

THE ROLE OF AMPHETAMINE METABOLISM IN TOLERANCE

BY

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a thesis submitted for the degree
of
Doctor of Philosophy
in the
University of London

September, 1974.

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Abstract

The investigations presented in this thesis attempt to clarify reports that the development of tolerance to amphetamines is causally related to the metabolic production of *p*-hydroxynorephedrine, a compound which fulfils the accepted criteria for noradrenergic false transmitter function. Using ^{14}C -labelled compounds, metabolic studies have been performed in man and other species with amphetamine and two of its derivatives, *p*-hydroxyamphetamine and ephedrine. Urinary metabolites have been identified and quantitated by chromatographic and reverse isotope dilution procedures. In some cases metabolic data have been obtained from human subjects and animals that were chronically dosed with amphetamine in order to determine whether alterations in drug metabolism with repeated exposure to the drug could account for the observed development of tolerance.

Selected pharmacological effects of amphetamine have been monitored under conditions of chronic dosage in two species, namely the rat and guinea pig. The development of tolerance to noradrenergically mediated effects of amphetamine has been confirmed in the rat and could be related to the formation of *p*-hydroxynorephedrine. In the guinea pig however, which, according to previous workers, does not metabolise amphetamine to the false transmitter, tolerance also develops.

Further evidence against the false transmitter hypothesis was provided by a series of experiments in which animals were pretreated with precursors of *p*-hydroxynorephedrine and subsequently challenged with amphetamine. The effects of amphetamine were attenuated in male but not female rats, despite the formation of similar amounts of *p*-hydroxynorephedrine in both sexes.

It is suggested on the basis of this work that tolerance to certain effects of amphetamine is not causally related to the metabolic production of *p*-hydroxy-

norephedrine and the conclusion is reached that the false transmitter hypothesis cannot adequately explain the acquisition of tolerance that develops to amphetamine on repeated exposure to the drug.

FOREWORD

The work described in this thesis was carried out between September, 1971 and June, 1974 in the Department of Biochemistry of St. Mary's Hospital Medical School.

I am indebted to both Professor W.S. Peart, without whose guidance this thesis would never have been embarked upon, and Professor R.T. Williams for his inspiration, advice and encouragement throughout this period. His scientific preeminence is an example for all.

I also wish to acknowledge the Medical Research Council for financial support in the form of a Junior Research Fellowship held between September, 1971, and March, 1974.

To Dr. Graham Dring, I extend my grateful thanks for his patience, practical advice and personal involvement in this project. His experience has been drawn upon continuously.

I have valued the many stimulating discussions with my colleague Dr. John Caldwell, who has also provided much constructive advice and assistance with many of the problems encountered. The statistical computer programmes provided by Mr. Jeffrey Idle have been invaluable in the interpretation of much data and to him I am very grateful.

I wish to thank Miss Sally Ashford and Miss Rene Anderson for their help in the preparation of illustrations and the typing of this thesis.

Finally, and most importantly, I wish to thank my wife for her patience and understanding during the work for and the preparation of this thesis.

PREFACE

"We now have statistical evidence that a very high percentage of addicts are 'ignited' during their post-adolescent and young adult period of life. These are periods of preparation for the greatest life challenges, and our desperate desire to fight addictive impulses should have its major point of assault at this time. How can we actually - if we are honest - have any success in freeing anyone from drug dependency if we are not able or not willing to eliminate the causes which have driven him to this dependency? All too frequently, we hear the unjust, superficial opinion that youth must be moved away from a particular drug to something else. Where is the positive response to the unfortunate conditioning which has created the tendency to addiction in the first place? The most immediate task must be to unravel and lay bare the causes of the neuroses which have created the problem. Not until we have done this can we expect to find the 'means and medicine' to help victims out of their dilemma".

ERNEST HARMS (editor)
Drugs and Youth,
Pergamon Press, 1973.

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PART I

CHAPTER ONE

INTRODUCTION

Chapter OneIntroduction

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Historical Perspectives and Scope of the Present Study

After the initial discovery of the pressor effects of suprarenal extracts (Oliver & Schaefer, 1885), the isolation of the active principle adrenaline (Abel & Crawford, 1897) and its subsequent synthesis (Stolz, 1904; Dakin, 1905), many structural variations on this compound were synthesised.

