benzo[^{14}C]nitrile

The above amide was intimately mixed with phosphorus pentoxide (3g) and heated under reduced pressure, whereby benzo[^{14}C]nitrile (0.88g) distilled at 162°/20mm.

[Carbonyl-^{14}C]Benzaldehyde

The above nitrile was reduced with stannous chloride and hydrochloric acid, according to the method of Stephen (1925), giving the aldehyde (0.77g).

1-Phenyl-2-nitro-[1-^{14}C]prop-1-ene

The aldehyde in benzene (10ml) was treated with nitroethane (4ml) as described by Blackburn and Burghard (1965). (Yield 0.89g).

1-Phenyl-[1-^{14}C]-propan-2-one

The above nitro compound as an oil was converted, without further purification, to this ketone (0.34g) according to the general method of Pearl and Beyer (1951).

(±)-2-Methylamino-1-phenyl-[1-^{14}C]propane hydrochloride

The 1-phenyl-[1-^{14}C]propan-2-one in absolute ethanol (30ml) and an ethanolic solution of methylamine (35%^{w}/w; 20ml) were placed in the reaction vessel of a hydrogenator. Palladium - charcoal (10%; 2g) was added and the mixture hydrogenated at 45 p.s.i. for 24 h. The catalyst was filtered off, and the excess methylamine and ethanol were removed from the filtrate by distillation at reduced pressure. The residual oil was then treated with a dry saturated ethereal solution (5ml) of HCl gas. White crystals (0.42g) of (±)-2-methylamino-1-phenyl-[1-^{14}C]propane hydrochloride separated on standing. It had m.p. 134-5° and specific activity of 2.36 μCi/mg. (Woodruff *et al.*(1940) give the m.p. of (±) methamphetamine hydrochloride as 135-136°). The radiochemical yield based on the Ba $^{14}CO_3$ was 10%.

Determination of purity of synthesised [^{14}C]-methamphetamine hydrochloride

Chromatography (see Table 2.1) in systems A,B and C gave a single ^{14}C peak of R_f 0.56, 0.93 and 0.80 respectively. In addition to this, isotope dilution analysis was performed.

Accurately weighed samples of [^{14}C]-methamphetamine hydrochloride (ca.1mg) and unlabelled methamphetamine hydrochloride (1.245g) were dissolved in water (15 ml) and 10M-NaOH (5ml) added. *p*-Toluene sulphonylchloride (1.75g) was dissolved in acetone (6ml), added, and the mixture boiled under reflux for 20 min., cooled by pouring into ice (10g) and the resulting precipitate filtered off. This was recrystallized to constant specific activity from aqueous ethanol, m.p. 82-84°. This isotope dilution assay showed the [^{14}C]-methamphetamine hydrochloride synthesised to have a radiochemical purity greater than 99%.

The derivative formed, *N*-*p*-toluenesulphonyl-*N*-methylamphetamine, appeared to be a new compound, and an authentic sample was prepared by standard methods. It was a white crystalline powder, m.p. 82-84° from

aqueous ethanol (found: C,67.1; H,7.05; N,4.6; S,10.3% $C_{17}H_2NO_2S$ requires C,67.1; H,6.9; N,4.6; S,10.6%).

(+)-1-(4'-Hydroxyphenyl)-2-methylaminopropane

Anisaldehyde (50ml) was mixed, with nitroethane (36ml), n-butylamine (2ml) and piperidine (3ml) in benzene (100ml), and the whole refluxed on a water bath for 12h, using a Dean-Stark trap to collect evolved water (12ml). The benzene and unused reagents were removed by evaporation at reduced pressure and the resulting 1-(4'-methoxyphenyl)-2-nitro-prop-1-ene recrystallized from ethanol, m.p.51-52° (Alles (1932) gives 43-44°). This nitro compound was converted to 1-(4'-methoxyphenyl)propan-2-one by treatment with Fe and HCl using the method of Pearl and Beyer (1951). The ketone distilled at 128-132°/2mm and the i.r. spectrum was identical with that of an authentic sample. This ketone (15g) was placed in the vessel of a catalytic hydrogenator with an ethanolic solution of CH_3NH_2 (33%^{W}/v : 60ml) and Palladium - charcoal (10% : 2g) added. The mixture was hydrogenated at 45 p.s.i. for 24h with agitation. The catalyst was filtered off, and excess methylamine and ethanol removed from the filtrate by rotary evaporation. The residual oil was treated with a dry saturated ethereal solution (40ml) of HCl gas. White crystals of (+)-1-(4'-methoxyphenyl)-2-methylaminopropane hydrochloride separated, which were filtered off and recrystallized from 90% aqueous acetone, m.p. 173-175° (Woodruff et al.(1940) give 178-179.5°).

This base hydrochloride (2g) was sealed in a Pyrex glass tube with 10M-HCl (10ml), the tube placed in an iron bomb and heated at 140° for 2h. After cooling the tube was opened and the contents adjusted to pH9, saturated with solid NaCl and extracted with ether (3x50ml). The bulked extracts were dried over anhydrous Na_2SO_4 and the ether removed by evaporation under reduced pressure. This gave white crystals of (+)-1-(4'-hydroxyphenyl)-2-methylaminopropane, which were recrystallized from methanol, m.p.161-2° (B.I.O.S. Final Reports give 162-3°).

Animals

Female Wistar albino rats, body weight 220 ± 15g and female Duncan-Hartley guinea pigs, body weight 250 ± 15g, were used.

Human Volunteers

Two healthy human volunteers participated in the trials, aged 24 and 31 years, weighing 77 and 70 Kg respectively.

Administration of Compounds

[^{14}C]-Methamphetamine hydrochloride was administered orally by stomach tube as an aqueous solution to the rat, and by intraperitoneal injection in aqueous solution to rats and guinea pigs. Human volunteers took the compound, dissolved in water, by mouth.

Human volunteers took [^{14}C]-amphetamine sulphate by mouth in a similar way.

Collection of Excreta

The animals were kept singly in metabolism cages; food and water being withheld for the first 24h to avoid contamination of the urine. After the first day, free access to food and water was given. Urine and faeces were collected daily for 5 days.

The human volunteers kept a normal intake of fluids and solid food. Urine and faeces were collected daily for 4 days.

Storage of Excreta

All excreta were kept at -20°, without pH adjustment, except during analytical procedures.

Spectra

I.r. spectra were determined using a Perkin-Elmer Infracord spectrophotometer. Mass spectra were obtained using a Varian CH5 mass spectrometer, the conditions used being ion source temperature 220° and beam energy 70 eV. Compounds were admitted through the direct inlet system.

Radiochemical Techniques

^{14}C in urine and faeces were determined according to Bridges (1967) using Packard Tri-Carb liquid scintillation spectrometers (models 3214, 3320 and 3375). Radiochromatogram scanning of paper and thin layer chromatograms was performed with a Packard radiochromatogram scanner (model 7200), identification of ^{14}C peaks being made by comparison of R_f values with authentic compounds (see Table 2.1). The ^{14}C content of peaks was estimated by planimetry, and also by cutting paper chromatograms into 1cm segments and counting these in the scintillation counter. With thin layer plates, 0.5 cm wide sections of the adsorbent were scraped off and counted similarly.

Guinea pig faeces were homogenized with 2 volumes of water in a Waring Blendor. The homogenate (20 ml) was made alkaline with 10M-NaOH (2ml) and an equal volume of H_2O_2 solution (100 vol%) added with a few drops of iso-octanol to control foaming. The mixture was kept at room temperature for 3 days until decolourized, and aliquots then assayed for ^{14}C by liquid scintillation spectrometry.

To identify faecal metabolites, the untreated homogenate was centrifuged and the radioactive compounds present in the supernatant separated by paper and thin layer chromatography and determined as before.

Isotope Dilution Procedures

Amphetamine, 4-hydroxyamphetamine, benzylmethyl ketone 4-hydroxynorephedrine, hippuric acid, benzoic acid and 4-hydroxy benzoic acid were

assayed by the methods described by Dring et al.(1970). About one-tenth of the daily urine output was used for each assay.

Methamphetamine

Methamphetamine hydrochloride (1g) was dissolved in urine and the solution adjusted to pH14 with 10M-NaOH. This was extracted with ether (3x100 ml), the extracts dried over anhydrous Na_2SO_4, evaporated to dryness and the residue tosylated as previously described (see Synthesis of [^{14}C]-Methamphetamine). The N-p-toluenesulphonyl-N-methylamphetamine was recrystallized from aqueous ethanol to constant specific activity (m.p.82-84°).

Norephedrine

This amine hydrochloride (1g) was dissolved in urine, which was made alkaline (pH14) by the addition of 10M-NaOH, saturated with solid NaCl and extracted with ether (3x100 ml). The bulked extracts were dried over anhydrous Na_2SO_4, evaporated to dryness, the residue taken up in 1M-NaOH (10 ml) and treated with benzoyl chloride (4 ml). The crystals of O,N-dibenzoylnorephedrine which formed were filtered off, washed with M-NaOH and water, and recrystallized to constant specific activity from 50% aqueous ethanol (m.p.166°, literature value 167-8°, see Heilbron and Bunbury, 1953).

4-Hydroxynorephedrine in Human Urine

Due to the large volumes involved, considerable difficulty was encountered in the assay of 4-hydroxynorephedrine in human urine by the above method of Dring et al.(1970). The following method was therefore adopted for human urine only.

The amine hydrochloride (1g) was dissolved in urine, this mixed with one-fifth of its volume of 10M-HCl and boiled under reflux for 2h. The solution was then evaporated to dryness. The residue was extracted with methanol (10 ml) and the solution filtered to remove salts. The methanol was evaporated off and the residue treated with 2.5 M-NaOH (15 ml) and benzoyl chloride (5 ml).

The mixture was shaken for 10 min and left for the derivative to solidify. When this occurred, the precipitate was filtered off, washed and recrystallized to constant specific activity as described above (m.p. 162-3°; corresponding to the dibenzoyl derivative of Dring et al.,1970).

4-Hydroxymethamphetamine

Equal volumes of urine and 10M-H were boiled under reflux together for 2h and 4-hydroxymethamphetamine free base (1g) dissolved in this hydrolysate. This was adjusted to pH9.5-10 with 10M-NaOH, saturated with solid NaCl and extracted with ether (3x50ml). The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was taken up in 2.5 M-NaOH (20ml) and p-toluene sulphonyl chloride (1.75g) in acetone (6ml) added, the mixture boiled under reflux for 20 min, cooled, poured into ice (10g) and the resulting oil extracted with ethyl acetate (3x20ml). The bulked extracts were washed with 1M-HCl, 1M-NaOH and water, and the ethyl acetate removed on the rotary evaporator. The residue was recrystallized from aqueous ethanol to constant specific activity (m.p. 108-110°).

This derivative, O,N-di (p-toluenesulphonyl)-4-hydroxy-N-methylamphetamine, appears to be a new compound and a sample was prepared by standard methods. It formed a white crystalline powder of the monohydrate, m.p. 108-110°, from aqueous ethanol (found: C,59.0; H,5.7; N,3.1; S,12.9%. $C_{24}H_{27}NO_5S_2.H_2O$ requires C,58.7; H,5.9; N,2.9; S,13.0%).

Chromatography

Table 1 gives R_f values of reference compounds used in this study, using the systems below. With systems A and B descending paper chromatography on either Whatman No.1 or No.3MM paper, was used. R_f values were the same on both papers.

System C was used for thin layer chromatography (t.l.c.), using glass plates 5 x 20cm or 20 x 20cm, spread with Alumina G (Merck A.G.,

Table 2.1 R_F Values and Colour Reactions of Methamphetamine and Related Compounds

Compound	R_F value in solvent: Paper A	Paper B	Thin layer C	Thin layer D	Colour with diazotized nitroaniline
N-Methamphetamine	0.57	0.92	0.81	0.24	none
Amphetamine	0.51	0.88	0.45	0.59	pale pink
4-Hydroxyamphetamine	0.32	0.79	0.39	-	purple
4-Hydroxymethamphetamine	0.35	0.82	0.64	-	purple
Ephedrine	0.52	0.86	0.70	0.30	pale pink
4-Hydroxyephedrine	0.17	0.80	0.15	-	purple
Norephedrine	0.39	0.82	0.18	0.67	pale pink
4-Hydroxynorephedrine	0.17	0.64	0.07	-	purple
Benzoic acid	0.93	0.33	0.00	-	none
Hippuric acid	0.82	0.20	-	-	none
Benzoyl glucuronide	0.60	0.09	-	-	none
4-Hydroxybenzoic acid	0.63	0-0.1	-	-	
Benzyl methyl ketoxime	0.86	0.88	0.88	-	none

Darmstadt, Germany) 0.25 mm thick, which were heated at 120° for 1h before use.

System D used similarly prepared plates, spread with Silica Gel G (Merck).

System A 2-Methyl-butan-2-ol/1, 1-dimethylpropanol/water/formic acid (5:5:10:2 by volume). (Alleva, 1966).

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

System C Methanol/Chloroform (1:1 by volume). (Noirfailise, 1966).

System D Methanol/acetone (1:1 by volume). (Moerman, 1964).

Paper chromatograms were run for about 35 cm from the origin and thin layer chromatograms about 10cm.

The locations of the compounds on the chromatograms were determined by the following methods.

Ultraviolet Light All the reference compounds used were seen as dark quenching spots when chromatograms were inspected under u.v. light at 254nm (Hanovia Chromatolite).

Diazotized p-nitroaniline Chromatograms were sprayed with diazotized p-nitroaniline, as described by Wickstrom and Salvesen (1952). Phenols gave strong blue-purple spots and amines gave paler red ones, neither of which were permanent.

Hippuric Acid Detection Hippuric acid was seen as an orange red spot after spraying with p-dimethylaminobenzaldehyde in acetic anhydride, and heating at 120° for 2 min (El Masry et al., 1956).

Benzoic Acid Detection Benzoic acid was detected with a spray of bromophenol blue in ethanol, showing as a blue spot (Opienska-Blauth et al., 1951).

Benzoylglucuronide Detection Benzoylglucuronide was visualized with the naphtharesorcinol spray of Bridges et al.(1965).

Benzylmethyl Ketoxime Detection A spray of aqueous cupric chloride (0.5%^{W}/v) was used to detect the ketoxime, which showed as a green colour growing more intense with time.

Hydrolyses of Urine

Urine was hydrolysed in two ways for chromatographic separation of the metabolites, with β-glucuronidase or with 5M-HCl.

Enzymic Hydrolysis

A suitable volume of urine was brought to pH5 with glacial acetic acid and incubated with an equal volume of β-glucuronidase solution (Ketodase; Warner-Chilcott, Eastleigh, U.K.) for 24h at 37^{o}. Two controls were set up, one with boiled enzyme and the other containing in addition phenolphthalein glucuronide to ensure that the enzyme was working. The solution was applied directly to chromatograms.

Acid Hydrolysis

Equal volumes of urine and 10M-HCl were boiled together under reflux for 2h. This mixture was cooled and neutralized before being applied to chromatograms.

Gas-Liquid Chromatography (g.l.c.) of Amines

An F & M Model 402 high resolution gas chromatographed (Hewlett-Packard Inc., Pasadena, Calif., U.S.A.) was used, with flame ionization detection. The column was of glass, length 6ft, i.d.3mm, packed with Chromosorb G-AW/DMCS (100-120 mesh) coated with SE-30(3%^{W}/w). The conditions used were: nitrogen carrier gas pressure 40 p.s.i., flow rate 50ml/min, air pressure 24 p.s.i., hydrogen pressure 20 p.s.i., injection

port temperature 240°. The temperature programming facility was employed, with an initial column temperature of 150°. After a delay of 8 min, the temperature rose by 5°/min to an upper limit of 185°, which was maintained.

Preparation of Acetylated Standards for g.l.c.

N-Acetylamphetamine

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

Acetylated amine derivatives for gas chromatography

Acetylderivatives of (+)-methamphetamine, (—)-ephedrine and (+)-norephedrine were prepared from the corresponding hydrochlorides as follows: The compound (20mg) was dissolved in water (10ml). 2.5 M-NaOH (2ml) was added to this, which was then saturated with solid NaCl, and extracted with ether (3x15ml). Pyridine (0.5ml) and acetic anhydride (0.5ml) were added to the bulked ether extracts and left to react at room temperature for 24h. The reagents were then removed in vacuo and the oily acetyl derivatives dissolved in ethylacetate for gas chromatography, which showed them to be essentially pure.

Preparation of Urine Extracts for g.l.c.

G.l.c. analysis has been used to detect methamphetamine and possible basic metabolites, amphetamine, ephedrine and norephedrine. N-Acetyl derivatives of these compounds were used to reduce volatility during the extraction procedure and to improve resolution on the column (see Anggard, 1970).

Urine (5-10ml) was made alkaline (pH14) with 10M-NaOH and saturated with solid NaCl. This was extracted with ether (3x10ml) which was dried over anhydrous Na_2SO_4 and treated with pyridine (0.5ml) and acetic anhydride (0.5ml) for 24h at room temperature. The reagents were removed at reduced pressure, the residue taken up in ethylacetate (0.5-1ml) and 5-10µl portions

of this injected onto the gas chromatograph column. Urine from animals of appropriate species which had not received drug solution was extracted in the same way.

The retention times of acetylated amphetamine, methamphetamine, norephedrine and ephedrine were 7.5, 10.6, 14.1 and 15.75 min respectively and calibration curves were constructed over the range 0.25-10μg. No substances interfering with the assay were found in urine.

Identification of (+)-methamphetamine and possible amine metabolites in the urine of guinea pigs using combined gas chromatography and mass spectrometry.

Gas chromatography-mass spectrometry (g.c.-m.s.)

A Varian Aerograph 1700 gas chromatograph was used in conjunction with a Varian MAT CH5 mass spectrometer. The gas chromatography column was of glass, length 5ft (2mm i.d.), packed with Chromosorb W-AW/DMCS coated with SE-30 (3%^{w}/w). Temperature programming was used, the column temperature being 185^{o} initially, rising after a delay of 2 min by 8^{o}/min to a maximum of 250^{o}, which was maintained. The injection port temperature was 220^{o}, the detector 250^{o}, the interface 265^{o}, the Biemann-Watson type molecular separator 240^{o}, the line of sight inlet line 200^{o} and the ion source temperature 180^{o}. The electron beam energy was 70eV and the helium carrier gas flow rate was 12ml/min at 40 p.s.i. 1μl samples of solution of reference compounds and urine extracts were injected onto the column.

Preparation of Urine Extracts for g.c.-m.s.

Amines were extracted from the 0-24h urine of guinea pigs dosed with [^{14}C]-methamphetamine hydrochloride (45 mg/kg) as described under g.l.c. analysis and acetylated in the same way. 1μl samples of the resulting solution were injected onto the column.

1μl samples of solution of reference compounds (1mg/ml in ethyl acetate) were also injected onto the column. These acetylated amines each gave a single peak, as recorded by the total ion current monitor of the mass spectrometer with retentions of amphetamine 2.0, methamphetamine 2.6, and norephedrine 4.25 min.

The mass spectrum of each peak was recorded and these indicated that both amphetamine and methamphetamine gave an N-acetyl derivative, and that norephedrine gave an O,N-diacetyl derivative.

No substance interfering with the analysis was found in urine.

G.l.c. of Benzylmethyl Ketoxime

The F & M Model 402 gas chromatograph was used. The 4ft glass column, i.d.3mm, was packed with W-98 (3.8%^{w}/w on Diatoport S (100-120 mesh). The conditions were, injection port temperature 120°, column temperature 90°, nitrogen pressure 40 p.s.i., flow rate 35 ml/min, air pressure 24 p.s.i. and hydrogen pressure 20 p.s.i. With this system retention times were benzylmethyl ketone 2.0 min and benzylmethyl ketoxime 8.0 min.

Preparation of Urine Extracts for g.l.c.

Urine (10ml) as such, and after hydrolysis with β-glucuronidase, was brought to pH 7.4 with a few drops of M-NaOH or M-HCl as appropriate and extracted with ethyl acetate (3x15ml). The extracts were evaporated to dryness at low temperature on a rotary evaporator, and the residue taken up in ethyl acetate (0.2-0.4ml) and 3μl portions injected onto the column. Control extracts were prepared from blank urine and no interfering material found when these were chromatographed under these conditions.

CHAPTER THREE

THE METABOLISM OF METHAMPHETAMINE IN THE RAT AND THE GUINEA PIG

Chapter Three — The Metabolism of Methamphetamine in the Rat and the Guinea Pig

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Introduction

In order to perform metabolic studies on a drug in animals and man it is necessary to have a highly specific and sensitive means of detecting that drug and its metabolites in body fluids. The best way to achieve this is by isotopically labelling one or more atoms in the drug molecule. With current techniques, this is most conveniently done by using radioactive isotopes, and in methamphetamine either radioactive carbon (^{14}C) or hydrogen (^{3}H; tritium) may be used. In general ^{14}C is preferable and was thus adopted for the present work. The position of the label within the molecule is critical as this decides the extent of degradation undergone which may be detected. With methamphetamine, labelling within the ring would be preferable, but is obviated on economic grounds. It was therefore decided to insert ^{14}C in the side chain next to the ring, enabling both ring hydroxylations and sidechain degradations to be detected.

Identification of Metabolites

Rat Urine

The urine (1 ml) was chromatographed on paper in solvent A and the chromatogram scanned for ^{14}C. Three broad peaks whose centres had R_f 0.0, 0.3 and 0.4 and a minor peak of R_f 0.89 were found (see Fig.3.1). Colour tests showed that three broad peaks contained respectively, glucuronides, phenolic substances and amines. The minor peak corresponded in R_f value to hippuric and benzoic acids. Hydrolysis of the urine with HCl or β-glucuronidase caused the peak of R_f 0.0 to disappear and give an equivalent increase in the peak of R_f 0.3 The peaks at R_f 0.49 and 0.89 were unaffected by acid or β-glucuronidase.

Fig 3.1 Radiochromatogram of 0-24 h Rat Urine after [^{14}C]Methamphetamine hydrochloride (45 mg/kg; 1.5 µCi) orally in System A

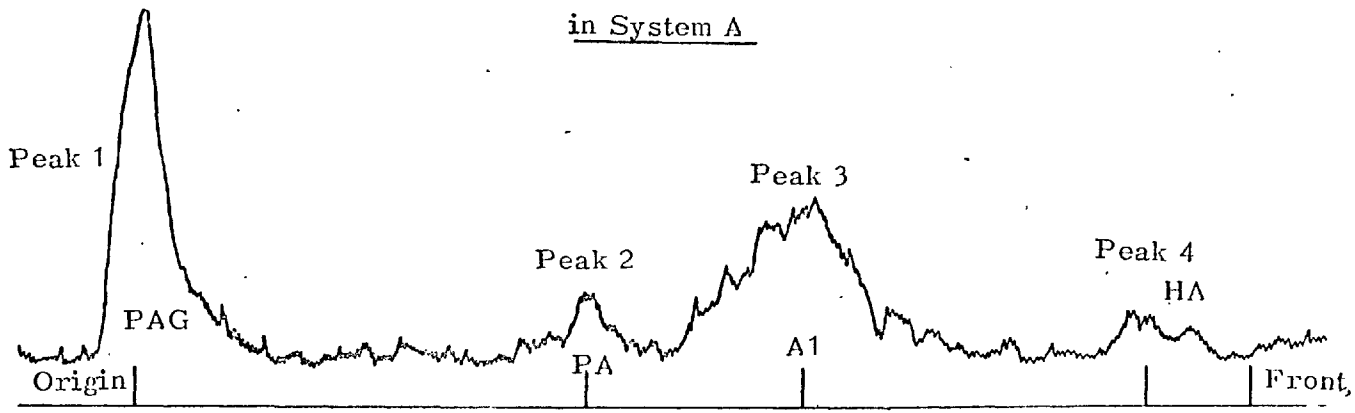
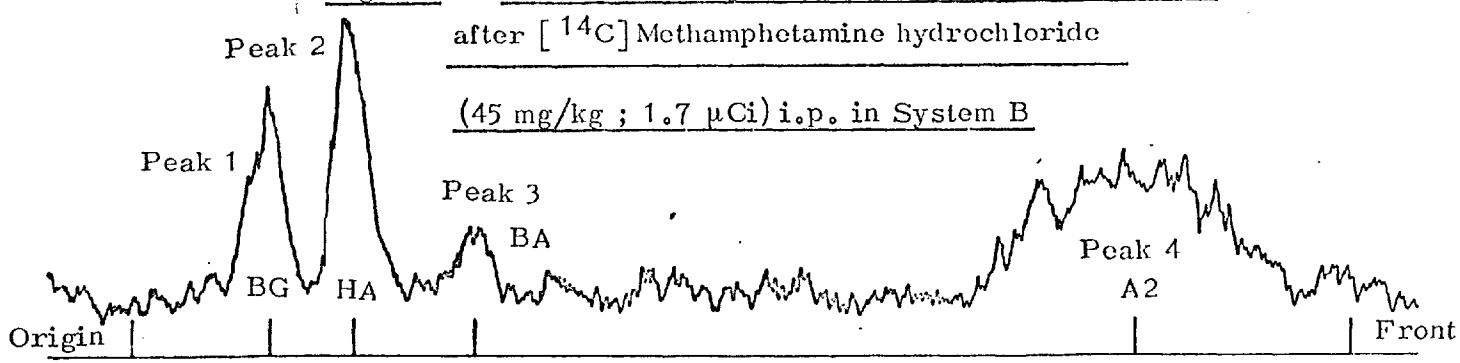


Fig 3.2 Radiochromatogram of 0-24 h Guinea Pig Urine after [^{14}C]Methamphetamine hydrochloride (45 mg/kg ; 1.7 µCi) i.p. in System B



PAG = phenolic amine glucuronides (see also Fig 3.3) PA = phenolic amines A1 = Amines (see also Fig 3.4)

BG = benzoyl glucuronide HA = hippuric acid

BA = benzoic acid A2 = Amines (see also Fig 3.5)

Fig. 3.3 Radiochromatogram of Eluate of Peak 1 in Rat Urine, after β-glucuronidase treatment in System C (see Fig 3.1)

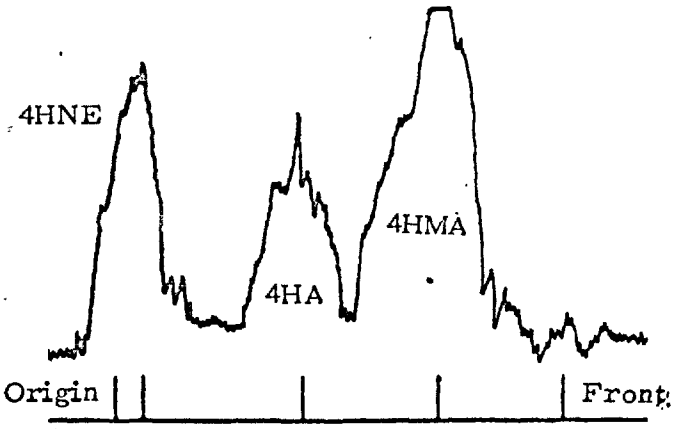


Fig 3.4 Radiochromatogram of Eluate of Peak 3 in Rat Urine in System C (see Fig 3.1)

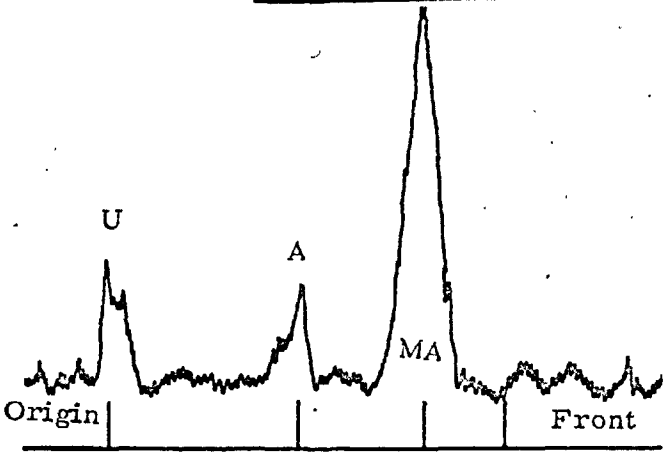
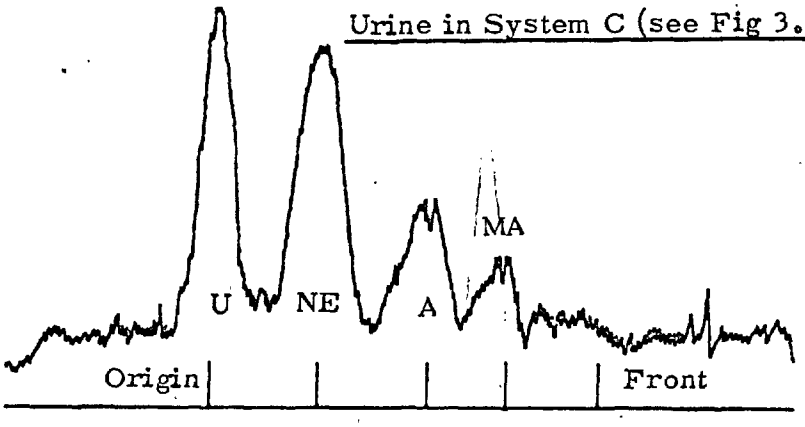


Fig. 3.5 Radiochromatogram of Eluate of Peak 4 in Guinea Pig Urine in System C (see Fig 3.2)



U = acidic/unknown

NE = norephedrine

A = amphetamine

MA = methamphetamine

4HNE = 4-hydroxynorephedrine

4HA = 4-hydroxyamphetamine

4HMA = 4-hydroxymethamphetamine

The material of R_f 0.0 above was eluted with water and chromatographed after hydrolysis with β-glucuronidase on thin layer in solvent C. Three radioactive peaks of R_f 0.04, 0.4 and 0.65 (see Fig. 3.3) were found all of which gave an intense purple colour for phenols with diazotized p-nitroaniline. These R_f values correspond to 4-hydroxynorephedrine, 4-hydroxyamphetamine and 4-hydroxymethamphetamine. These compounds were estimated by scintillation counting after scraping 0.5 cm sections of the absorbent from the thin layer plate as described previously (see Chapter 2).

The peak of R_f 0.3 in solvent A was eluted with water and rechromatographed as above in solvent C. Again three peaks of R_f 0.04, 0.4 and 0.65 were obtained corresponding with the above three phenols released by β-glucuronidase from the peak of R_f 0.0 in solvent A. These were estimated as before.

The peak of R_f 0.49 in solvent A was eluted with water and rechromatographed in solvent C to yield three radioactive peaks of R_f 0.0, 0.43 and 0.8 (see Fig. 3.4). The peak of R_f 0.43 gave a pale pink colour with diazotized nitraniline and corresponded to amphetamine whose identity was confirmed by gas liquid chromatography. The material at R_f 0.8 gave no colour with diazotized nitroaniline and corresponded to methamphetamine also confirmed by gas liquid chromatography. The material of R_f 0.0 was an acidic substance whose identity is at present unknown.

The ^{14}C peak of R_f 0.89 in solvent A suspected to be hippuric and benzoic acids was too small to estimate accurately. Labelled benzoic acid in the urine was therefore estimated by isotope dilution directly on the original urine. Isotope dilution was also used to analyse for 4-hydroxynorephedrine, 4-hydroxymethamphetamine and 4-hydroxybenzoic acid.

Guinea Pig Urine

Dose 45 mg/kg

The urine was chromatographed in solvent A as above and gave two

broad peaks whose centres had R_f 0.38 and 0.79.

The peak of R_f 0.38 gave a pale pink colour with diazotized p-nitroaniline but was unaffected by treatment with β-glucuronidase. The peak was eluted with water and rechromatographed on thin layer in solvent C whereby it resolved into 4 peaks of R_f 0.0, 0.2, 0.47 and 0.79. The first was an acidic unknown compound and the succeeding peaks in order corresponded with norephedrine, amphetamine and methamphetamine.

The urine was chromatographed on paper in solvent B. It gave 4 peaks (see Fig. 3.2), three sharp ones of R_f 0.1, 0.18, and 0.28 corresponding to benzoylglucuronide (positive naphtharesorcinol test), hippuric acid (p-dimethylaminobenzaldehyde test) and benzoic acid (bromophenol blue test), and a broad one R_f 0.82. The peak of R_f 0.1 disappeared on treatment with β-glucuronidase and that at 0.28 correspondingly increased. On hydrolysis with HCl, the peak at 0.1 disappeared and at 0.18 diminished with a corresponding increase in that at 0.28. The broad peak of R_f 0.82 in B was eluted with water and rechromatographed on thin layer in solvent C. It gave the same pattern of 4 peaks as did the original material of R_f 0.38 in A rechromatographed in solvent C, and therefore consisted of the unknown, norephedrine, amphetamine and methamphetamine (see Fig. 3.5). The presence of benzylmethyl ketone and norephedrine was shown by isotope dilution, and that of methamphetamine, amphetamine and norephedrine by g.l.c.

Demonstration of the Presence of Amphetamine and Norephedrine with g.c.-m.s.

The acetylated extracts from guinea pig urine (see Chapter 2) gave two peaks as recorded by the total ion current monitor of the mass spectrometer, with retention times 2.0 and 4.25 min (see Fig. 3.6). The first corresponded to N-acetylamphetamine and the second to O,N-diacetylnorephedrine. The mass spectrum of each peak was recorded and these corresponded almost exactly with the mass spectra of the authentic compounds (see Figs. 3.7 & 3.8).

Fig 3.6 Gas-liquid chromatography of samples for gas chromatography - mass spectrometry

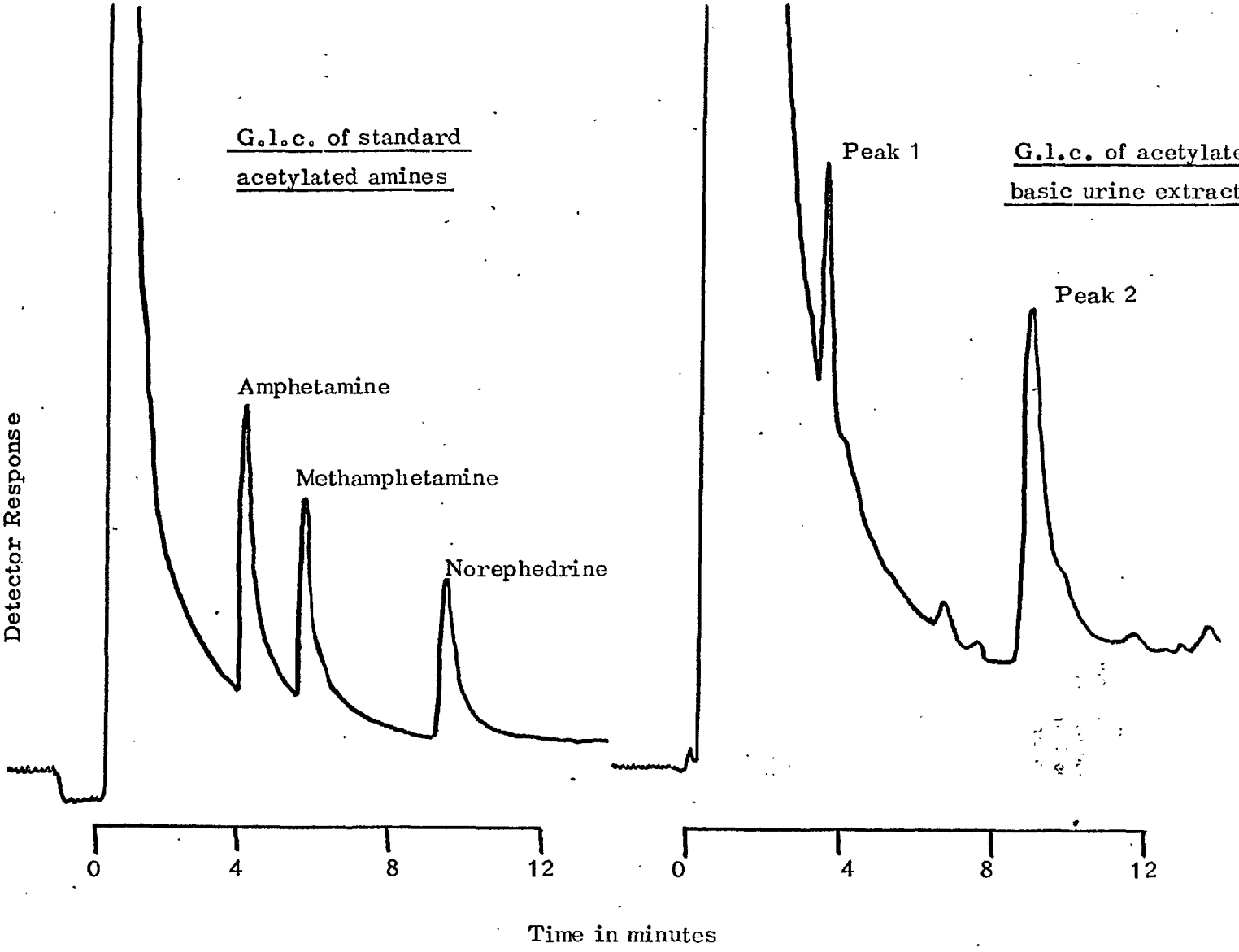


Fig 3.7A Mass spectrum of N-Acetyl amphetamine via G.C. - M.S. retn. time 2.0 min

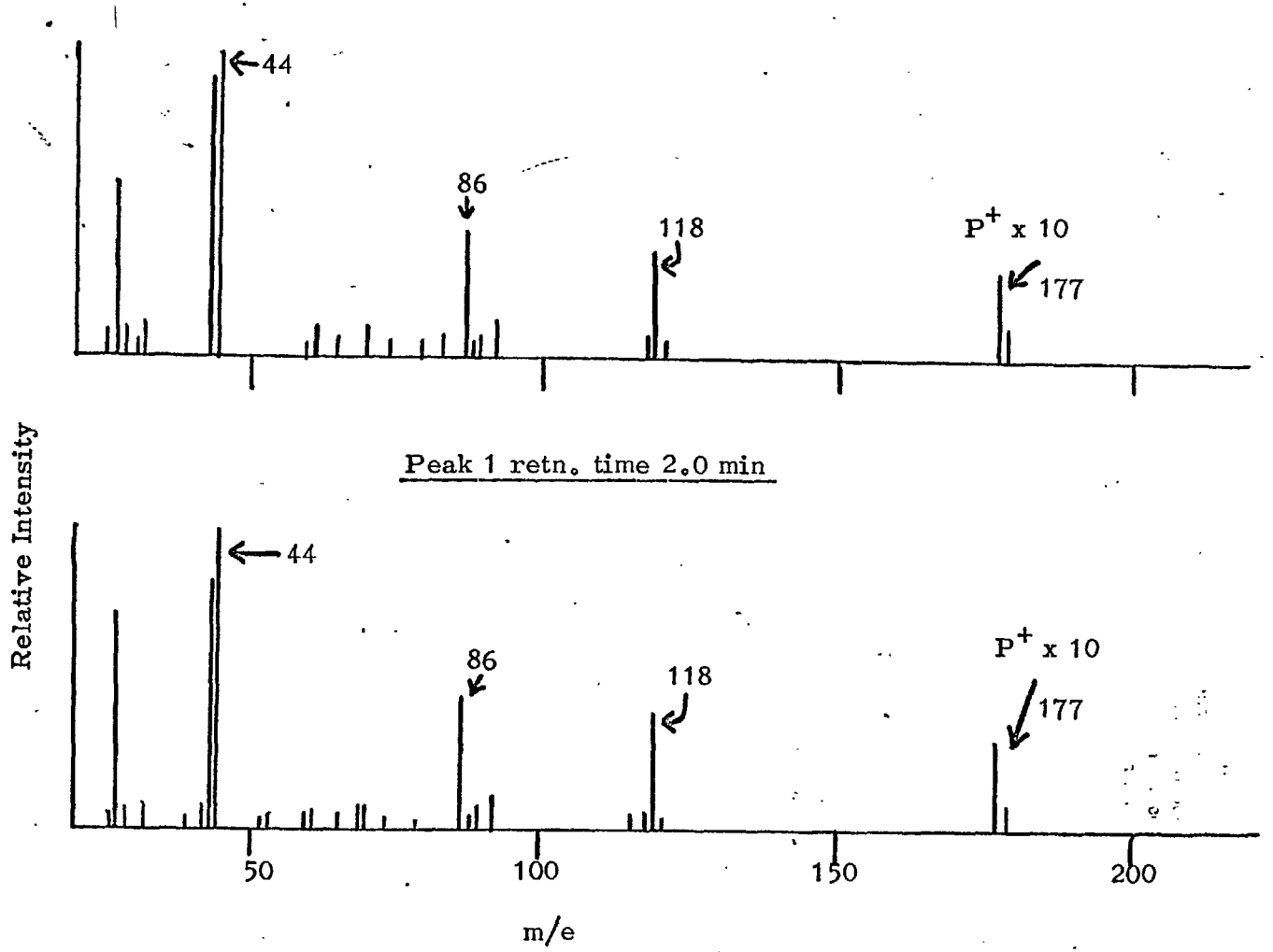
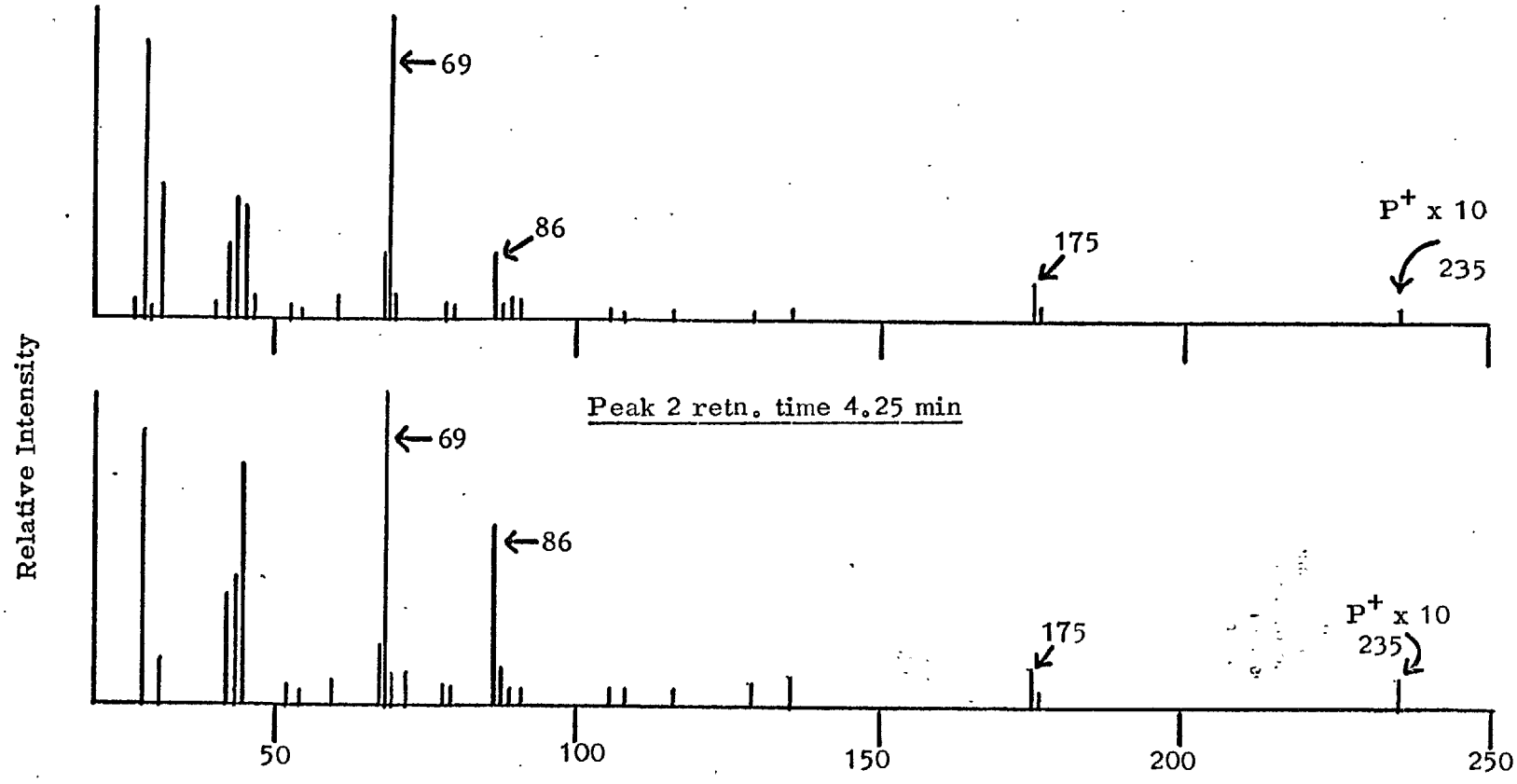


Fig 3.8 A Mass spectrum of O,N-diacetylnorephedrine via G.C. - M.S. retn. time 4.25 min



B

No peak corresponding to N-acetylmethamphetamine was seen so it appears that the present compound is excreted in urine only to a very small extent. This is in agreement with results from other analytical methods.

Guinea Pig Faeces

The supernatant obtained by centrifuging homogenates of faeces produced after a dose of 45 mg/kg was examined for metabolites in the same way as urine.

Guinea Pig Urine

Dose 10 mg/kg

The 0-24h urine was chromatographed in System A, giving two ^{14}C peaks, at R_f 0.38 and 0.79. Neither peak was affected by β-glucuronidase treatment, but the peak of R_f 0.38 was diminished by acid hydrolysis, indicating the presence of a volatile metabolite(s).

The urine was also chromatographed in System B and gave four ^{14}C peaks of R_f 0.1, 0.18, 0.3 and 0.82. The peak at R_f 0.1 corresponded to benzoyl glucuronide, that at R_f 0.18 to hippuric acid and that at R_f 0.3 to benzoic acid.

The peak of R_f 0.82 contained insufficient radioactivity to elute, so compounds suspected of being under this peak, methamphetamine, amphetamine and norephedrine, were assayed by isotope dilution and g.l.c. Benzylmethyl ketone was sought by isotope dilution before and after hydrolysis, and benzylmethyl ketoxime was assayed by g.l.c. before and after -glucuronidase treatment.

Isolation of Unknown Metabolite from Guinea Pig Urine

Guinea pig urine (after a dose of 45 mg/kg) was made strongly acid with 10M-HCl, extracted with ether, the extracts dried over anhydrous Na_2SO_4 and the ether removed on a rotary evaporator. The residue was taken up in ethanol, chromatographed on Whatman 3MM paper in System B and the paper scanned for ^{14}C. Four peaks were seen, with R_fs of 0.1, 0.2, 0.31 and 0.83, corresponding to benzoyl glucuronide, hippuric acid, benzoic acid and the acidic unknown. The other metabolites running around R_f 0.8 in System B are amines and therefore would not be extracted from acidic solution, so the residual material running at R_f 0.83 is concluded to be the acidic unknown. The area containing the unknown was eluted with water and the solution concentrated, rechromatographed in System C and the t.l.c. plates scanned for ^{14}C. Initially, one peak was seen, of R_f 0.0 which corresponded with the unknown, but on repeated t.l.c. two peaks were seen, one of R_f 0.0 as before and a new peak of R_f 0.9. The new peak increased in size the longer the eluate had been standing, which suggested that the unknown was decomposing or rearranging under the conditions of the experiment. The absorbent bearing the peak of R_f 0.9 was scraped off, packed in a Pasteur pipette with a glasswool plug in the neck and eluted with water. The water was adjusted to pH 5 with 1M-HCl and extracted with ether, which was dried over anhydrous Na_2SO_4. The ether was removed and the residue taken up in methanol, and this solution used for mass spectrometry by direct insertion. The mass spectrum of the isolated material is given in Fig. 3.9.

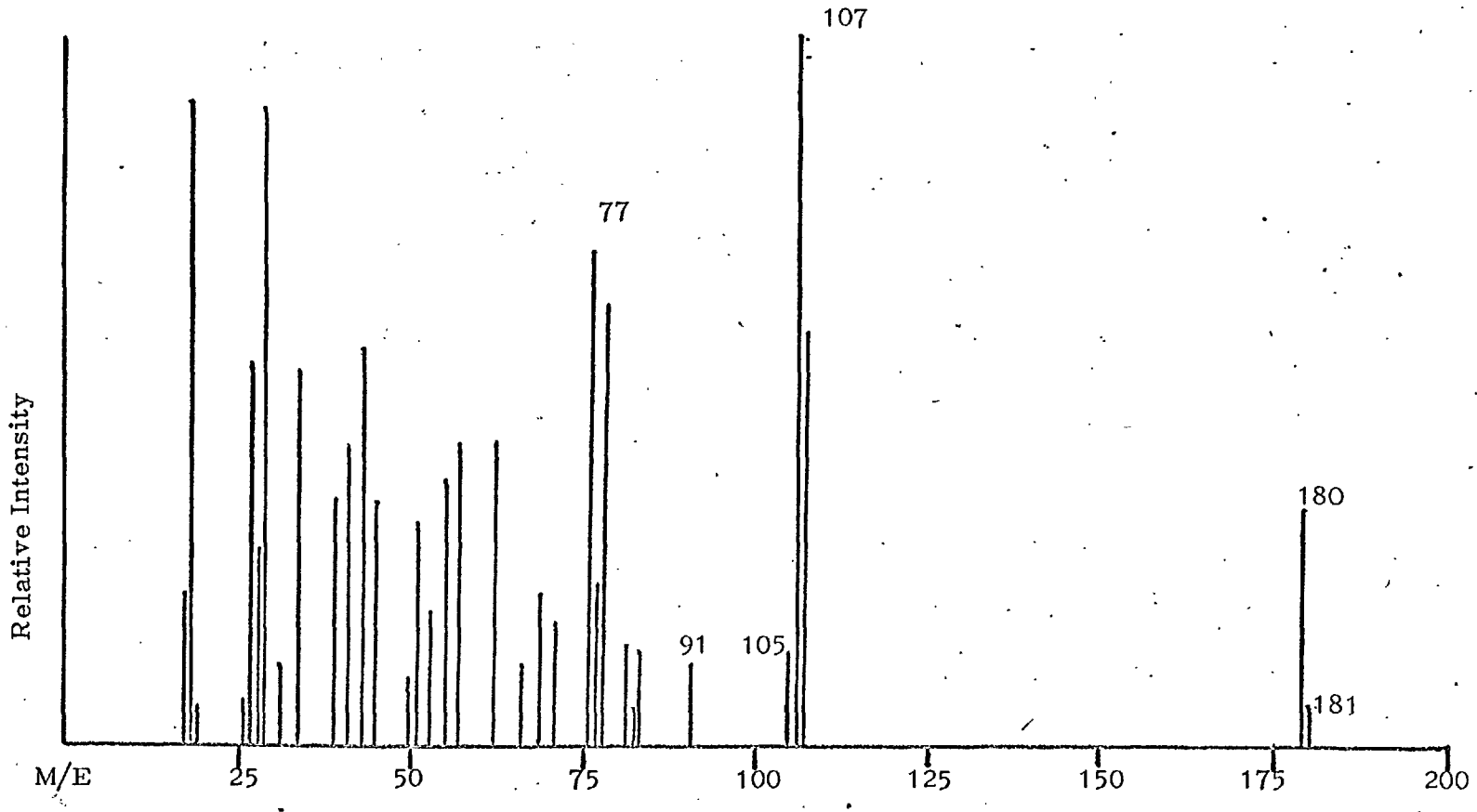
Results

Rat

The excretion of ^{14}C after the administration of [^{14}C]-methamphetamine hydrochloride after both oral and i.p. injection is shown in Table 3.1. The total recovery in both cases is almost identical, about 83% of the

Fig 3.9 Mass Spectrum of Unknown Metabolite from Guinea Pig Urine

(Dose 45 mg/kg ; 1.7 μCi : Extraction as described in the text)



dose being recovered, 81% in the urine and 2% in the faeces. The rate of excretion is, however, dependent on the route of administration at this dose level, ^{14}C being eliminated much more slowly after oral dosage than after i.p. injection.

In the rat, the metabolic reactions undergone by methamphetamine include aromatic hydroxylation, sidechain hydroxylation, N-demethylation and sidechain degradation, and the metabolites found in urine are shown in Table 3.2. The major reaction is aromatic hydroxylation, the major metabolite being 4-hydroxymethamphetamine (31% of the dose) with 4-hydroxyamphetamine (5.9%) and 4-hydroxynorephedrine (15.8%) also excreted. The total aromatic hydroxylation is thus about 53% of the dose in 2 days after dosage. These three phenolic metabolites are excreted mainly as conjugates with glucuronic acid.

The second most significant metabolic reaction from a quantitative view point is N-demethylation, giving rise to amphetamine (3.1%), 4-hydroxyamphetamine (5.9%) and 4-hydroxynorephedrine (15.8%), and will be the first step in side chain degradation to benzoic acid (3.5%). The total N-demethylation thus amounts to about 28% of the dose. By contrast, complete sidechain breakdown amounts to only 3.5% of the dose. Total benzoic acid was measured by isotope dilution, but chromatography indicated that this was excreted at least in part as hippuric acid.

The extent of sidechain hydroxylation is given by the output of 4-hydroxynorephedrine. Either ephedrine or norephedrine could be precursors of this metabolite, but neither were detected.

It must also be noted that about 11% of the dose was excreted unchanged as methamphetamine.

The relative proportions of the metabolites on day 1 and day 2 differ, and this follows expectations in that the extent of metabolism increased with a longer stay within the animal. Less unchanged

Table 3.1 The elimination of ^{14}C by the rat after administration of [^{14}C]Methamphetamine hydrochloride

[^{14}C]Methamphetamine hydrochloride (45 mg/kg) in water was administered to rats either orally (8.5 μCi/kg) or by i.p. injection (1.5 μCi/kg). Values given are % of ^{14}C dose in excreta; for oral dosage, averages of 3 animals, with ranges in parentheses, and for i.p. injection, individual figures for 2 animals.

	% of ^{14}C dose	
Route of administration	p.o.	i.p.
In urine on day 1	46.8(35.2-58.0)	69.2, 81.3
2	27.0(18.2-33.0)	5.1, 1.6
3	7.0(3.2-9.2)	2.4, 0.9
Total	80.8(77.4-85.5)	77.7, 83.3
In faeces on day 1	0.6(0-1.8)	1.9, 2.0
2	1.0(0.7-1.2)	0.3, 0.2
3	0.3(0.1-0.6)	0.8, 0.7
Total	1.9(1.3-3.3)	3.1, 2.8
Total excretion	82.7(78.7-88.8)	80.8, 86.6

Table 3.2 · The metabolites of [^{14}C]-Methamphetamine in rat urine

Dose 45 mg/kg ; 8.5 μCi/kg orally (see Table 3.1). The first two days urine were analysed as described in the text, and the results are expressed as averages of 3 animals with ranges in parentheses.

Metabolite sought		% of ^{14}C dose in that form		
		Day 1	Day 2	Total
Methamphetamine		10.5(5.3-17.9)	0.5(0.0-0.8)	11.0
Amphetamine		2.0(0.7-2.6)	1.1(0.8-1.4)	3.1
4-Hydroxymethamphetamine	Free	2.9(0.9-6.4)	0.0	
	Conjugated	12.7(3.8-23.7)	15.8(10.2-22.4)	
	Total	15.6(10.2-24.6)	15.8(10.2-22.4)	31.4
4-Hydroxyamphetamine	Free	1.0(0.4-1.9)	0.0	
	Conjugated	2.8(1.1-3.7)	2.1(1.8-2.3)	
	Total	3.8(3.0-4.2)	2.1(1.8-2.3)	5.9
Norephedrine		n.d.*	n.d.*	
4-Hydroxynorephedrine	Free	1.7(1.3-2.3)	0.0	
	Conjugated	7.7(2.0-14.5)	6.4(3.0-10.1)	
	Total	9.4(4.3-16.1)	6.4(3.0-10.1)	15.8
Benzoic acid (free + conjugated)		2.4(0.7-4.1)	1.1(0.7-1.8)	3.5
Unknown		3.9(2.9-5.0)	0.0	3.9
Total of above metabolites		47.4(35.3-59.3)	26.9(18.2-33.0)	74.3
Total ^{14}C in urine examined		46.8(35.2-58.2)	26.9(18.2-33.0)	73.7

*n.d. = not detected

methamphetamine was found on day 2, but quantities of 4-hydroxymethamphetamine, 4-hydroxyamphetamine and 4-hydroxynorephedrine on day 2 were very similar to those on day 1. Benzoic acid levels were, however, low on day 2, and no unknown metabolite appeared to be excreted on the second day.

4-Hydroxybenzoic acid was assayed by isotope dilution, but was absent.

Guinea Pig

Table 3.3 shows the excretion of ^{14}C after the administration of [^{14}C]-methamphetamine by i.p. injection to the guinea pig at two dose levels. The total recovery at both dose levels is very similar, about 85% being excreted in three days, with almost all the radioactivity appearing on the first day. However, the distribution of ^{14}C between urine and faeces varies with dosage. At 10 mg/kg, the ^{14}C was found almost exclusively in the urine (81%) with a very small amount in the faeces (3%), but at 45 mg/kg, 69% of the dose was recovered in the urine with some 18% in the faeces.

Table 3.4 shows that, at both levels examined, the guinea pig metabolizes methamphetamine in a completely different manner to the rat. Most striking in the absence of aromatic hydroxylation, and also the virtually complete N-demethylation. Appreciable faecal excretion is only seen at the higher dose level.

With a dose of 45 mg/kg, the main metabolic reaction appears to be N-demethylation, amounting to 64% of the dose in 24h, giving rise to amphetamine (3%), norephedrine (14%) and also being the first step in sidechain breakdown to benzylmethyl ketone (3%) and benzoic acid (32%). The degradation of the sidechain is also extensive, as indicated by the small quantity of benzylmethyl ketone found after hydrolysis and by the amount of benzoic acid excreted, this being found in urine unchanged and as glycine and glucuronic acid conjugates. The sidechain hydroxylation

Table 3.3 The elimination of ^{14}C by the guinea pig after administration of [^{14}C] Methamphetamine hydrochloride

[^{14}C] Methamphetamine hydrochloride in water was administered to guinea pigs at 2 dose levels, 45 mg/kg; 8.5 μCi/kg, and 10 mg/kg; 1.3 μCi/kg. Values given are % of ^{14}C dose in excreta , averages of 3 animals with ranges in parentheses.

	% of ^{14}C dose	
Dose, mg/kg	45	10
In urine on day 1	64.7(39.1-81.3)	81.2(76.5-83.
2	3.5(2.3-4.7)	1.5(0.8-2.8)
3	0.8(0.3-1.3)	-
Total	69.0(43.2-86.3)	82.7(77.1-86.6)
In faeces on day 1	18.1(5.1-28.3)	2.7(2.1-3.5)
2	0.3(0.2-0.4)	0
3	0.03(0-0.1)	-
Total	18.4(5.5-28.7)	2.7(2.1-3.5)
Total excretion	87.8(71.9-99.9)	85.4(79.2-90.1)

Table 3.4 Urinary and faecal metabolites of [^{14}C]-methamphetamine in the guinea pig

Two dose levels were used, 45 mg/kg; 8.5 μCi/kg and 10 mg/kg; 1.3 μCi/kg (see Table 3.3). After the larger dose, both urine and faeces were examined for metabolites, but after the smaller dose only the urine. The results for urine are expressed as averages, with ranges in parentheses; only two animals excreted sufficient ^{14}C in the faeces for analysis, and individual values are therefore quoted.

	% of dose			
	Day 1 after dose of 45 mg/kg			Day 1 after dose of 10 mg/kg
	Urine	Faeces	Total	Urine
Methamphetamine	2.1(1.5-3.3)	1.3(2.0,0.6)	3.4	0.4(0.0-1.1)
Amphetamine	3.1(0.7-6,7)	9.8(12.0,7.6)	12.9	3.6(1.6-4.9)
Norephedrine	13.3(5.5-21.1)	5.6(5.8, 5.3)	18.9	1.3(0.0-2.0)
Benzoic acid (free)	4.1(3.1-5.6)	0.0	-	21.6(12.4-26.1)
Benzoylglucuronide	10.3(7.2-13.9)	0.0	-	23.4(10.1-34.0)
Hippuric acid	16.1(12.2-20.3)	0.0	-	18.6(15.4-22.1)
Total benzoic acid	30.5(25.0-38.5)	0.0	30.5	63.4
Benzyl methyl ketone (after hydrolysis)	1.9(0.7-2.3)	-	1.9	10.9(0.0-18.6)
Benzyl methyl ketoxime before hydrolysis	-	-	-	n.d.*
Benzyl methyl ketoxime after β-glucuronidase	-	-	-	n.d.*
Unknown	12.8(6.3-22.0)	7.9(8.5,7.4)	20.7	-
Total ^{14}C of above metabolites	63.7(39.0-81.6)	24.6(28.3, 20.9)	88.3	79.8(73.7-83.3)
Total ^{14}C in excreta examined	64.7(39.1-81.3)	24.6(28.3, 20.9)	89.3	81.2(76.5-83.8)

*n.d. = not detected

measured by the output of norephedrine, is considerable. The excretion of unchanged methamphetamine is very small (2%).

At this higher dose level, the faecal excretion of ^{14}C is much higher than in the rat, and the metabolites in faeces are shown in Table 3.5. Very little unchanged methamphetamine was found (about 1%), about half the faecal ^{14}C being amphetamine (10% of the dose). A significant amount of norephedrine (6%) was present.

As will be pointed out later, sidechain hydroxylation is a highly significant metabolic option from the pharmacological viewpoint, and the presence of norephedrine has been shown by isotope dilution and g.c.-m.s., both of which offer a conclusive identification of this material.

At the lower dose level examined (10 mg/kg), the main metabolic reaction was again N-demethylation, giving rise to amphetamine (3.6%) and leading to benzoic acid (64%). The degradation of the sidechain to benzoic acid is even more extensive than at the higher dose, and the acid is again found unchanged and conjugated with glucuronic acid and glycine. At this dose level, benzylmethyl ketone was detected as a metabolite in considerable quantity after acid hydrolysis. This is a volatile material, and, as it is absent before hydrolysis, is probably present as an involatile precursor. Peaks were found on radiochromatograms which were of similar size to the amount of benzylmethyl ketone found and as isotope dilution studies revealed that no other compounds with similar R_f values were present in the quantities indicated, these peaks probably represented the precursor of benzylmethyl ketone. Comparison of R_f values, and g.l.c. studies before and after β-glucuronidase, showed that this precursor was not benzylmethyl ketoxime.

Sidechain hydroxylation, a most significant metabolic route at the higher dose, is scarcely seen at the lower dose, norephedrine being detected in two or three animals examined, being in these about 2% of the dose. The Faecal ^{14}C was so low (2%) as to preclude investigation of compounds therein at this lower dose level.

Table 3.5 The metabolitesof [^{14}C]-Methamphetamine in guinea pig faeces

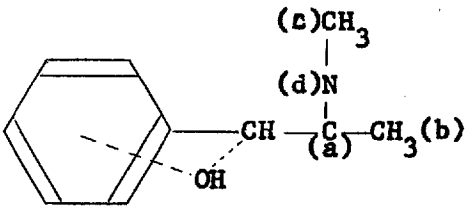
Dose 45 mg/kg ; 8.5 μCi/kg by i.p. injection. Two of three guinea pigs excreted sufficient faecal ^{14}C for analysis, individual values being quoted.

Compound	% of dose in that form
Methamphetamine	2.0, 0.6
Amphetamine	12.0, 7.6
Norephedrine	5.8, 5.3
Unknown	8.5, 7.4
Total metabolites	28.3, 20.9
Total ^{14}C in faeces	28.3, 20.9

The Nature of the Unknown Metabolite in Guinea Pig Urine

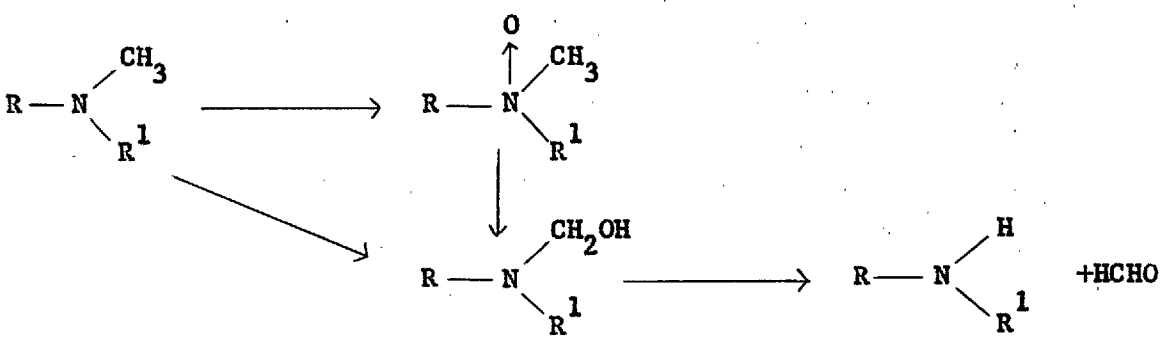
From the mass spectrum shown (Fig. 3.9.) a partial elucidation of the structure of this metabolite is possible. The parent ion is apparently at m/e 180, measured by peak matching against perfluorokerosene to be 180.102032, which corresponds to $C_{10}H_{14}NO_2$ (180.102452). However, here the "nitrogen rule" must be invoked. This states that a molecule of even molecular weight almost certainly contains no nitrogen or an even number of nitrogens, which is due to nitrogen, unlike almost all other atoms, having an odd valency but an even molecular weight. This suggests that the large peak at m/e 180 is not the parent ion, but is P-1, and that the molecular ion is the smaller peak at m/e 181, which corresponds to $C_{10}H_{15}NO_2$.

The cracking pattern obtained gives some clues as to the structure of the metabolite. Peaks at m/e 77, 78 and 79 probably correspond to $C_6H_5^+$, $C_6H_6^+$ and $C_6H_7^+$ and indicate the presence of a benzene ring. The peak at m/e 91 is perhaps $C_7H_7^+$, indicating a tropylium ion rather than a benzylic cation. Peaks at m/e 105 and 107 probably show the presence of a hydroxyl group on the carbon next to the ring in the sidechain. These peaks are characteristic of compounds with carbon on the oxygen next to the ring (Reisch et al., 1968) and are assigned $C_6H_5C{=}O$ (105) and C_6H_5CHOH (107). It must be mentioned that m/e 107 could also be due to $C_7H_7O^+$, a phenyl tropylium ion. It is therefore not possible on this evidence to assign the position of the hydroxyl group exactly, but the absence of ring hydroxylation of both amphetamine and methamphetamine in the guinea pig and the occurrence of sidechain hydroxylation in this species makes the sidechain hydroxylated form more likely. The absence of any peak between thepeaks of m/e 180 and 107, suggests the loss of a fragment of m/e 73 in one piece. This probably results from the loss of the sidechain by β-fission, a process common to phenylalkylamines (Beckett et al., 1967), and indicates the sidechain to be C_3H_7NO, locating the second oxygen in this fragment. In addition, this suggests that the amphetamine skeleton is intact;



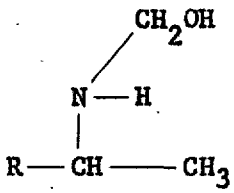
The letters (a), (b), (c) and (d) in the sidechain indicate possible locations of this second oxygen. Location of this at (a) would give the carbinolamine, which is a suggested intermediate in deamination (Brodie et al., 1958; Parli et al., 1971) but these compounds are held to be too unstable to exist except as transient forms, so that this is unlikely to be the location of the second oxygen. Oxygenation at (b) is not likely in the light of current ideas on metabolism of these compounds, and Dring et al. (1970) were unable to detect phenylalanine as a metabolite of amphetamine in the rat, so this location also seems improbable.

It is generally thought that N-dealkylation involves oxidative attack by enzymes on either (or both) the N atom or the adjacant carbon in the group to be removed, and that the intermediates then undergo enzymic or spontaneous rearrangement to give the amine and an aldehyde (Parke, 1968):

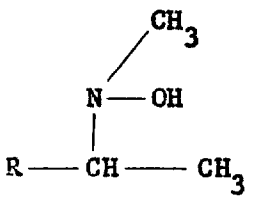


Instances have been reported of the urinary excretion of stable conjugates of these intermediates e.g. the glucuronide of the N-hydroxymethyl metabolite of diphenamid (McMahon and Sullivan, 1965), and the O-glucuronide of 2-acetamidofluorene (Cramer et al., 1960).

This then makes both positions possible locations for the oxygen:



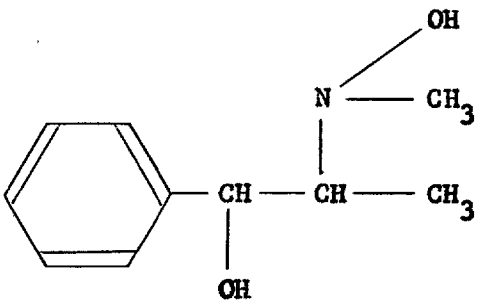
1



2

The material whose mass spectrum was obtained was extracted from solution at pH 5-6 and so is not basic as the alcoholic form would be. However, hydroxylamines of the type 2, substituted in a similar way have pK_a values of between 3 and 5 (Bissot et al., 1957) and probably would be extracted under the conditions used.

From the evidence of the mass spectrum and the inferences made as outlined it can be suggested, albeit very tentatively, that the unknown metabolite may be



which is present in urine as a precursor, perhaps as a weak conjugate or as some other structural isomer. Chromatography would appear to show that the unknown metabolite in the urine of both rat and guinea pig is the same material.

Discussion

The excretion of ^{14}C after the administration of [^{14}C]-methamphetamine to the rat is similar after both oral dosage and i.p. injection. The total recovery in both cases is almost identical, about 83% of the dose being

recovered, 81% in the urine and 2% in the faeces. The rate of excretion, however, is dependent on the route of administration at this dose level, radioactivity being voided much more slowly after oral dosage than after i.p. injection. This is in contrast to the findings of Ellison et al.(1966) for amphetamine, who showed that ^{14}C elimination was almost identical for oral and i.p. administration in a number of species. The difference observed for methamphetamine could possibly be due to the faster absorption of the drug after i.p. injection giving a correspondingly faster excretion.

In the guinea pig, the elimination of ^{14}C after [^{14}C]-methamphetamine dosage by i.p. injection is similar at both dose levels investigated with regard to extent and rate, but the route of elimination varies with the dose as does the metabolism of the drug. With a dose of 10 mg/kg, the excreted ^{14}C (84%) is almost exclusively in the urine with only 3% in the faeces, but at a dose of 45 mg/kg, the faecal excretion becomes more important (18%) with 69% in the urine. Dring et al. (1970) found virtually no ^{14}C in the faeces of guinea pigs after [^{14}C]-amphetamine administration with a dose of 10 mg/kg. The difference in excretion route is most probably a reflection of the differences in metabolism between the two dose levels tested.

There is a very great difference between the metabolic fate of methamphetamine in the rat and the guinea pig, and in addition there are marked differences between the metabolism of amphetamine and methamphetamine in these two species.

There are four main routes of metabolism for methamphetamine: a) ring hydroxylation, b) sidechain hydroxylation, c) N-demethylation and d) sidechain breakdown, proceeding by oxidative deamination to benzylmethyl ketone, and then, by further oxidation, to benzoic acid.

In the rat, all four of these routes are seen at the dose level employed. Aromatic hydroxylation is the main metabolic reaction, the major metabolite being 4-hydroxymethamphetamine. The next metabolite in order

of quantity is 4-hydroxynorephedrine, with small amounts of 4-hydroxy-amphetamine. The 4-hydroxynorephedrine found is the result of both ring and sidechain hydroxylations, and N-demethylation, and may be formed in a number of ways, suggestions being shown in Fig. 3.10.

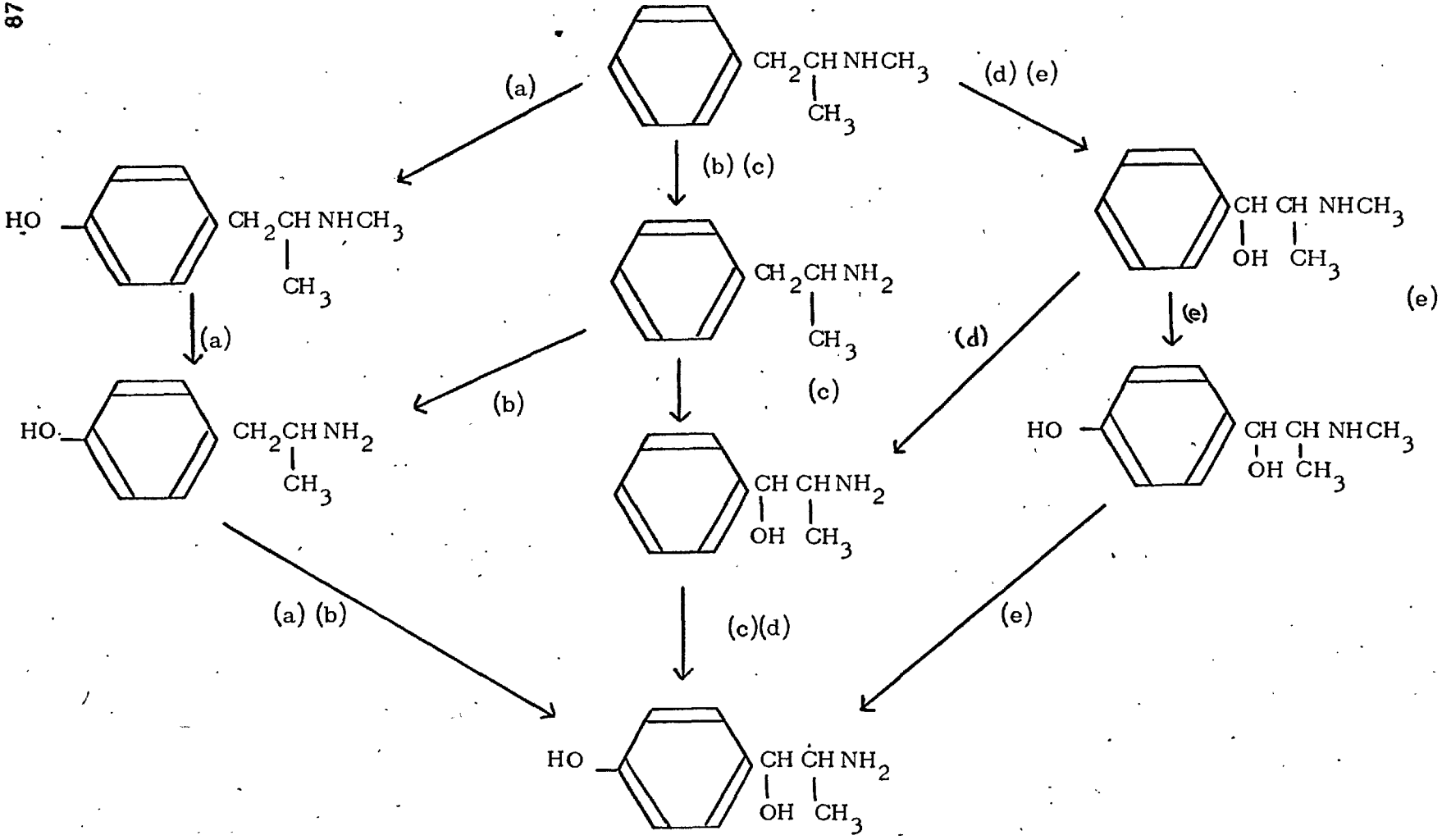
Of the five possibilities indicated, (a) and (b) are the most likely as all the intermediates are found in rat urine. However, the other routes are feasible, and evidence for (c) is given by the finding of Dring et al. (1970) that both 4-hydroxynorephedrine and norephedrine are metabolites of amphetamine in the rat.

Route (d), the initial sidechain hydroxylation of methamphetamine to ephedrine is feasible, and although never observed "in vivo", Creveling et al.(1962), using a purified dopamine-β-hydroxylase preparation from beef adrenals, reported the reaction as occurring "in vitro". Both Nagase et al. (1967) and Bralet et al.(1968) have found that ephedrine is ring hydroxylated by the rat, and N-demethylated, giving 4-hydroxyephedrine and 4-hydroxy-norephedrine (see Chapter 1, Table 1.2). Although the intermediates along this pathway have not been detected in urine, it is not inconceivable that some at least of the 4-hydroxynorephedrine originated by this series of reactions. Route (e) is similarly feasible.

Other routes can be postulated for the origin of this metabolite, but are less likely than those mentioned. It is most likely that several pathways contribute to the production of this compound.

The amount of benzoic acid found in the urine (3%) is very similar to that found by Dring et al.(1970) after amphetamine administration (3% of dose also). It is possible to suggest that the absence of 4-hydroxy-benzoic acid is due to the ability of the ring hydroxylated metabolites to be excreted as their glucuronic acid conjugates, rather than undergoing further metabolic breakdown.

Fig 3.10 Possible Pathways for the Formation of 4-Hydroxynorephedrine from Methamphetamine by the Rat



Amphetamine, the N-demethylated metabolite of methamphetamine will not be so rapidly excreted as the conjugate and thus may be further metabolised to benzoic acid. As an alternative to this, the enzyme system for benzoic acid may be at a very low level in the rat and be saturated by very small amounts of substrate.

It is thus seen that amphetamine is extensively metabolised by the rat and it is of interest to consider the efficiency of the metabolism as a detoxication mechanism. Methamphetamine is a potent CNS stimulant, as is amphetamine, and the introduction of the ring hydroxyl group reduces the CNS effect and toxicity, but increases the pressor activity of the compound. This may be seen in the following data, for the rat.

	Pressor Activity(a)	Threshold CNS stimulant dose(b)	LD_{50}(c)
Methamphetamine	275	-	30-40
Amphetamine	250	0.3	30-40
4-Hydroxymethamphetamine	250	-	300-400
4-Hydroxyamphetamine	100	80	300-400

(a) amount equivalent to unit dose of adrenaline (Graham et al.,1945)
(b) doses in mg/kg s.c. (Schulte et al.,1941)
(c) doses in mg/kg i.p. (Graham et al.,1945)

Considering these four compounds, N-demethylation of methamphetamine does not change the toxicity or the pressor effect of the drug appreciably. However, when combined with aromatic hydroxylation, in the production of 4-hydroxyamphetamine, the toxicity decreases tenfold, there is a 100fold drop in the CNS stimulant activity but a doubling of the pressor action. The aromatic hydroxylation of methamphetamine decreases the toxicity but has very little effect on the pressor activity. It thus appears that the toxicity depends on the presence or absence of the ring hydroxyl group, but that the pressor effect is governed by both this group and N-substitution.

The consequences of side chain hydroxylation are most important, and will be dealt with at greater length in Chapter 6.

In the guinea pig only three of the four main metabolic reactions appear to be present, there being little or no aromatic hydroxylation. This is in accord with expectations from the findings of Dring et al.(1970) in this species for amphetamine. The metabolism has been examined at two dose levels, and there is a marked difference between the high and low dose.

At the higher dose level, the major reaction is N-demethylation (only 3% of the dose appearing unchanged) as a preliminary to sidechain breakdown to benzoic acid and its conjugates (32%) and sidechain hydroxylation, giving norephedrine (20%). Amphetamine, the product of N-demethylation, is also a significant metabolite and the unknown is present in considerable quantity. A small amount of benzylmethyl ketone is present as an acid-labile precursor.

As with the rat, it is of interest to consider the possible origin of the sidechain hydroxylated metabolite, in this species norephedrine. Norephedrine has not been detected as a metabolite of amphetamine in the guinea pig (Dring et al.,1970; Lewander, 1971c) but the dose levels were less than in the present experiments. Creveling et al.(1962) have reported that ephedrine is produced from methamphetamine "invitro" by dopamine- -hydroxylase preparations, and Axelrod (1953,1955b) showed that guinea pigs, and also guinea pig liver microsomes "in vitro" readily demethylate ephedrine to norephedrine (see Chapter 1, Table 1.2). Ephedrine has not been detected as a urinary metabolite of methamphetamine but this does not preclude the possibility of it being an intermediate in the production of norephedrine.

In the guinea pig at the lower dose level (10 mg/kg) the metabolic pattern differs markedly from that seen previously at a dose of 45 mg/kg. The major reaction is again N-demethylation (less than 1% excreted unchanged) as a preliminary to all other metabolites. Amphetamine is a minor

metabolite (3%) and norephedrine present in even smaller amounts (2%) which is in marked contrast to the higher dose level. Benzoic acid and its conjugates account for most of the excreted ^{14}C, some 64% of the dose, but at this dose an acid-labile precursor of benzylmethyl ketone is a significant metabolite (11%).

Dring et al.(1968,1970) found that in rabbit urine there was present an acid-labile and alkali-labile precursor of benzylmethyl ketone after amphetamine dosage, and, as mentioned in Chapter 1, this appeared to be the sulphate of the enolic tautomer of the ketone. The peak on chromarograms which appeared to be the precursor in guinea pig urine corresponds in R_f to Dring's enol sulphate, and this may be the same conjugate.

A great deal of attention has been focussed on the mechanism of the deamination of amphetamine over the last 18 months. Hucker et al.(1970) have reported that the major metabolite of amphetamine in guinea pig liver microsomes is benzylmethyl ketoxime, and Parli et al.(1970) have also detected this oxime as an amphetamine metabolite in rabbit liver microsomes (see Chapter 1, especially Fig. 1.6). Experiments have shown that if benzylmethyl ketoxime is hydrolysed with acid in the same way as urine, there is a quantitative conversion to the ketone. This oxime is therefore a possible precursor of the ketone found as a urinary metabolite after acid hydrolysis in this work. Comparison of R_f values shows that the oxime is not present free, but it may be conjugated. Hucker et al.(1971), however, fed benzylmethyl ketoxime to rabbits and recovered less than 2% unchanged, all conjugated with glucuronic and sulphuric acids. G.l.c. analysis revealed that guinea pig urine contained no benzylmethyl ketoxime, either before or after β-glucuronidase hydrolysis, so it appears that this ketoxime is probably not the precursor of the ketone found in the urine after acid hydrolysis.

Sidechain Hydroxylation

The sidechain hydroxylation of methamphetamine metabolites by the

rat, giving 4-hydroxynorephedrine, is of great pharmacological importance, as the introduction of the alcoholic hydroxyl group enables the metabolite to be taken up by nerve cells, be retained in the noradrenaline-containing cell fractions and be released in place of noradrenaline on nerve stimulation. The same reaction in the guinea pig, the production of norephedrine, may be of similar significance.

This metabolic reaction is closely related to the biosynthetic pathway of noradrenaline (see Table 1.1) in that it is analogous to the β-hydroxylation of dopamine to noradrenaline.

Levin and Kaufman (1961) showed that dopamine-β-hydroxylase, the enzyme converting dopamine to noradrenaline, is not specific for dopamine but will hydroxylate a wide range of substrates structurally related to noradrenaline. Kaufman and Freidman (1965), in a review, have concluded that the structural requirement for β-hydroxylation is a basic nucleus of either a phenethylamine or a phenylisopropylamine. 3- or 4-Hydroxylation of the ring greatly increases enzyme activity, but N-methylation decreases it.

Goldstein and Contrera (1962) showed that, among many compounds, 4-hydroxyamphetamine was a substrate for the enzyme "in vitro", and in a later paper, Goldstein et al.(1964) reported in addition the β-hydroxylation of amphetamine to norephedrine by this enzyme "in vitro". They also report that the β-hydroxylation of these amphetamines "in vitro" is stereospecific for the (+)-isomer.

Goldstein et al.(1965) have demonstrated that this β-hydroxylation also occurs "in vivo", and that it is similarly stereospecific, 4-hydroxynorephedrine being found only in rat heart after (+)-amphetamine. In similar experiments, Thoenen et al.(1966) demonstrated that both norephedrine and 4-hydroxynorephedrine are formed from (±)-amphetamine by the cat, and released from the spleen, an organ rich in sympathetic nerves, on sympathetic stimulation. Evidence for the release of 4-hydroxynorephedrine under these conditions is excellent, but that for norephedrine release is somewhat equivocal.

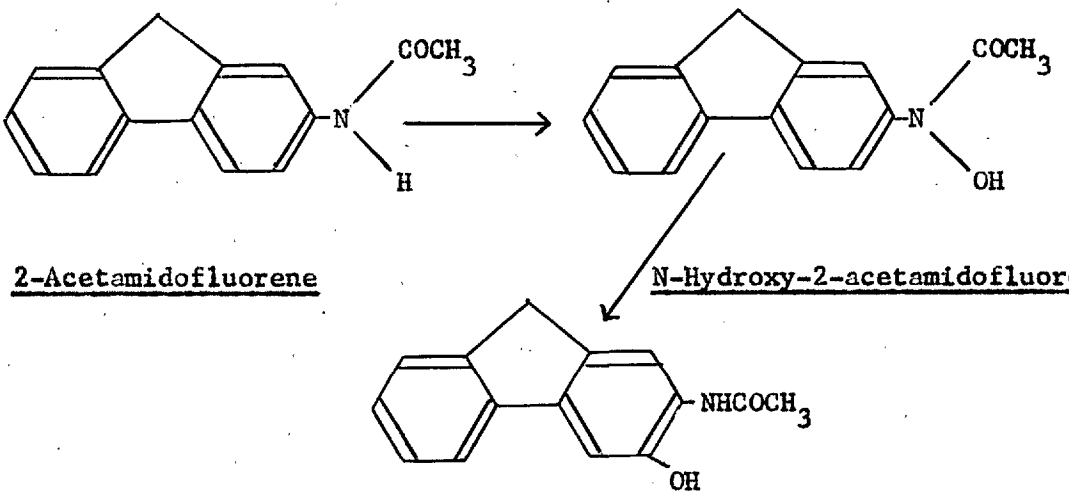
Iversen et al.(1966) have shown that 4-hydroxynorephedrine is present in rat heart after administration of 4-hydroxyamphetamine, and that the amount formed is greatly reduced by immunosympathectomy. This appears to indicate that the β-hydroxylation and the adrenergic nerve endings are very closely related. Lewander (1971b) has found that both 4-hydroxy-norephedrine and norephedrine are present in rat brain after amphetamine dosage, suggesting that the CNS has the capacity to carry out this reaction in the same way as the peripheral nerve fibres. This is important as Lewander has also found that the ring hydroxylated amphetamine penetrates the blood-brain barrier less well than does amphetamine itself.

It is now well established that although noradrenaline is the principal neurotransmitter in adrenergic neurones, a variety of other materials may act as "false transmitters" under certain circumstances. These materials willbe stored and released in exactly the same way as noradrenaline, but their action at the post-synaptic receptor will be very different, being usually much less active than the endogenous transmitter. The presence of "false transmitters" thus causes an alteration of sympathetic function, which is in all observed cases an impairment. 4-Hydroxynorephedrine derived from 4-hydroxyamphetamine has been suggested to act in this way (see Gill et al.,1967).

It would be expected from the literature referred to that the side-chain hydroxylation of methamphetamine seen in both rat and guinea pig is carried out by dopamine-β-hydroxylase, but a consideration of the quantities involved and, in the guinea pig, the dose dependence of the reaction, renders this open to question. If an animal of 200g receives a dose of 45 mg/kg and excretes 20% of the dose as a sidechain hydroxylated metabolite, this will amount to some 2mg of that metabolite, which is a large amount to be produced by an enzyme located solely in the adrenergic neurones. In addition, in the guinea pig it has been shown that the amount of sidechain hydroxylation is dependent on the dose of the drug, increasing markedly with higher dose. If the hydroxylation was occurring solely in the adrenergic neurone, then this dose dependence suggests that proportionally more of the drug was either

penetrating into the nerve tissue, or that that activity of the metabolising enzymes within the nerve was rising, as the dose was increased. The situation is very hard to explain when only the nerve tissue and plasma are considered, but a more meaningful discussion is possible if a third compartment for drug distribution and metabolism is considered. This compartment is the liver and its drug metabolising enzymes. At the lower dose level, the drug is almost completely metabolised and the only metabolic reactions observed are those involved in sidechain breakdown, namely N-demethylation, deamination and benzoic acid production. The total production of amine metabolites is very small (<5%) and very little norephedrine detected (1%). However, at the higher dose level the metabolism is much less complete, about 4% being unchanged with 15% as amphetamine and 18% as norephedrine. It thus appears that at the higher dose the enzyme systems for deamination and sidechain breakdown are being saturated and therefore the plasma concentration of amines is higher, and if these enter the nerve endings and undergo sidechain hydroxylation, the amount of norephedrine produced will rise. This, however, is not convincing as it fails to explain the increase of the proportion of the amine metabolites excreted as norephedrine which is seen at the higher dose. In addition norephedrine is not a metabolite of amphetamine in the guinea pig (Lewander, 1971c) and if the explanation were then ephedrine would be expected as a minor metabolite of methamphetamine despite its subsequent N-demethylation.

It is therefore of interest to consider an alternative mechanism of sidechain hydroxylation. As has been outlined previously, one possible route for N-demethylation is through oxidative attack on the nitrogen, yielding an N-hydroxide, and an interesting rearrangement of N-hydroxides has been observed in the metabolism of 2-acetamidofluorene. This is initially N-hydroxylated and then is isomerised to the corresponding o-hydroxyacetamide (Booth and Boyland, 1964).

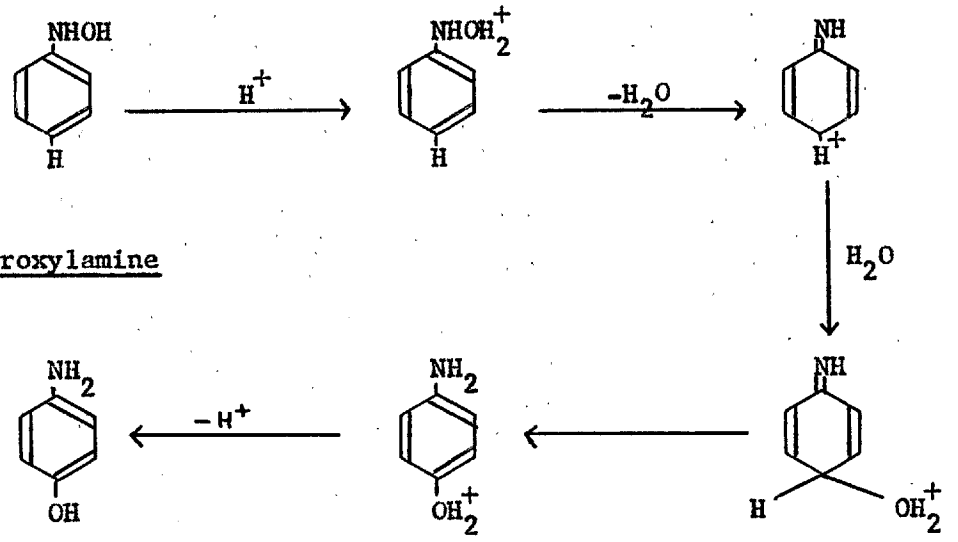


2-Acetamidofluorene

N-Hydroxy-2-acetamidofluorene

3-Hydroxy-2-acetamidofluorene

This is carried out by the soluble fraction of the liver, but similar rearrangements have been reported as chemical reactions, e.g. the isomerisation of phenylhydroxylamine to 4-aminophenol in dilute acid. The mechanism of this has been postulated to be as follows (Hughes and Ingold, 1951).

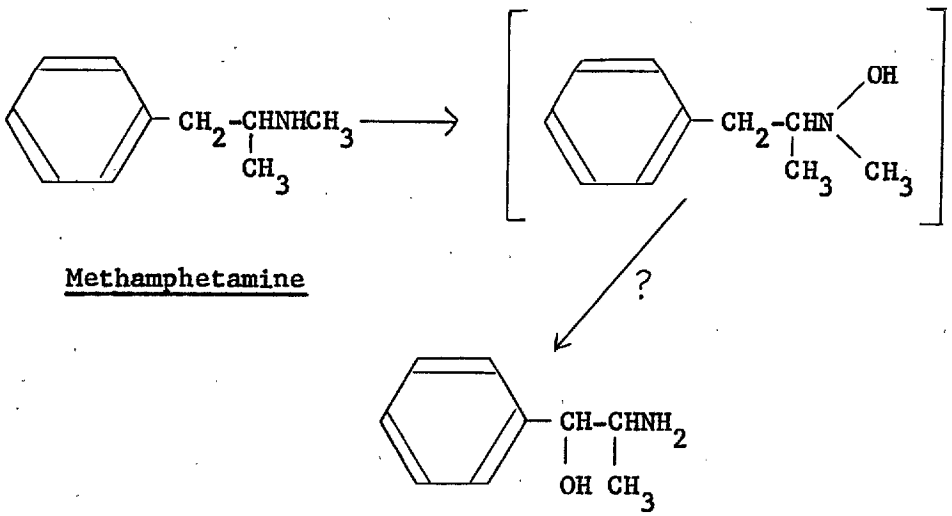


Phenylhydroxylamine

4-Aminophenol

Although these arrangements have only been observed in aromatic systems, it is not ridiculous to postulate a similar rearrangement of an

N-hydroxide of methamphetamine to give a sidechain hydroxylated product, as the methylene group next to the ring is in some ways a participant in an aromatic system. Such a rearrangement would involve loss of the methyl group to yield norephedrine, and in view of the vast increase in sidechain hydroxylation resulting from N-methylation of amphetamine it could be that the methyl group is involved in such a rearrangement.



Methamphetamine

Norephedrine

If it is indeed true that tissues other than adrenergic nerve endings are able to hydroxylate putative transmitters in the sidechain, then this may be of significance when considering the sites for, and mechanisms of, noradrenaline synthesis.

CHAPTER FOUR

BILIARY EXCRETION OF AMPHETAMINE AND METHAMPHETAMINE IN THE RAT

Chapter Four — The Biliary Excretion of Amphetamine and Methamphetamine in the Rat

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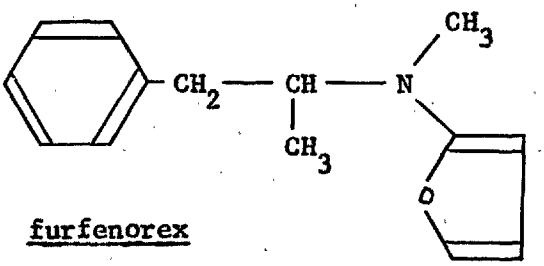
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Introduction

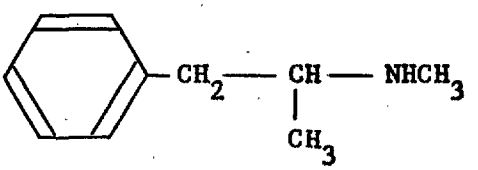
It is now well established that the bile may be an important route of excretion for a variety of drugs and foreign compounds (see Smith, 1966; 1970) and there is now some information on the multitude of factors affecting biliary excretion (see Millburn, 1970). No detailed work has been performed on the biliary excretion of the amphetamine group of drugs, but a number of brief notes in the literature indicate that this may occur.

In studies of the tissue distribution of methamphetamine labelled with ^{14}C in the N-methyl group, Vree and Van Rossum (1970) obtained autoradiograms of whole mice showing that radioactivity concentrated in the gall bladder to a far greater extent than in the surrounding liver tissue. Boissier et al.(1970) report that methamphetamine, both when produced as a metabolite of the anorectic drug, Furfenorex, by the rat, and when administered as itself, is excreted in rat bile and found in the duodenal contents.



furfenorex

(N-2-furyl methamphetamine)

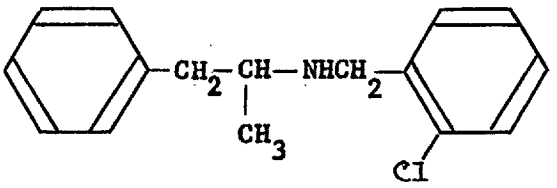


methamphetamine

The biliary excretion was not quantified and it is stated that the drug is not excreted in faeces but undergoes enterohepatic circulation, being readily reabsorbed in the gut.

Thomasset (1970) found that, in rats, 4.3% of a dose of [^{14}C]-amphetamine was excreted in the bile in 8h, and showed that the biliary radioactivity was almost exclusively composed of 4-hydroxyamphetamine.

In other experiments, the same worker found that 15% of a dose of [^{14}C]-clobenzorex was excreted in rat bile in 8h.



clobenzorex

(N-o-chlorobenzylamphetamine)

The principal biliary metabolites were conjugates of 4-hydroxy-amphetamine and clobenzorex or a hydroxylated derivative, with small amounts of clobenzorex, amphetamine and hippuric acid.

Beckett and Rowland (1965a) reported that the bile of a patient with a biliary fistula contained no unchanged amphetamine after the oral administration of (+)-amphetamine sulphate.

A full study of the biliary excretion of amphetamine and meth-amphetamine was carried out in the rat, to elucidate this phenomenon further.

Methods

(i) Preparation of Rats with Biliary Fistulae

Female Wistar albino rats, weight 200g ± 10g, were anaesthetized with thiopentone (Intraval, May and Baker Ltd., Dagenham, Essex), 70 mg/kg in water, given by i.p. injection. Cannulae were inserted in the common bile duct by the method of Abou-el-Makarem et al. (1967). The rats were kept for 24h in restraining cages (Bollman, 1948) with free access to a solution of dextrose (4.3%^{w}/v) and Na Cl (0.18%^{w}/v), and the animals kept warm with suitable lamps above the cages. Bile was collected in graduated tubes and urine in polythene trays under the cages.

Both labelled drugs were administered by i.p. injection due to the difficulty of giving oral doses by stomach tube after surgery. Both

drugs are powerful CNS stimulants and experiments showed that if the rats were dosed while still unconscious they awoke rapidly in a very agitated state and died quickly. Accordingly, they were allowed to recover from anaesthesia and left for 2h before injection of the drugs.

(ii) Radiochemical Techniques

Total ^{14}C in bile was determined by liquid scintillation spectrometry as previously described for urine and faeces. The nature of the urinary and biliary ^{14}C after both drugs was determined by radiochromatography as previously described (see Chapter 3).

The solvent systems used were A (3-methylbutanol:2-methylbutan-2-ol:water:formic acid;5:5:10:2 by vol) for paper chromatography and C (methanol:chloroform;1:1 by vol) for t.l.c.

Identification of Metabolites

(i) Amphetamine

Radiochromatograms of the 0-24h urine of biliary cannulated rats were prepared, using system A. Two major ^{14}C peaks were found, R_f 0.00 and 0.49, and two minor ones, R_f 0.26 and 0.81. Treatment with β-glucuronidase caused the large peak at R_f 0.00 to disappear, giving an increase in the peak at R_f 0.26, which corresponds to 4-hydroxyamphetamine. The large peak at the origin therefore appears to be the glucuronide of 4-hydroxyamphetamine. The large peak at R_f 0.49 corresponds to amphetamine, and the minor one at R_f 0.81 to hippuric acid (see Fig.4.1). The 0-24h bile was similarly chromatographed and the same four peaks found except that the one at the origin was the major peak, and the other three very minor ones (see Fig.4.2). The metabolites were quantified by cutting the papers into strips and the radioactivity determined by liquid scintillation spectrometry.

Fig 4.1 Radiochromatogram of 0-24 h Rat Urine after [^{14}C] Amphetamine sulphate (7.5 mg/kg ; 1.5 μCi) i.p., to a biliary cannulated rat, in System A

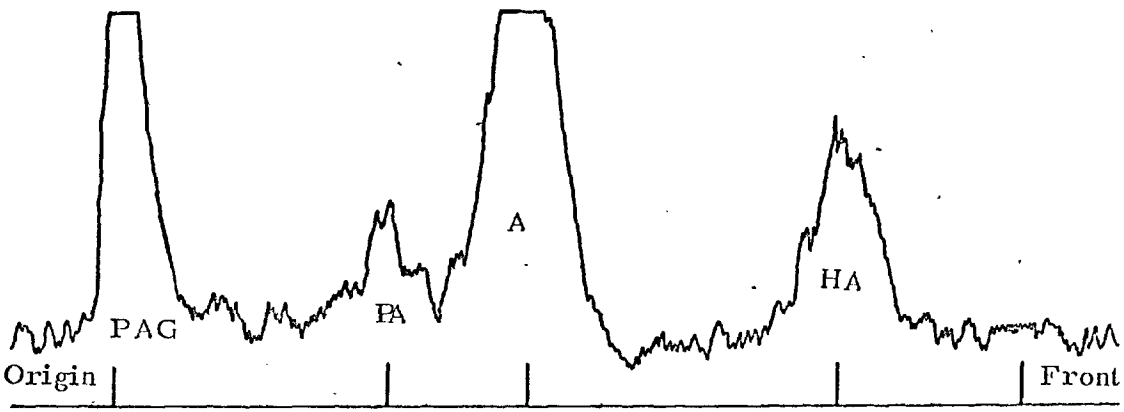
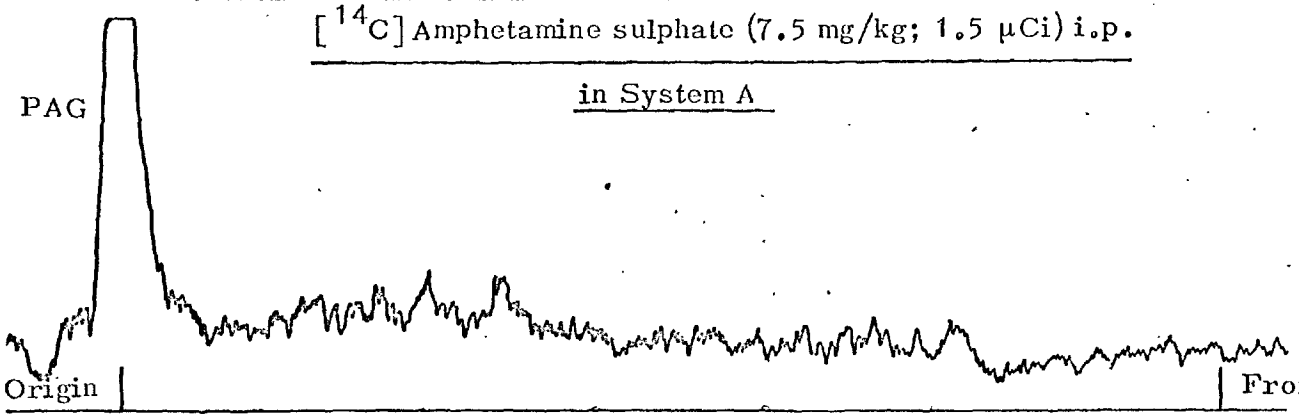


Fig 4.2 Radiochromatogram of 0- 24 h Rat Bile after [^{14}C] Amphetamine sulphate (7.5 mg/kg; 1.5 μCi) i.p. in System A



PAG = 4-hydroxyamphetamine glucuronide PA = 4-hydroxyamphetamine A = amphetamine HA = hippuric acid

(ii) Methamphetamine

The urine and bile were chromatographed on paper in solvent A and then on thin layer in solvent C as described in Chapter 3, and then scanned for ^{14}C. The bile (1ml used) gave, in solvent A, a broad peak of R_f 0.00 and three very minor peaks at 0.3, 0.49 and 0.82. On hydrolysis with HCl or β-glucuronidase, the peak of R_f 0.00 disappeared with an equivalent increase at R_f 0.3, whilst those at 0.49 and 0.82 were unaffected. Before hydrolysis the peak at R_f 0.00 gave a naphtharesorcinol test for glucuronic acid and that at 0.3 after hydrolysis gave positive tests for phenols. The material of R_f 0.0 was eluted with water and then hydrolysed with β-glucuronidase. The hydrolysate was chromatographed on thin layer in solvent C in which three ^{14}C peaks were found of R_f 0.04 (4-hydroxynorephedrine), 0.4 (4-hydroxyamphetamine) and 0.65 (4-hydroxymethamphetamine). The first peak contained enough material to give a purple colour for a phenol with diazotised p-nitroaniline. These peaks were scraped off the chromatogram and their ^{14}C estimated by scintillation counting.

The peak before hydrolysis of R_f 0.3 in solvent A was eluted with water and chromatographed on thin layer in system C. Three peaks, R_f 0.04, 0.4 and 0.65, were found corresponding to the above three phenols.

The peak of R_f 0.49 in system A was eluted similarly and chromatographed in solvent C. Two ^{14}C peaks were obtained, one at R_f 0.45 corresponding to amphetamine and the other at 0.8 corresponding to methamphetamine. The peak at R_f 0.82 in solvent A corresponds to hippuric acid.

Results

The excretion of amphetamine and its metabolites in the biliary cannulated rat over a period of 24h is shown in Table 4.1. In this period 68-70% of the dose is excreted in the urine and about 15% in the bile. The bile contains small amounts of amphetamine (0.9%) and hippuric acid (0.1% of the dose), but the main biliary metabolite is the glucuronide of

Table 4.1 The biliary excretion of amphetamine and its metabolites in the rat

[^{14}C] Amphetamine sulphate (7.5 mg/kg; 11.5 μCi/kg) was injected intraperitoneally in water into biliary cannulated rats. Urine and bile were collected as described in the text. The values quoted are the average for 3 rats with ranges in parentheses.

Material estimated		% of dose excreted in 24 h* Urine	Bile
Total ^{14}C excreted		69.1(68.6-70.1)	15.5(11.5-19.2)
Amphetamine		24.4(18.2-29.5)	0.9(0.7-1.0)
4-Hydroxyamphetamine	free	4.7(3.7-5.7)	2.2(1.2-2.8)
	conjugated	33.8(28.8-37.6)	12.4(8.2-17.0)
	Total	38.5(33.6-43.3)	14.6(10.7-18.2)
Hippuric acid		6.3(4.3-7.0)	0.1(0.1-0.1)
Sum of metabolites		69.2(68.6-70.1)	15.6(11.5-19.1)

*In the next 24 h little further ^{14}C was excreted and in two of the rats the values were, urine 0.9 and 2.8% and bile 0.2 and 0.3% of the dose.

Total recovery (urine + bile) in 24 h = 84.6(80.1-87.8)%

Table 4.2 The biliary excretion of methylamphetamine and its metabolites in the rat

[^{14}C]Methamphetamine hydrochloride (7.5 mg/kg ; 1.25 μCi/kg) was injected intraperitoneally in water into biliary cannulated rats. Urine and bile were collected as described in the text. The values quoted are the average of 3 animals with ranges in parentheses.

Material estimated		% of dose of ^{14}C excreted in 24 h	
		Urine	Bile
Total ^{14}C excreted*		53.7(43.5-70.0)	18.1(13.5-20.8)
Methamphetamine		16.2(9.2-27.4)	0.5(0-1.1)
Amphetamine		22.0(19.9-25.5)	1.8(0.3-2.6)
4-Hydroxymethamphetamine	free	1.1(0.5-1.8)	tr
	conjugated	5.3(5.0-5.5)	1.0(0-2.5)
	total	6.4(5.9-7.3)	1.0(0-2.5)
4-Hydroxyamphetamine	free	1.1(0.3-2.0)	0.1 trace
	conjugated	3.2(2.8-3.5)	0.9(0.7-1.0)
	total	4.3(3.1-5.4)	1.0
4-Hydroxynorephedrine	free	0.1(0-0.2)	1.8(1.0-3.8)
	conjugated	1.9(1.7-2.1)	11.6(8.4-14.2)
	total	2.0(1.8-2.1)	13.4(9.4-16.0)
Hippuric acid		0.0	0.4(0.3-0.5)
Unknown		2.9(2.4-3.3)	n.d.
Sum of metabolites		53.8(43.6-69.9)	18.1(13.5-20.9)

*About 6% more ^{14}C was excreted in the next 24 h in the urine.

4-hydroxyamphetamine (12.4%) together with a smaller amount of free 4-hydroxyamphetamine (2.2%).

Table 4.2 shows the results obtained with methamphetamine. In 24h about 54% of the dose is excreted in the urine and 18% in the bile. The main biliary metabolite in this case is the glucuronide of 4-hydroxynorephedrine which amounts to nearly 12% of the dose.

Discussion

Amphetamine

The excretion of amphetamine in the urine and bile of biliary cannulated rats, with its metabolites, is summarized in Table 4.3. In the cannulated animal there is an increased urinary excretion of unchanged amphetamine (25% of dose) compared with the intact rat (13%) and there is consequently a decrease in the output of total 4-hydroxyamphetamine. It would appear that the cannulation of the rats under thiopentone anaesthesia has to some extent inhibited the aromatic hydroxylation of amphetamine. It is probable that this is an effect of thiopentone since a similar inhibition of the hydroxylation of amphetamine has been observed in rats pretreated with certain anti-depressants and barbiturates (see Chapter 1).

These results are in agreement with those of Thomasset (1970) as to the biliary metabolites, but the total biliary excretion in these experiments is much higher. This may be due to this work using female rats and Thomasset using male rats. Female rats are known to excrete 2-3 times as much tartrazine in the bile as males (Gregson et al.,1972) so that the sex of the rats may be the cause of the discrepancy.

In the intact rat, little ^{14}C is excreted in the faeces after [^{14}C]-amphetamine, but the output of ^{14}C in the bile and urine of cannulated rats in one day is roughly the same as the output of ^{14}C in the urine of intact rats in two days. This suggests that the biliary metabolites of

Table 4.3 The metabolites of amphetamine in bile-duct cannulated and intact rats

The figures quoted are taken from Table 4.1 and Dring et al (1970) and given to the nearest whole number.

		Cannulated rats % of dose			Intact rats* % of dose
Metabolite	Mol. wt.	Urine	Bile	Total in 1 day	Urine in 2 days
^{14}C		69	16	85	85
Amphetamine	135	24	1	25	13
4-Hydroxyamphetamine					
free	151	5	2	7	7
conjugated	327	34	12	46	53
total	-	39	14	53	60
Hippuric acid	179	6	0	6	3
Sum of metabolites		69	16	85	76

*Data of Dring et al (1970)

amphetamine (some 16% of the dose) are reabsorbed and subsequently excreted in the urine. The main biliary metabolite appears to be the O-glucuronide of 4-hydroxyamphetamine which could be reabsorbed as such or after hydrolysis to the free phenol by the gut flora and/or secretions (see Smith and Williams, 1966). β-Glucuronidase activity is widely distributed among the intestinal bacteria (Hawksworth et al.,1971).

Methamphetamine

The summarized data for methamphetamine is shown in Table 4.4. As far as the metabolites are concerned the main difference between the cannulated animals and the intact ones is in the urinary output of amphetamine which is 22% of the dose in the cannulated rats and only 3% in the intact ones. The output of methamphetamine (17%) is only slightly higher in the cannulated as compared with the intact rats (11%). It would appear that, as suggested before, the ring hydroxylation of amphetamine is inhibited by the thiopentone used as an anaesthetic but not the demethylation of methamphetamine to amphetamine. It would appear also that there is an inhibition of the ring hydroxylation of methamphetamine for the output of total 4-hydroxymethamphetamine is only 7% in the cannulated compared with 19% in the intact rats. The output of total 4-hydroxyamphetamine is low and similar (5% and 6%) in both types of rats. The output of total 4-hydroxynorephedrine is also similar in cannulated and intact rats (15% and 16%). This would suggest that thiopentone anaesthesia tends to inhibit the ring hydroxylation of amphetamine and methamphetamine, but not the subsequent reactions of sidechain hydroxylation to give 4-hydroxynorephedrine or the demethylation of methamphetamine.

In the intact rat, very little ^{14}C is excreted in the faeces after [^{14}C]-methamphetamine (see Chapter 3) but in cannulated rats some 18% of the dose appears in the bile. A comparison of the data (Table 4.4) for cannulated and intact rats suggests that the metabolites excreted in the bile are reabsorbed and excreted in the urine. The main biliary metabolite

Table 4.4 The metabolites of methamphetamine in bile-duct cannulated and intact rats

The figures quoted are taken from Table 4.2 and Chapter 3 and given to the nearest whole number

Metabolite	Mol. wt. of metabolite	Cannulated rats % of dose			Intact rats* % of dose Urine	
		Urine	Bile	Total in 1 day	Day 1	Day 1 + 2
^{14}C	-	54	18	72		
Methamphetamine	149	16	1	17	10	11
Amphetamine	135	22	2	24	2	3
4-Hydroxymethamphetamine						
Free	165	1	tr	1	3	3
Conjugated	341	5	1	6	13	16
Total	-	6	1	7	16	19
4-Hydroxyamphetamine						
Free	151	1	tr	1	1	1
Conjugated	327	3	1	4	3	5
Total	-	4	1	5	4	6
4-Hydroxynorephedrine						
Free	167	tr	2	2	2	2
Conjugated	343	2	11	11	9	14
Total	-	2	13	15	11	16
Hippuric acid	179	0	0.4	0.4	2	4
Unknown	-	3	0	3	4	4
Sum of metabolites	-	54	18	72	47	74

*Data from Chapter 3. tr = trace

of methamphetamine is the glucuronide of 4-hydroxynorephedrine (12% of the dose) together with smaller amounts of the conjugates of 4-hydroxy-methamphetamine (1%) and 4-hydroxyamphetamine (1%). As mentioned before, these could be reabsorbed as such or after deconjugation in the gut.

This is in complete contrast to the work of Boissier et al.(1970) who report the excretion of significant quantities of methamphetamine in rat bile. However, the results obtained in this work accord far more closely with expectations based on other work in the literature, as will be seen.

The Main Biliary Metabolites

In an important paper in this field, Millburn et al.(1967) postulated that for significant biliary excretion (i.e. 5-10% of the dose) in the rat, compounds should be polar and possess a molecular weight of not less than 325±50. In the case of amphetamine, the only metabolite that meets these requirements is the O-glucuronide of 4-hydroxyamphetamine which is polar and has a molecular weight of 324. As shown in Table 4.3, its biliary excretion is 12% of the dose and therefore fulfils the above hypothesis. Very much smaller amounts of amphetamine (m.wt. 134) and hippuric acid (m.wt. 179) are found in bile, these being much smaller molecules, in accordance with Abou-el-Makarem et al.(1967) who showed that small aromatic molecules, among them hippuric acid, are poorly excreted in rat bile.

In the case of methamphetamine, there are three metabolites (see Table 4.4) which could meet the above criteria for significant biliary excretion, namely the glucuronides of 4-hydroxyamphetamine,-methamphetamine (m.wt. 341) and -norephedrine (m.wt. 343). However, only the last is excreted in bile to a significant extent (13% of dose), but it is formed in greater amounts than the other two.

The results show that although the 4-hydroxyamphetamine and

4-hydroxymethamphetamine glucuronides the urine:bile ratio is about 3:1 (after both amphetamine and methamphetamine), that for 4-hydroxynorephedrine glucuronide, resulting from methamphetamine, is about 1:6. This suggests that this compound has a higher affinity for biliary excretion than the other glucuronides. As the molecular weights of all three are approximately the same it would appear that the addition of the sidechain hydroxyl group may be responsible for this, especially since the possession of this group changes the physico-chemical characteristics of the aglycone markedly as compared with the corresponding deoxy compound (Vree et al., 1970).

Such differences in extent of biliary excretion between closely related molecular structures have been observed previously, and examples include compounds which differ by a free hydroxyl group, e.g.

Compound	% of dose in 3h rat bile
4-Hydroxybiphenyl glucuronide	56
4-glucuronosido-4'-hydroxybiphenyl	92 (Millburn et al., 1967)
Phthalyl sulphathiazole	19
Hexahydrophthalyl sulphathiazole	66 (Hirom, 1970)
Salicylic acid	1.5
Protocatechuic acid	14 (Williams et al., 1965)

It can be seen, therefore, that the biliary excretion of the metabolites of both amphetamine and methamphetamine in the rat fits in well with the hypothesis previously outlines.

The phenolic amine metabolites of both amphetamine and methamphetamine, although less toxic than the unchanged drugs, have a marked pharmacological activity (see Chapter 3). Since these metabolites may undergo enterohepatic circulation they will be cleared from the body more slowly than those undergoing strictly renal excretion. On repeated dosage with either drug, then, the phenolic amine metabolites may be accumulated in the body of the rat much more than would be expected if they were eliminated exclusively via the kidney.

The demonstration of an enterohepatic circulation of a drug does not, however, necessarily imply a prolongation of the pharmacological effect, even though the drug's life in the body is prolonged. The synthetic hormone stilboestrol is excreted in the bile of rats as a monoglucuronide conjugate and, after undergoing an enterohepatic circulation, it is eliminated in the faeces (Fischer et al.,1966). The glucuronide, after biliary excretion, is hydrolysed to stilboestrol by the gut flora (Clark et al.,1969). The stilboestrol can be reconjugated by the intestinal wall (Fischer and Millburn, 1970) as well as by the liver. The net effect of the two sites of metabolism is to confine the drug to the portal blood and intestine so that no free drug escapes into the systemic circulation. This is seen in the plasma half-life of stilboestrol being virtually identical in both cannulated and intact animals (Dr. P. Millburn-personal communication). In this instance, then, the enterohepatic circulation of this compound does not prolong its pharmacological activity, and it may be that this is the case with the amphetamines also.

It was observed in Chapter 3 that the guinea pig excretes a considerable amount of administered radioactivity in the faeces after a dose of methamphetamine at the higher dose studies (45 mg/kg). No explanation for this could be found, but it might be suggested that the faecal metabolites were being excreted in the bile prior to final elimination. The metabolites of methamphetamine found in the guinea pig range in molecular weight from 122 (benzoic acid) to 298 (benzoyl glucuronide). Preliminary indications suggest that the minimum molecular weight for significant biliary excretion in the guinea pig is about 400 (Abdel-Aziz et al., 1971) and it is therefore unlikely that the material found in guinea pig faeces had undergone biliary excretion prior to final elimination.

It is clear that in the rat an enterohepatic circulation of some metabolites of amphetamine and methamphetamine can occur to a certain extent. However, this is unlikely to occur in man, since the molecular weight threshold for biliary excretion is probably in the region of 500 (Millburn, 1970) which is much higher than that of any of the metabolites of these drugs.

CHAPTER FIVE

THE METABOLISM OF METHAMPHETAMINE AND AMPHETAMINE IN MAN

Chapter Five — The Metabolism of Methamphetamine and Amphetamine in Human Volunteers

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Introduction

The investigations carried out in animals have relied on chromatography as the prime method for the identification of metabolites, with isotope dilution, g.l.c. and g.c.-m.s. used as confirmatory techniques. In human studies, however, only small amounts of radioactivity can be given and the large urine volume results in a urine with insufficient activity for radiochromatography. Accordingly, isotope dilution was the major analytical method, with chromatography of certain extracts also employed.

As in animal studies, sidechain hydroxylated metabolites of methamphetamine were found in human urine, and these are of considerable pharmacological importance. Both the metabolites found, norephedrine and 4-hydroxynorephedrine, could be formed from amphetamine also, and a re-examination of the fate of amphetamine in man was undertaken, with especial reference to these compounds.

Identification of Metabolites

(i) Methamphetamine

Urine (50 ml) was adjusted either to pH 1 (with 10M-HCl) or to pH 14 (with 10M-HCl), saturated with solid NaCl and extracted with ether (3 x 75 ml). The bulked extracts were dried over anhydrous Na_2SO_4 and the ether removed at reduced pressure. The residue was taken up in ethanol (2 ml) and this used for radiochromatography. Extracts of alkaline urine contained 21-30% of the dose and on chromatography in system C, 3 radioactive peaks were seen, one large, with R_f 0.83, corresponding to methamphetamine, and two minor peaks with R_fs 0.47 and 0.99, corresponding to amphetamine and an unknown amine. In system D, the same 3 peaks were seen, a major one with R_f 0.22, corresponding to methamphetamine and two minor peaks of R_f 0.60 and 0.95, again corresponding to amphetamine and an unknown amine respectively.

Extracts of acidified urine contained 5-8% of the dose, and on chromatography in system A, one ^{14}C peak was seen, corresponding to hippuric acid. When chromatographed in system B, however, the extracts gave two radioactive peaks, a larger one of R_f 0.26 corresponding to hippuric acid and a smaller one of R_f 0.86 being an unknown acid.

All other possible metabolites not extracted in the above procedures were assayed by isotope dilution direct on the urine, after acid hydrolysis where apprpriate. In addition, the presence of hippuric acid was confirmed by isotope dilution.

(ii) Amphetamine

Isotope dilution was used as the principal analytical method, and 4-hydroxyamphetamine, norephedrine, 4-hydroxynorephedrine, benzylmethyl ketone and benzoic acid were measured with the previously described methods.

Amphetamine was estimated by extraction and chromatography. Alkaline urine was extracted as described for methamphetamine and a concentrate in ethanol obtained. Chromatography in system C gave a single radioactive peak, R_f 0.45 corresponding to amphetamine, and in system D one peak also was found, with R_f 0.61, again indicating amphetamine.

Results

(i) Methamphetamine

The excretion of radioactivity by two males after oral administration of ^{14}C -methamphetamine hydrochloride is shown in Table 5.1. The ^{14}C is eliminated largely in the urine, so the faeces were not examined. About 60% of a dose was excreted in the first 24 h, with another 20% appearing on day 2.

As in animal studies, four main metabolic pathways for methamphetamine were seen, N-demethylation, sidechain and ring hydroxylations and deamination, leading to sidechain breakdown. The major radioactive material found in urine was unchanged methamphetamine (about 23%) and the major metabolic reaction observed was ring hydroxylation, giving 4-hydroxymethamphetamine (15%), 4-hydroxyamphetamine (1%) and 4-hydroxynorephedrine (1-2%). N-demethylation totalled some 16% of the dose, only 2-3% of amphetamine being excreted. The amount of deamination was small, 6% of the dose, 1-2% of benzylmethyl ketone being found after hydrolysis, and 4-5% of hippuric acid being detected. Two minor unknown metabolites were detected on chromatograms, both being 1-2% of the dose. The unknown amine had R_f 0.99 in system C and 0.93 in system D, and the unknown acid had R_f 0.86 in system B. The results are shown in Table 5.2.

(ii) Amphetamine

Table 5.3 gives the excretion of ^{14}C by two male volunteers after oral administration of [^{14}C]-amphetamine sulphate, with data from previous work included for comparison. The ^{14}C is eliminated largely in the urine (over 80% in 2 days) with about 60% on the first day. The results for subject LGD are virtually identical with values obtained in similar experiments in 1966.

The major radioactive material found in the urine was unchanged amphetamine (18-20%) and the major metabolic reaction was sidechain breakdown giving benzoic acid (28% after acid hydrolysis). Ring hydroxylation was observed to a very small extent, giving 4-hydroxyamphetamine and 4-hydroxynorephedrine, and LGD produced twice as much of these metabolites (4.8%) as JC (2.7%). A small amount of an acid-labile precursor of benzylmethyl ketone was found (1-2%). Sidechain hydroxylation was observed, also to a minor degree, giving norephedrine (2-3%) and 4-hydroxynorephedrine (0.3%). The data is recorded in Table 5.4. Again the results obtained for LGD are very similar to those obtained in 1966, for those metabolites measured in both studies.

Table 5.1 The elimination of ^{14}C by two male volunteers after administration of [^{14}C]-methamphetamine

Each volunteer took 20 mg of [^{14}C]-methamphetamine hydrochloride (3 μCi) in water by mouth. Figures given are % of ^{14}C dose in the urine. Faeces were not examined.

Subject	JC	LGD
Body weight (kg)	77	70
In 0–24 h urine	67.8	54.9
24–48 h	21.1	23.2
48–72 h	5.5	7.4
72–96 h	1.2	2.8
Total ^{14}C recovery	96.6	88.3

Table 5.2 The metabolites of [^{14}C]-methamphetamine in human urine

Dose and route as in Table 5.1. Values given are % of dose administered excreted as that metabolite. The 0-24 h urine was examined, and for both subjects this had pH 6.7.

Compound	Subject JC	Subject LGD
Methamphetamine	27.2	18.1
Amphetamine	2.2	3.2
Norephedrine	1.7	2.4
4-Hydroxyamphetamine (total)	1.1	1.0
4-Hydroxymethamphetamine (total)	14.1	15.8
4-Hydroxynorephedrine (total)	1.2	2.2
Benzylmethyl ketone (before hydrolysis)	0	0
(after hydrolysis)	2.1	0.8
Hippuric acid	3.8	5.6
Unknown amine C	1.8	1.0
Unknown acid D	0.7	3.1
Total metabolites	55.9	53.2
Total radioactivity in 0-24 h urine	68.8	54.9

Table 5.3 The elimination of ^{14}C by two male volunteers after administration of [^{14}C]-amphetamine

Each volunteer took 20 mg of [^{14}C]-amphetamine sulphate (3μCi) in water by mouth. Figures given are % of ^{14}C dose in urine. Faeces were not examined. The third column gives results of a previous study on one of the volunteers (LGD) performed in 1966 (Dring et al., 1970) and is included for the purposes of comparison. The dose in the earlier study was 5 mg, 6 μCi.

Subject	JC	LGD	LGD (1966)
Body weight	77	70	70
In 0–24 h urine	56.9	63.6	61.3
24–48 h urine	24.5	21.1	23.8
Total ^{14}C recovery	81.4	84.7	84.1

Table 5.4 The metabolites of [^{14}C]-amphetamine in human urine

Dose and route as in Table 5.3. The values given are % of dose administered excreted as that metabolite. The 0-24 h urine was examined, and had pH 6.5 (JC) and 6.7 (LGD). The third column contains results of a previous study on one of the volunteers (LGD) performed in 1966 (see Table 5.3 for details).

	Subject		
Compound	JC	LGD	LGD (1966)
Amphetamine	18.0	20.0	22.9
4-Hydroxyamphetamine (total)	2.4	4.4	6.0
Norephedrine	2.2	2.6	-
4-Hydroxynorephedrine (total)	0.3	0.4	-
Benzylmethyl ketone (before hydrolysis)	0	0	-
(after hydrolysis)	0.9	1.7	1.3
Benzoic acid (total)	28.2	27.1	21.8
Total metabolites	52.0	56.2	52.0
Total radioactivity in 0-24 h urine	56.9	63.6	61.3

Discussion

The results obtained show that after both methamphetamine and amphetamine, radioactivity is found almost exclusively in the urine (81-96% recoveries) with about 60% in the first day after dosing. The pH of the urine was virtually constant in all four experiments, so the well-known influence of urine pH on excretion rate of the amphetamines (Beckett et al., 1965) was not of great significance in this case.

The metabolic pattern of these two closely related drugs in man is seen to be very different. The amount of unchanged drug recovered is very similar, for amphetamine 20% and for methamphetamine 23%. However, the amount of ring hydroxylation is very greatly increased by N-methylation, this reaction occurring to a small extent with amphetamine (2-4%) but being the major metabolic route of methamphetamine, accounting for some 17-18% of the dose. The amount of the precursor of benzylmethyl ketone produced from both drugs is very similar, 1-2% in both cases, but the extent of sidechain breakdown to benzoic acid is very different. The production of benzoic acid is the major metabolic route of amphetamine in man, accounting for some 20% of the dose, while this is at a very low level for methamphetamine, only amounting to 4%. Sidechain hydroxylation was found for both these drugs to a small extent. Norephedrine was produced in similar quantities (2%) from amphetamine and methamphetamine, and 4-hydroxynorephedrine amounted to 0.3% for amphetamine and 1-2% for methamphetamine. The difference in amount of 4-hydroxynorephedrine is probably a reflection of the difference in extent of aromatic hydroxylation of the two drugs.

The remarkable increase in ring hydroxylation resulting from the N-methylation of amphetamine is of great importance. The aromatic hydroxylation of amphetamine in 4 human subjects is given by Smith and Dring (1970) and a fifth subject (JC) can now be added. The figures are:

Subject	% of dose as 4-hydroxyamphetamine
RLS*	0.5
RFL*	0.7
SF*	1.9
LGD*	6.0
LGD**	4.4
JC**	2.4

* Dose 5 mg ** Dose 20 mg

Smith and Dring (1970) have concluded that there appear to be marked individual variations in the excretion of this metabolite, which could have a genetic basis. Data from the present study confirms that LGD is able to ring hydroxylate amphetamine to a greater extent than the other subjects, although JC has the next highest production of this metabolite. Whether there is any significance in these apparent individual variations in the production of this metabolite cannot be assessed on present data.

The ring hydroxylation of methamphetamine is of much more significance as a metabolic route than for amphetamine. The total hydroxylation is much greater (17-18%) and the values for the two subjects examined are very similar, unlike amphetamine. The N-demethylation of methamphetamine is very small, and thus the major ring hydroxylated metabolite is 4-hydroxymethamphetamine. The ratio of N-methylamine to noramine is very similar for both ring hydroxylated and non-hydroxylated compounds, indicating the small extent of N-demethylation. The figures are:

	% in that form		
Methamphetamine (MA)	20-25		
Amphetamine (A)	2-3	MA/A (approx)	10
4-Hydroxymethamphetamine (HMA)	15		
4-Hydroxyamphetamine + 4-Hydroxy-norephedrine (HA+HNE)	2-3	HMA/HA+HNE (approx)	7

It is thus seen that the N-demethylation is not greatly influenced by the ring hydroxylation of methamphetamine to any appreciable extent. The poor demethylation of methamphetamine would be expected as the amount of amphetamine excreted after both methamphetamine (Beckett and Rowland, 1965b) and ethylamphetamine (Beckett et al.,1969) has been shown to be low (see Chapter 1).

Previous work on the metabolism of amphetamines has shown that N-substitution increases the extent of metabolism of the racemic compounds. About 30-40% of a dose of amphetamine is excreted unchanged (Beckett and Rowland, 1965a; Dring et al.,1970), but after methamphetamine only some 25% of the dose is excreted as unchanged drug plus amphetamine. Studies on the N-substituted amphetamine, N-3-chloropropylamphetamine, have shown that the extent of ring hydroxylation is greatly increased (Long, 1970) by this structural modification. Beckett et al.(1969) have concluded that the extent of the metabolism of the amphetamines is increased by N-substitution, and that the extent of metabolism rises with the size of the N-alkyl group. The following table shows that this is true and that a pathway of major significance in the explanation of these observations is ring hydroxylation.

	% of dose as	
	Unchanged drug	Ring hydroxylated metabolites
Amphetamine	30-40	0.5-6
Methamphetamine	20-25	15
N-3-Chloropropylamphetamine	< 1	37

Accompanying this increase in the importance of ring hydroxylation with N-alkylation is a concomitant decrease in the extent of benzoic acid production.

Sidechain hydroxylation giving norephedrine and 4-hydroxynorephedrine has been detected after both amphetamine and methamphetamine.

Sjoerdsma and von Studnitz (1963) detected 4-hydroxynorephedrine as a metabolite of 4-hydroxyamphetamine in man, to the extent of 4-9% of the dose. Subsequent to this, Gill et al.(1967) found that the administration of 4-hydroxyamphetamine to normotensive subjects impaired sympathetic function and attributed this to the formation of 4-hydroxynorephedrine from the drug. As outlined in Chapter 3, this metabolite is able to act as a false transmitter at adrenergic nerve endings, thus giving a partial sympathetic blockade. Dring et al.(1966) first reported the formation of 4-hydroxyamphetamine from amphetamine by man, and in semiquantitative experiments Cavanaugh et al.(1970) observed that 4-hydroxynorephedrine is a metabolite of amphetamine in man. The present work is in agreement with the findings of Cavanaugh et al., the ratio of 4-hydroxynorephedrine to 4-hydroxyamphetamine being about 1:10 in their experiments and 1:5-1:10 in this work. The production of norephedrine from amphetamine is observed for the first time and the ratio of norephedrine to amphetamine is, as for their ring hydroxylated analogues, about 1:10.

The sidechain hydroxylation of methamphetamine giving the norephedrines does not appear to be related to the 4-hydroxyamphetamine and amphetamine production as above, but is in the same relationship to the corresponding N-methylated compound. Thus the ratio of norephedrine to methamphetamine + amphetamine is about 1:10, and similarly that of the 4-hydroxy analogues of these compounds is about 1:10.

It is therefore possible to suggest that, when the amphetamines undergo sidechain hydroxylation, the ratio of sidechain hydroxylated metabolite to its deoxy analogue will be about 1:10.

It is of interest to consider possible reasons for the amount of sidechain hydroxylated metabolite being related to the amount of N-methylamines found, rather than the levels of noramines, which are the products of sidechain hydroxylation. Man is apparently a very poor demethylaterof this compound as compared with rat and guinea pig, and there is a profound physicochemical difference between the primary and secondary amines. The

secondary amines have very similar pK_as to the primary amines, but are markedly more lipid soluble (Vree et al.,1969). In Chapter 3 the possible pathways for the sidechain hydroxylation have been elaborated, and work in the literature would suggest that this reaction is mediated by dopamine-β-hydroxylase. In animals, it can be suggested that this not necessarily true, the doubts hingeing on the apparent dose dependence of the reaction in the guinea pig and the quantities of drug undergoing this reaction. However, in man at the dose level used, these objections do not appear to be important and it seems reasonable to assume that the sidechain hydroxylation is carried out by dopamine-β-hydroxylase located in sympathetic nerve endings. Nerve tissue contains a very high proportion of lipid material and thus the more lipid soluble secondary amines will accumulate within the nerve to a greater extent than the primary amines. If the nerve tissue is able to N-demethylate as well as sidechain hydroxylate these compounds, then the two reactions may be linked by virtue of the lipid solubility of the materials. The ability of brain tissue to N-demethylate pethidine and morphine has been reported previously (Clouet,1964).

Several authors have suggested a causal link between the production of false transmitters from amphetamines and the development of tolerance to these agents (Gill et al.,1967; Brodie et al.,1970; Cavanaugh et al.,1970) and the present study has shown that false transmitter metabolites are produced from both amphetamine and methamphetamine by man. The amounts formed from each drug are similar in the case of norephedrine, but some five times more 4-hydroxynorephedrine is formed from methamphetamine than from amphetamine. As 4-hydroxynorephedrine fulfils the criteria for a false transmitter better than does norephedrine (see Musacchio et al.,1965; Kopin,1968) then it may be that the higher production of this metabolite is related to the qualitative differences between amphetamine and methamphetamine when given chronically (Kosman and Unna,1968). It appears that the stimulant effects of amphetamine are unaffected by prolonged administration, whereas methamphetamine rapidly loses its stimulant properties under these conditions.

It may be, therefore, that the production of this metabolite, 4-hydroxy-norephedrine, is in some way related to the very great dangers of methamphetamine from the viewpoint of drug dependence (Kramer et al.,1967; O'Connor,1968).

CHAPTER SIX

DISCUSSION

Chapter Six Discussion

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Methamphetamine and Drug Dependence

Drug dependence is at present pandemic in civilised society and despite the presence of tolerated pockets of such usage since prehistory. the current situation has arisen during the last 20 years. As a preliminary to a consideration of methamphetamine in detail, it is first necessary to look at the field in general.

The term drug is used for any chemical substance which alters mood, perception or consciousness and is misused to the apparent detriment of society (Laurie,1967). Since prehistoric times, both primitive and civilised man has utilized a number of agents with these properties for a variety of purposes - escapism, pleasure, divination, magic, prophesy, warfare, rituals and orgies (Louria,1968). Manifold examples can be given of this type of drug usage. Probably the best known is the use of hallucinogenic mushrooms by Central and South American Indians in religious activities. Usage of what are now considered dangerous drugs has already been extensive in the underdeveloped countries. In the Orient, there is pandemic dysentery and the constipating effect of the opiates is used to combat this; the drug also causes lack of awareness of hunger and physical discomfort. In South America, cocaine, from the chewing of coca leaves, has been used since prehistory for very similar purposes. Regrettably, when European nations have annexed such territories, they have seen fit to encourage such drug use for their own ends, rather than remedying the causes underlying the problem. In Bolivia, Amerindian gold miners were given coca leaves while the Conquistadores sent the gold home to the King of Spain, while in the 19th. century, the British fought two Opium Wars (1839-1842 and 1856-1860) with China to retain the right to move opium into China from India. The opium was used for the purposes previously outline.

In general, Western society has accepted this use of dangerous drugs around the world, especially as it has been principally in the backward countries. As late as the Second World War, Indian soldiers in the Allied

forces who were opium users received a ration of the drug regularly. In the last 20 years, however, the endemic nature of this drug usage has changed, reaching epidemic proportions in many parts of Western society, especially among young people. The U.S.A., Japan, the Scandinavian countrie and Great Britain have all been hit severely by this, and, despite the sensational reporting of the problem in the mass media, the situation in the U.K. is much better than in many other nations.

Drug dependence has been defined on behalf of W.H.O. by Eddy et al. (1965) as "a state of psychic or physical dependence, or both, on a drug, arising in a person following the administration of that drug on a periodic or continuous basis". They define seven categories of dependence by the types of agents producing them:

1 - Opiates

2 - Barbiturates and Ethanol

3 - Amphetamine

4 - Cocaine

5 - Cannabis

6 - Hallucinogens, especially LSD

7 - Khat (an East African plant with mild stimulant properties)

All these have a high abuse potential in that they have potent actions on the psyche, stimulant or depressant, which make them attractive to various people depending on their mental state. The effects will depend on the personality of the subject: this is well exemplified by ethanol, which has an inbitory effect on the brain, especially on the higher centres, and thus allows the cruder instincts to overcome the conscious control normally exercised.

Amphetamine dependence, according to Eddy et al., is characterised by (a) a variable psychic dependence; (b) no physical dependence and, consequently, no characteristic abstinence syndrome, though withdrawal will be followed by a state of mental and physical depression as the organism escapes from persistent stimulation; and (c) slow development of a consider able degree of tolerance to many effects by the CNS, though not participated

in equally by all parts of the CNS, so that nervousness and sleeplessness persist and psychotoxic effects such as hallucination and delusion occur. Although it is now obvious that the amphetamine drugs are very dangerous, and have no real therapeutic application (Dunlop,1970), it has taken some 35 years for this to become well-known. In the U.S.A., Davidoff and Reifenstein (1937) and Guttmann and Sargent (1937) in the U.K. both warned that the euphoriant action of these drugs, at that time recently introduced, would lead to habituation and dependence. In 1939, Guttmann reported that tolerance was developed to these agents. However, they remained on sale to the general public without prescription until 1955 and not until 1968 were they further restricted on a voluntary basis, and their prescription discouraged. Methamphetamine is only available to hospitals in the U.K. since October 1968 (Lancet Editorial, 12th Oct.,1968) and it is now very doubtful whether it has any place in orthodox therapy. Abuse continues, nonetheless, and has been, and still is, a most serious problem, particularly in the countries mentioned previously.

The basis of amphetamine abuse is the mood elevation produced and the state of well being induced, and both frequency and size of dose are increased to maintain this CNS stimulation. With large doses, the psychotoxic effects often lead to aggressive and dangerous behaviour,anti-social in nature, and a syndrome has been observed which is clinically indistinguishable from schizophrenia (Connell,1958).

The ability of the amphetamines to induce tolerance to the CNS stimulation is a quality possessed by very few other drugs with similar actions, and this permits doses of many hundreds of times the therapeutic amount to be given, if dosage has been sufficiently prolonged to build up tolerance. Although tolerance develops to the stimulant effect, nervousness and insomnia continue and increase with dose. It is essential to distinguish between the "spree abuse" of teenagers, used to stay awake at weekends and pop festivals, and the true dependence associated especially with i.v. injection of the drug.

Of all the amphetamines, methamphetamine is probably the most dangerous (O'Connor,1968) and is particularly associated with injection rather than oral dosage. The large doses giving rise to psychotic symptoms and bizarre behaviour, especially sexual activities, are almost exclusively given by injection (Rylander,1971). The pattern of methamphetamine abuse has been described by Kramer et al.(1967). Subjects begin by taking oral doses, rising from the therapeutic level of 10-20 mg three times a day to some 150-200 mg per day. To improve the quality of the drug experience further, the subjects then progress to i.v. injection. Tolerance develops very quickly and dose size and frequency increase rapidly. Doses are injected frequently (about every 2h) for several days, the size varying from 100mg-1g. The highest recorded daily intake under these conditions is 15g. Toxic symptoms then manifest themselves, giving profound lethargy and paranoia. To control these, dosage is stopped and in many cases barbiturates or opiates used to control the paranoia. Lack of food and sleep also exaggerates the psychic symptoms. Exhaustion results in sleep for 18h-5 days. On waking, there remains a pronounced lethargy, which predisposes to renewed drug use, and the cycle repeats itself. Tolerance to the drug is complete after about 5 days of dosing, and the desirable effects disappear, so the persons inject very large doses in syrupy solution which give a large bolus of the drug to obtain the stimulant effect, which goes on dilution in the circulation.

If an attempt is made to give up drug use on this scale, an abstinence syndrome is seen, involving lethargy, exhaustion and sleep of a duration suggesting that it is due to neurochemical changes induced by the drug.

Animal experiments have shown that this paradoxical depression resulting from prolonged dosage with a CNS stimulant is not confined to man, and this work has been reviewed by Kosman and Unna (1968). They conclude that chronic administration of methamphetamine to rats and mice leads to a depression of activity, and Utena et al.(1959) have also commented that prolonged dosage of guinea pigs with methamphetamine leads to passive

behaviour and loss of response to external stimuli. On withdrawal of the drug from all three species, the observed hypoactivity continues and lasts for up to three weeks. Despite the similarity of the effects of amphetamine and methamphetamine, it is of interest to note that there is conclusive evidence that the stimulant effect of amphetamine does not decrease with repeated dosage (Kosman and Unna,1968) and there is no depression on withdrawal.

Methamphetamine Tolerance - an Hypothesis

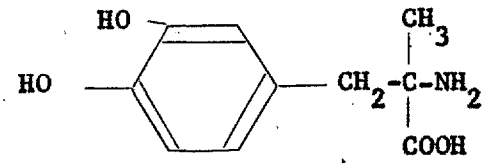
An attempt can now be made to discuss some of the observed effects of methamphetamine and amphetamine administration in the light of the metabolic data recorded in this thesis and elsewhere.

In mammals, noradrenaline is the principal mediator at the post-ganglionic terminal of sympathetic nerves, but other amines with closely related structures can replace noradrenaline and act as false transmitters and impair the function of the adrenergic nerve endings, as previously outlined in Chapter 3.

The two main methods of identifying false transmitters are (a) by location of the false transmitter in the noradrenaline-containing fraction of nerve endings, and (b) by the liberation of the false transmitter on stimulation of sympathetic nerves, after previously dosing with the false transmitter or a precursor. It is possible to consider two approaches to the accumulation of a false transmitter in nerve endings. A false transmitter is by definition an amine with structure closely related to the true transmitter, noradrenaline, which is able to be stored, released and taken up again by the nerve ending in the same way as noradrenaline (see Chapter 1). The accumulation of such materials is possible because of the non-specificity of the storage, release and uptake processes, and can be brought about by dosage with the false transmitter or, due to the non-specificity of the noradrenaline synthesizing enzymes, by the administration of a suitable precursor, which may be an amino acid or an amine.

The Amino Acid Approach

The synthesis of noradrenaline is markedly stereospecific for L-amino acids, but a variety of unnatural L-amino acids may be converted to false transmitters by virtue of the low substrate specificity of DOPA decarboxylase and dopamine-β-hydroxylase. False transmitters thus derived from amino acids may accumulate in the adrenergic nerve terminals in the brain and in the periphery and may interfere with sympathetic function. Secondary effects of the displacement of the true transmitter may influence noradrenaline synthesis rates (Kopin, 1971). Some of the pharmacological effects of dosage with unnatural amino acids may be explained on the basis of the adrepergic blockade created in this way. The best example of this is α-methyl DOPA, the widely used hypotensive drug.



This was originally introduced as a DOPA decarboxylase inhibitor, but is now thought to act through its metabolite, α-methyl noradrenaline, produced from it in the same way as is noradrenaline from DOPA (see Fig.1.1).

Amine Approach

Amino acids may be precursors of false transmitters, and in the same way, suitable amines may be taken up, stored and released as such or after metabolism to a false transmitter.

The example of 4-hydroxyamphetamine has been given before in this thesis: this amine is converted to 4-hydroxynorephedrine, which is thought to be responsible for the loss of adrenergic function seen on prolonged dosage with 4-hydroxyamphetamine, and in support of this, Rango et al.(1970) have found that 4-hydroxynorephedrine has a hypotensive effect when given orally to hypertensive patients. Amphetamine itself may be metabolised to 4-hydroxynorephedrine which could perhaps act in the same way.

Metaraminol (3-hydroxynorephedrine) may be taken up in the sympathetic nerve endings and released on nerve stimulation, giving an impairment of adrenergic function sufficiently great to give a useful antihypertensive effect in man (Crout,1966).

Evidence abounds in the literature that the sidechain hydroxylated metabolite of methamphetamine and amphetamine, 4-hydroxynorephedrine is able to act as false transmitter (see Musacchio et al.,1965; Thoenen et al.,1966). As well as this, all the amine metabolites and both unchanged drugs are excellent substrates for the noradrenaline uptake pump of sympathetic nerves, and can also disrupt adrenergic function by acting at this site (see Iversen, 1967).

Evidence for Norephedrine being a False Transmitter

The evidence for norephedrine being able to act as a false transmitter is somewhat equivocal. The structural requirements for storage within the catecholamine storage granules have been outlined in Chapter 1, and it has been shown that the β-hydroxyl group in the sidechain of transmitter materials is essential for their storage. The granule consists of dopamine-β-hydroxylase, a nucleoside triphosphate usually ATP, calcium ions, chromogranins and the transmitter molecules. "In vitro" work by Da Prada et al. (1971) appears to indicate that the granule structure depends on nucleotide-transmitter aggregates which are stabilized by expanded intragranular proteins (the chromogranins).

Weiner and Jardetzky (1964) have studied the ATP-catecholamine interaction, and conclude that the basis of this is ionic linking between ATP and the amino group of the transmitter, and hydrogen bonding involving the sidechain hydroxyl group of the amine. Thoenen et al.(1966) report that although they were unable to detect norephedrine release from cat spleen on sympathetic stimulation, this metabolite of amphetamine was present in homogenates of the organ, and they do not preclude the possibility of it being released on nerve stimulation. The quantities of norephedrine in their experiments were very small, and may thus have escaped detection.

Norephedrine may be produced both "in vivo" and "in vitro" from amphetamine by the action of dopamine-β-hydroxylase, and is therefore able to interact with this protein constituent of the granule. On this evidence, norephedrine has structural features which could enable it to participate in granule formation and it is therefore possible to suggest that it may perhaps be a false transmitter, with a lesser affinity for the various processes involved in this action than 4-hydroxynorephedrine.

When methamphetamine is taken, it has many actions at the sympathetic nerve ending alone. It releases noradrenaline, inhibits MAO, thus prolonging the life of the released noradrenaline, and may have some action direct on the postganglionic receptor membrane. It acts directly on the noradrenaline uptake pump, competing with the noradrenaline and prolonging the active life of the transmitter within the synapse. In addition to these effects of the unchanged drug, the metabolites, amphetamine and the ring and sidechain hydroxylated products, have all or some of these actions. Iversen (1967) has measured the relative affinities of a number of these compounds for the noradrenaline uptake pump.

Compound	Relative affinity for uptake site (phenethylamine = 100)
(±)-4-hydroxyamphetamine	610
(±)-amphetamine	240
(+)-methamphetamine	165
(±)-norephedrine	55
(-)-noradrenaline	407
(-)-adrenaline	110

The false transmitter metabolites will be formed, probably within the nerve ending, and be stored there in place of noradrenaline, causing a stoichiometric reduction in the noradrenaline stores (Brodie et al., 1970). These false transmitters will then be released on nerve stimulation and either taken up again into the nerve ending, or lost into the circulation, from

which it may be excreted unchanged, metabolised further and then excreted, or taken up into other nerve cells. The uptake of the false transmitter molecules from the circulation into nerve endings is suggested by the findings that metaraminol (Crout et al.,1964) and ephedrine and its 4-hydroxylated metabolites (Bralet et al.,1971) may be taken up from the circulation into the adrenergic nerve endings. This will result in the dissipation of the false transmitter, and the net effect of the drug will be a stimulation due to the noradrenaline released, and the prolongation of the life of this noradrenaline by the mechanisms outlined.

It is, however, possible to suggest that this situation will be markedly altered when repeated doses are given. As more drug is presented for metabolism, more false transmitters will be formed which will replace more and more noradrenaline, and begin to affect the function of the sympathetic nerves. This will be especially so if the sidechain hydroxylation reaction is dose dependent in other species in the way it is in the guinea pig (see Chapter 3). With higher and higher doses the inhibition of MAO will be greater, as will be the interference with the noradrenaline uptake pump.

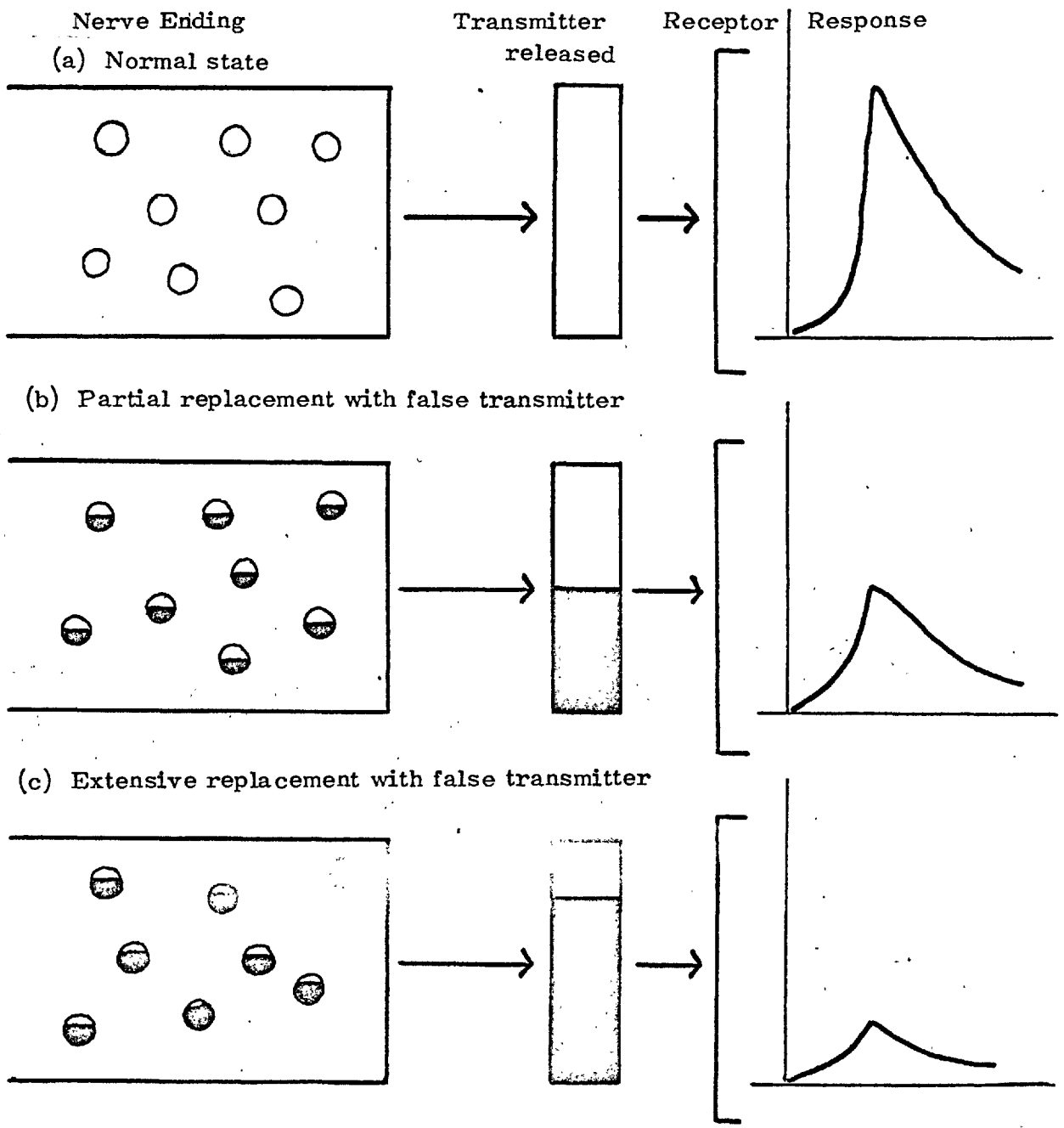
When MAO is inhibited, the lifetime of noradrenaline in the synapse is increased, and it would be expected that this would give a stimulant effect, with CNS excitement and elevated blood pressure among its effects. However, if an MAO inhibitor is given for a period of time (in humans, 2-3 weeks) then it has a sedative effect and gives a clinically useful reduction in blood pressure. Compounds whose prime action is to inhibit MAO find a wide clinical application for these actions (Goodman and Gilman,1965). Sjoerdsma et al.(1959) reported that when MAO is inhibited the excretion of a number of amines, such as tyramine, is increased. Kakimoto and Armstrong (1962) demonstrated that chronic MAO inhibition is associated with an accumulation of octopamine in the tissues of the rabbit, and Kopin et al. (1965) have shown that this octopamine is able to function as a false transmitter, and that the replacement of noradrenaline by this amine explains the apparent sympathetic blockade seen after MAO inhibition.

The significance of MAO inhibition by methamphetamine and amphetamine is great, as with both drugs a characteristic pattern of noradrenaline metabolites is seen, with suppressed levels of deaminated catechols and increased methylated metabolite levels (Cook and Schanberg,1970; Iversen et al.,1966).

On repeated dosage, then, there will be more and more false transmitters formed from the drug; in addition, it is possible to suggest that the endogenous false transmitters resulting from MAO inhibition may also contribute significantly to an interruption of adrenergic nerve function. When each dose of the drug is given, the methamphetamine discharges the materials stored in the sympathetic nerve endings, which will be a mixture of false transmitter and noradrenaline, the proportion of the false transmitter rising with the length of exposure to the drug and increasing dose size. The excitation resulting therefore progressively decreases, as the proportion of false transmitter rises, until it becomes minimal (see Fig.6.1). As normal body function depends on the balance of sympathetic to parasympathetic stimulation in the autonomic nervous system, then the blockade of one wing leads to the predominance of the other, in this case the parasympathetic, resulting in a general behavioural and physical depression. It has been shown (Brodie et al.,1970) that the effects of 4-hydroxynorephedrine as a noradrenaline-depleting agent are long lasting.

In animals, this is identical with the behavioural pattern on prolonged dosage with methamphetamine. In humans, the depression and lethargy grow so great that sleep ensues. It has been found that, in chickens, methamphetamine dosage increases the activity of tyrosine hydroxylase, the rate limiting step in noradrenaline biosynthesis (Mandell and Morgan,1970). This is presumably due to a lowering of the free noradrenaline within the neurone by the drug competing for dopamine-β-hydroxylase, and this activating the negative feedback loop for tyrosine hydroxylase (see Chapter 1), this involving, perhaps, new enzyme synthesis (Mueller et al.,1969).

Fig 6.1 The Effect of Replacement of the Natural Mediator with a False Transmitter on the Efficiency of Synaptic Transmission



When the drug is withdrawn the false transmitters and other materials interrupting nerve function will be cleared from the body, and there will be a rapid rise in noradrenaline levels, due to the high level of tyrosine hydroxylase in the neurone. It has been suggested that such large increases in noradrenaline in the CNS cause behavioural depression (Mandell and Spooner, 1968). This is therefore a possible explanation for the depression seen in man after awakening from the withdrawal sleep, and this depression predisposes to further stimulant use. The rapid repletion of noradrenaline stores means that when the drug use recommences, there will be a stimulant effect due to noradrenaline release. This will further reinforce the continued use of the drug. When more and more doses are taken, however, the stimulant effect will disappear as the nerve function is altered and the pattern of events outlined before repeats itself.

It has been stated previously that the tolerance which develops to the amphetamines is not general throughout the CNS. The anorexigenic effect is very short lived, while insomnia persists with prolonged dosage. It appears that methamphetamine is different from amphetamine in that it loses its CNS stimulant activity on repeated administration. The hypothesis outlined in this chapter explains some aspects of tolerance to methamphetamine on the basis of the production of false transmitter metabolites from the drug. It is known that the metabolites are not produced from amphetamine in the same quantity, and this can be suggested to be the reason for the differing effects of the two drugs when given chronically.

Tolerance to the anorectic effect of amphetamine and methamphetamine is developed quickly, and it may be that the mechanism of this tolerance is different from that causing behavioural depression. It has been suggested that false transmitters arising from inhibition of MAO play a role in the aetiology of tolerance, and it may be that this mechanism is responsible for the development of those tolerances common to both drugs (both of which are MAO inhibitors and thus give rise to these endogenous false transmitters) while the behavioural depression found only after methamphetamine is due to the accumulation of false transmitters derived from the drug.

Limitations of this Hypothesis

Having stated a case for the results of this work being of significance in explaining tolerance produced to methamphetamine, it is necessary to give some of the more obvious limitations.

It has been assumed that the central and peripheral adrenergic neurones are identical and equally accessible to the drug and its metabolites. The ability of the various compounds to penetrate into the brain is different, unchanged amines crossing from plasma into brain easily and the ring and sidechain hydroxylated metabolites crossing much less well (Lewander,1971a).

Secondly, and more important, the whole discussion is based on the assumption that methamphetamine and its metabolites act solely on the adrenergic nerve endings. As has been indicated, methamphetamine inhibits MAO, but at least three other neurotransmitters, dopamine,tryptamine and 5-hydroxytryptamine (see Hebb,1970) are substrates for this enzyme also, and the function of nerve endings using these transmitters may be influenced greatly by the action of methamphetamine. In addition, the activity of methamphetamine on the release and function of the inhibitory transmitters recently found in the CNS (see Hebb,1970) has not been considered.

It is not known whether the present views on the role of noradrenaline within the CNS are correct. In the final analysis, we cannot at the moment say where or how a drug acts, only measure various gross parameters of its action, which may or may not be of significance in interpreting its true mode of activity. The present thesis describes work which began as an exercise in drug metabolism, investigating the effect of chemical structure on drug metabolism, and which has yielded results which have been used in the construction of an hypothesis which attempts to explain the genesis of tolerance to methamphetamine.

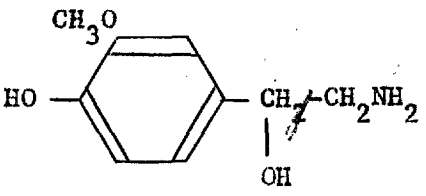
It has been said that the test of an hypothesis is not whether it is true, but whether it generates good experiments, and it can be suggested that the hypothesis outlined here is open to proof or disproof by experiment.

However, despite a perfect knowledge of the biochemistry and pharmacology of the drugs of dependence, and however good the treatment devised as a result of this knowledge, which may come about in the future, we will be no nearer to an understanding of the reasons which cause a person to use drugs, and to return to drug use after a cure. Perhaps if this understanding is ever obtained, we will know more about people and society as well as about drugs, and through this knowledge come to abolish the factors, whatever they be, which bring about drug abuse.

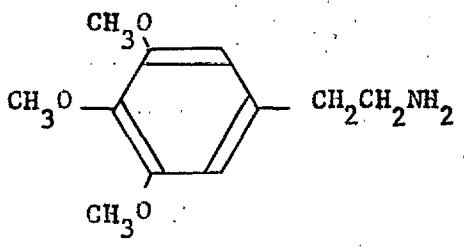
Schizophrenia and the Amphetamine Psychosis

A number of compounds have been found to give, in man, CNS effects very similar to those seen in acute schizophrenia, that is visual and auditory hallucination, thought disorder, withdrawal and paranoia. Such agents have been called psychotomimetic, as they are able to mimic the psychotic state. The most potent of these materials is lysergic acid diethylamide (LSD) but a wide variety of materials have this action. As has been noted earlier, one of the psychotoxic effects of the amphetamine is the production of a psychosis clinically indistinguishable from schizophrenia, and this perhaps suggests that the amphetamine and psychosis and schizophrenia have a common aetiology.

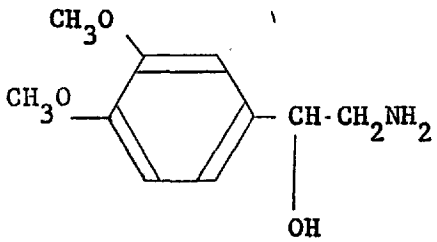
In 1952, Osmond *et al*. postulated that schizophrenia might be due to an abnormal *O*-methylation of noradrenaline, to give a psychotomimetic material structurally related to the potent hallucinogen, mescaline.



normal noradrenaline metabolite



mescaline



abnormally methylated noradrenaline metabolite

Subsequent to this, Friedhoff and Van Winkle (1962) tentatively identified such an abnormally methylated amine in the urine of schizophrenics, the so-called "pink spot" thought to be 3,4-dimethoxyphenylethylamine, but the validity of this finding is now very doubtful.

A wide variety of the structural congeners of mescaline have now been investigated for psychotomimetic properties, and among them are a number of methoxylated amphetamines. In studies on rats, 4-methoxyamphetamine has been shown to be a very potent behaviour disrupter, second only to LSD (Smythies et al.,1967), and in man, it has five times the potency of mescaline as a hallucinogen (Shulgin et al.,1969). It has therefore been suggested that the amphetamine psychosis is caused by an abnormal methylation of 4-hydroxyamphetamine produced from the drug (Angrist et al.,1970), but these workers were unable to detect 4-methoxyamphetamine in the urine of patients given amphetamine in doses large enough to provoke the psychosis. This finding, of course, does not entirely rule out this possibility for the mechanism of the psychosis, as the drug could be methylated and subsequently demethylated before excretion. Three enzymes capable of O-methylating monophenols have been found in the body: the first is the O-methylation of N-acetyl-5-hydroxytryptamine to form the hormone melatonin, which occurs solely in the pineal gland (Axelrod and Weissbach,1961), and the second is the enzyme from liver which is able to O-methylate diiodophenols (Tomita et al.,1964). The third enzyme is phenol-O-methyl transferase, which is located principally in liver and lung (Axelrod and Daly,1968). It is thus possible that such a transient O-methylation could occur, and be the cause of the psychosis.

Another explanation could be due to the disruption of CNS function, caused by the drug and its metabolites as suggested previously, giving rise to the psychosis. The brain contains some 13 billion neurones, most of which interact with each other within the brain, rather than carrying information to and from the brain. If the substitution of a false transmitter for the endogenous noradrenaline in the peripheral neurones causes an adrenergic blockade evidenced by hypotension and other clinical signs, then the effects of this substitution within the CNS will be similarly profound. One effect of such disruption in the brain could be production of a psychosis.

It is of interest to observe that Leak (1958) has stated that the amphetamine psychosis is associated more with the use of methamphetamine than amphetamine.

Concluding Remarks

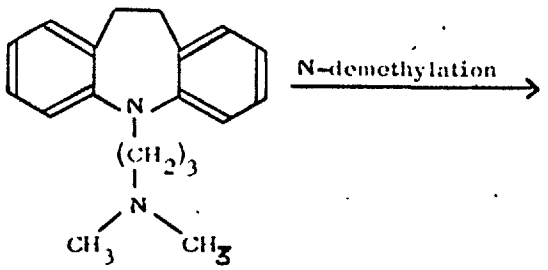
When the pharmacology and toxicology of drugs and foreign compounds is being considered, two important questions must be asked. The first obvious question is "What does the drug do to the body?", which the pharmacologist must answer. The second is "What does the body do to the drug?", and this is the domain of the biochemist. The toxicologist, of course, must concern himself with both sides of the problem. The study of the metabolism of a foreign compound in the body may help to explain its pharmacological action, toxicity and may also aid in finding structural modifications to diminish the toxic effect without removing the usefulness of the compound.

The metabolism of a drug in the body may be of significance to its pharmacology in several ways: it may (i) cause its activation, by converting it to an active drug from an inactive precursor, (ii) cause a change in the nature of the pharmacological activity, (iii) cause a deactivation and excretion of the drug, (iv) convert the drug into toxic products giving rise to adverse side effects. Examples of these are shown in Fig.6.2.

Fig 6.2

Examples of the pharmacological implications of drug metabolism from Parke (1972)

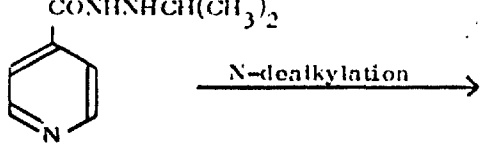
Activation



Imipramine

Desmethylimipramine (antidepressant)

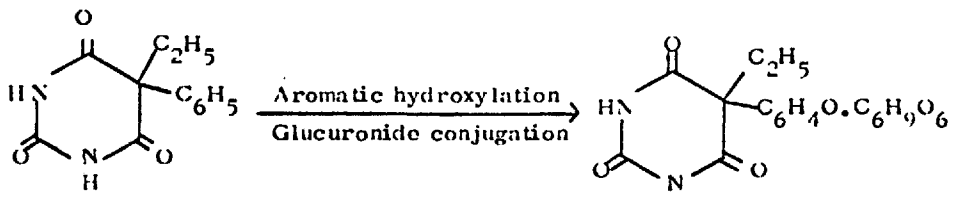
Change of activity



Iproniazid (MAO inhibitor)

Isoniazid (antitubercular)

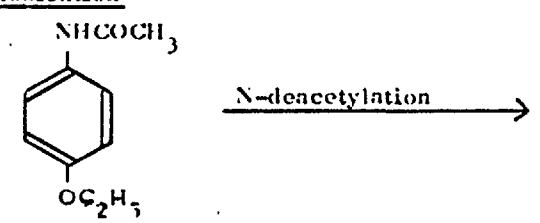
Deactivation



Phenobarbitone (hypnotic)

4'-Hydroxyphenobarbitone glucuronide (inactive)

Intoxication



Phenacetin (Analgesic)

p-Phenetidin (Methaemoglobin-former)

Studies of drug metabolism are now an essential part of the procedure for the evaluation of the safety and efficacy of drugs (W.H.O.,1966) and these studies must be done in laboratory animals and man. The aim of such studies is fourfold (Parke,1972):

(i) To establish the pharmacokinetics of the drug and its metabolites concerning absorption, distribution, tissue accumulation and excretion.

(ii) To find a species which metabolizes the drug in the same way, both quantitatively and qualitatively, as man, for use in toxicological testing.

(iii) To determine the pharmacology of the drug and its metabolites and to find which molecular species are responsible for the desired effects and which give the toxic effects, if any.

(iv) To contribute towards the design of safer and more effective drugs.

The pathways of drug metabolism can be broadly divided into two major categories: biotransformations (Phase 1 reactions) which include oxidation, reduction, hydroxylation and hydrolysis, all enzymic reactions in which a new functional group is introduced into the drug, or an existing functional group is modified; and conjugations (Phase 2 reactions), which are enzymic suntheses in which functional groups of the drug molecule are coupled with a new group, such as methyl, acetyl, sulphate, glucuronic acid or one of a range of amino acids.

From the results obtained in this work, it is possible to see how the metabolism of methamphetamine is related to that of other compounds of like structure in man, rat and the guinea pig, as outlined in Chapter 1.

In the rat, the prime route of metabolism of this class of compounds is ring hydroxylation, this being seen in amphetamine, mephentermine, N-3-chloropropylamphetamine and, to a lesser extent in ephedrine. As would be expected, ring hydroxylation is an important metabolic reaction of methamphetamine in the rat (Table 6.1). The rat is also known to N-dealkylate such compounds, e.g. ephedrine, mephentermine and N-3-chloropropylamphetamine, and this is the major metabolic pathway of methamphetamine. As with

Table 6.1 Summary of Metabolic Reactions of Methamphetamine

Species	Man	Guinea Pig		Rat
Dose mg/kg	0.29	10	45	45
Route of administration	p.o.	i.p.	i.p.	p.o.
Reactions	% of dose			
Unchanged	23	0.5	3	11
Aromatic hydroxylation	18	0	0	53
N-demethylation	14	79	64	28
Sidechain hydroxylation	4	1	19	16
Deamination	6	74	33	4
Benzoic acid production	5	63	31	4

amphetamine and ephedrine, the ring hydroxyl group is largely coupled with glucuronic acid in a Phase 2 reaction. Side chain breakdown is at a low level for methamphetamine, as it is for other compounds. However, the amount of side chain hydroxylation seen after methamphetamine is very much higher than for other compounds, and would not have been predicted from a comparison with these.

The guinea pig metabolizes methamphetamine by N-demethylation, which is also seen for ephedrine, then by sidechain breakdown to benzoic acid (Table 6.1). This is seen for amphetamine, but not for ephedrine, where the sidechain hydroxyl group stabilizes the molecule to metabolism. The metabolism of methamphetamine in the guinea pig appears to be dose dependent, with the deaminated product benzylmethyl ketone being an important metabolite at a low dose (Table 6.1) and norephedrine being excreted at a high dose, this being virtually absent at a lower dose. The route of excretion also varied with dose, being in the urine at a lower dose, where the metabolism is almost complete, but being in both urine and faeces at a high dose, where metabolism was less extensive. Ring hydroxylation also appeared to be absent, as would be expected from results for amphetamine and ephedrine, where this reaction is at a very low level (see Chapter 1, Tables1.

In man, the metabolism of methamphetamine is very different from amphetamine itself, but appears to follow patterns seen in less complete studies on other N-alkylated amphetamines. The major metabolic reaction is ring hydroxylation (Table 6.1), whereas for amphetamine this is at a very low level. N-3-chloropropylamphetamine is ring hydroxylated to a very great extent in man (see Chapter 1) and methamphetamine lies between this and amphetamine with respect to this reaction:

	Ring hydroxylation in man (% of dose)
Amphetamine	2
Methamphetamine	18
N-3-chloropropylamphetamine	37

The amount of side chain breakdown to benzoic acid in man after methamphetamine (Table 6.1) is hugely different from that seen after amphetamine. This is the major metabolic pathway for amphetamine (see Chapter 1) but is very minor for methamphetamine. The amounts of sidechain hydroxylation and ketone production are very similar after the two drugs. Man appears to dealkylate these compounds poorly compared with the rat and guinea pig. This low dealkylation is also seen after ephedrine.

It is thus seen that, in the case under consideration, the metabolism of methamphetamine in the two animal species used is very different from that seen in man. Table 6.1 summarizes that data, and shows that man lies between the rat and guinea pig with respect to ring hydroxylation of methamphetamine, is like the rat in benzoic acid production and is lower than both species in N-demethylation. On this evidence it would appear that the rat and the guinea pig are poor experimental animals for use in pharmacological investigations of methamphetamine. This is especially important as both species have been used in investigations of the tolerance produced by this drug, and may suggest that such results cannot be extrapolated to man in a realistic way. If this were a new compound for therapeutic use, it would be necessary, therefore, to seek another species whose metabolism more closely resembles that of man, in order to perform the essential pharmacological, behavioural and toxicological investigations.

This study underlines the great interspecies variations seen in drug metabolism, and further has shown that the metabolism of closely related drugs in the same species, while showing many similarities also exhibits marked differences. This does not augur well for the purposes of prediction of metabolism of as yet uninvestigated compounds, and it will be necessary to investigate many more drugs of this type before valid guidelines can be laid down.

The results obtained have made possible the construction of an hypothesis for the development of tolerance to methamphetamine. This has

has interpreted the characteristic pattern of tolerance in the light of the metabolic data in this thesis, with reference to the many relevant behavioural, neurochemical and pharmacological investigations recorded in the literature. This hypothesis is open to test, although the techniques required for this lie far away from those employed in this work. As has been pointed out, drug dependence gives rise to great concern in society today, and although the suggestions made in this thesis may not be completely correct it is hoped that the results obtained may aid in giving an insight into this most serious problem.

APPENDIX

ORGANIC MASS SPECTROMETRY

For the last year of this study, a mass spectrometer with ancillary gas chromatograph has been available, and use of this has been made in the confirmation of urinary metabolites, the partial elucidation of an unknown metabolite and to provide identification of the products of certain syntheses.

Organic Mass Spectrometry

The use of mass spectra to analyse chemical compounds was proposed very early in this century by J.J. Thompson. The application of physical techniques to investigations of organic compounds was then in its infancy and practical difficulties precluded their exploitation. In the early 1930's, some experiments showed that hydrocarbon gases fragmented in characteristic ways when bombarded by ions as in the mass spectrometer, and it was discovered that unknown compounds could be identified by comparison with known materials and, further, that the structure of a compound could be assigned on mass spectral evidence alone.

Improvements in instrumentation led to investigation of compounds containing atoms other than C and H, and of volatile liquids as well as gases. The petroleum industry was the first to exploit this sensitive technique, but now mass spectrometry (m.s.) is used very widely throughout the chemical industry and in research. The literature of organic m.s. has grown explosively since 1960, and is now most comprehensive (see Beynon et al.,1968).

The volatility and stability of a compound are most important in determining its suitability for m.s.. With recent instruments, solid samples may be inserted directly into the ion source. Involatile materials such as carbohydrates, salts and pepetides can be converted to volatile derivatives more suitable for mass spectrometry. The mass spectrometer consumes only very small amounts of material during the recording of a spectrum ($<<1\mu$ g) so the reliable handling of microgram quantities of material in a very pure state is essential. T.l.c. may be used to purify very small amounts of compounds and g.l.c. permits the handling of even smaller amounts (see Foster, 1969).

When milligram quantities of materials are available, during syntheses or in metabolic studies where large doses may be given, m.s. may be used in the same way as other forms of spectroscopy, notably i.r., for identification. Where microgram quantities only are available, m.s. provides the only positive identification possible of such small amounts at the present time.

Gas chromatography - Mass spectrometry

G.l.c., like other forms of chromatography, is a separation technique which requires in addition the use of a detection method to show the presence of compounds. This most usually achieved by the use of flame ionization or electron capture detectors.

For m.s., especially high resolution m.s., small, very pure samples must be delivered into the ionization chamber in a very pure state. G.l.c., with the mass spectrometer in the place of the detector, provides a convenient means of doing this. The combination became feasible in 1964, when both Ryhage and Watson and Biemann devised separators which concentrate the column eluates. These remove the carrier gas but leave the compound to be analysed in the m.s. The combination was first used for the identification of natural products and endogenous urinary compounds, and was first applied to drug metabolism by Hammar et al.(1968) in a study on the fate of chlorpromazine. Since then, the technique has been applied to a variety of problems in this field in two ways: (a) to allow detection of very small amount of materials by the use of the mass spectrometer as a g.l.c. detector. This can give identification of these materials, often by single or multiple ion monitoring (Brooks and Middleditch, 1971); and (b) to allow positive identification of metabolites initially detected by other methods.

The present study provides yet another example of the use of the mass spectrometer, alone and in combination with g.l.c., in the partial elucidation of the structure of a previously unknown metabolite in the methamphetamine in the guinea pig, and in giving conclusive evidence of the presence of norephedrine in guinea pig urine after methamphetamine dosage. The last is

particularly important as other workers have denied the occurence of this metabolite in the guinea pig (Beckett et al.,1971) and because of the pharmacological implications which can be suggested for this metabolite.

The mass spectra of five compounds, three taken by g.l.c.-m.s. and two by direct insertion, are given in Table A.1 and Fig. A.1 gives probable identities of the major peaks.

Table A.1 Mass Spectral Data of Compounds of relevance in this study

Figures given are m/e values for the characteristic major peaks, with relative intensities in parentheses. Spectra (a), (b), and (c) were recorded using g.c.-m.s. and (d) and (e) via the direct inlet system.

(a) N-Acetylamphetamine 177 (2.5) 118 (27) 86 (42) 44 (100) 43 (88)

(b) N-Acetylmethamphetamine 191 (2.2) 100 (72) 58 (17) 13 (52)

(c) O,N-Diacetylnorephedrine 235 (0.3) 175 (9) 86 (23) 69 (100) 44 (36) 43 (39)

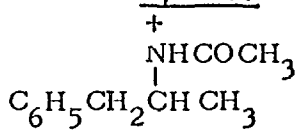
(d) Benzylmethyl ketoxime 149 (4) 91 (12) 69 (12) 57 (20) 44 (95)

(e) p-Toluenesulphonylmethamphetamine 303 (0.3) 214 (16) 213 (39) 212 (100) 157 (8) 156 (16) 155 (96) 91 (92) 77 (5)

Fig. A.1 Probable nature of peaks in the mass spectra recorded in Table A.1

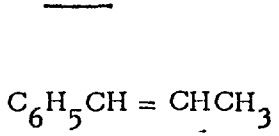
(a) N-Acetylamphetamine

m/e 177

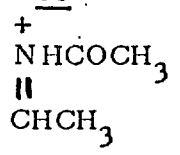


(parent ion)

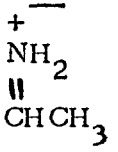
118



86

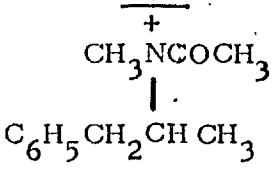


44



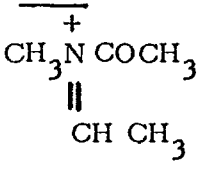
(b) N-Acetylmethamphetamine

191

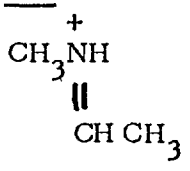


(parent ion)

100

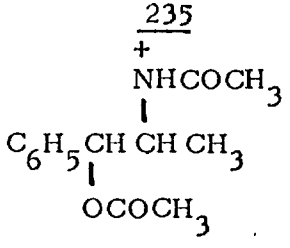


58



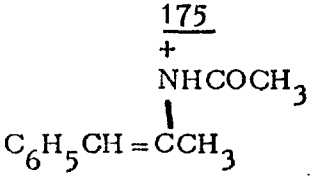
(c) O,N-Diacetylnorephedrine

235

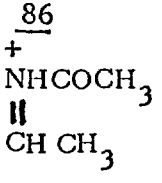


(parent ion)

175



86



69

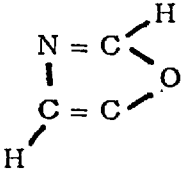
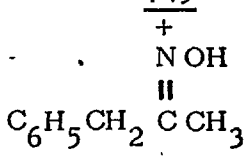


Fig. A.1 continued

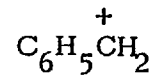
(d) Benzylmethyl ketoxime

149

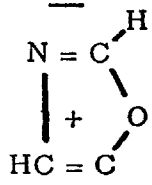


(parent ion)

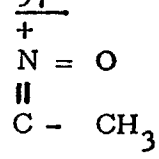
91



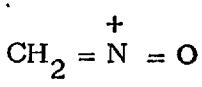
69



57

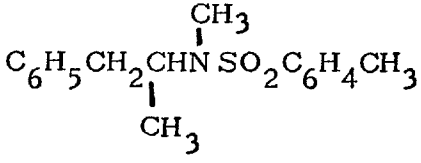


44



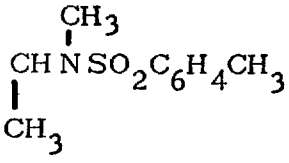
(e) p-Toluenesulphonylmethamphetamine

303



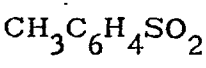
(parent ion)

212



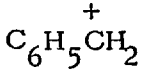
+ 213, 214-isotope contributions from sulphur

155



+ 156, 157-isotope contributions from sulphur

91



77



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