In the elegant study of Barger and Dale (1910), the structure activity relationships of many biologically active amines were reported. These authors introduced the term 'sympathomimetic' for those amines whose actions resembled stimulation of the true sympathetic nervous system. Optimal sympathomimetic activity was afforded by a structure consisting of a benzene ring with a two carbon side chain and a terminal primary or secondary amino group. Activity was increased by the substitution of hydroxyl groups in the 3' and 4' positions on the aromatic ring, and by an alcoholic hydroxyl group on the β -carbon of the side chain.

Piness, Miller and Alles (1930) noted the sympathomimetic properties of amphetamine, although this drug had been synthesised some four decades previously (Edeleano, 1887), and the drug was introduced therapeutically as a bronchodilator, respiratory stimulant and analeptic (Alles, 1933). By 1935, the central stimulant effect of amphetamine had led to the use of this drug in the treatment of depressive states, narcolepsy, obesity and fatigue (Bett, Howells & Macdonald, 1955) and a little later the problems of tolerance became apparent (Guttman, 1939).

The literature contains many references to studies on the metabolism and pharmacology of amphetamines, with a notable accentuation on the possible mechanisms by which these drugs produce their pharmacological effects (vide infra). In recent years the problems of drug abuse have stimulated research into the underlying mechanisms of tolerance and dependence. With respect to amphetamine,

a drug which, with repeated dosage in man, can produce a psychotic state clinically indistinguishable from schizophrenia (Connell, 1958), an opportunity exists for a study of the mechanisms by which this psychosis may develop. Interpretation of the biochemical and pharmacological correlates of this state may ultimately lead to a better understanding of the pathogenesis of schizophrenia.

The studies on drugs of the amphetamine class which will be reviewed in this introduction can be fairly well categorised. Earlier work on drug biotransformation revealed the formation of metabolites which have themselves been shown to possess pharmacological activity and which almost certainly contribute to the action of amphetamine (Freeman & Sulser, 1972; Taylor & Sulser, 1973) and possibly to the tolerance that develops to this drug with repeated exposure (Brodie, Cho & Gessa, 1970). Many such studies have been performed in laboratory animals and, in view of the widespread species variations in drug metabolism (Williams, 1964), extrapolation of such data to the human situation must be carefully considered.

Most pharmacological investigations have been concerned with the mechanisms by which amphetamine interacts with endogenous catecholamines (vide infra). It is apparent, as will be reviewed later, that many of these studies have been performed in the rat, a species which metabolises amphetamine extensively by ring hydroxylation to form active metabolites, thus the pharmacological profile of amphetamine in the rat may be unrepresentative of other species including man. Nevertheless, detailed research upon the effects of both amphetamine and its metabolites on catecholamine disposition in this species have greatly contributed to our understanding of the mechanism of action of this drug.

The delineation of neuronal pathways within the brain and the development of stereotactic techniques have also added to our understanding of the mode of action of amphetamine.

Following the discovery that some metabolites of amphetamine can not only alter the storage of endogenous catecholamines but also be released on nerve stimulation, the concept of the false neurotransmitter was proposed and has been implicated in the development of tolerance to amphetamines (Brodie, Cho & Gessa, 1970).

The purpose of the work presented in this thesis was therefore to investigate further this proposed relationship between the metabolic formation of false neurotransmitters and the development of tolerance.

In the first half of the thesis, metabolic studies are presented in which possible precursors of the false neurotransmitter, *p*-hydroxynorephedrine, namely *p*-hydroxyamphetamine, amphetamine and ephedrine, have been administered to several species including man, and the urinary excretion of metabolites determined. The metabolic production of the false transmitter and its relationship to the development of tolerance to these drugs is discussed.

In the latter part of the thesis the validity of the false transmitter hypothesis has been tested by two series of experiments. In the first series, the development of tolerance to amphetamine is compared and contrasted in two species, only one of which produces the false transmitter metabolically. In the second study, metabolic precursors of *p*-hydroxynorephedrine were administered to animals that were subsequently challenged with amphetamine. If the false transmitter hypothesis is valid, then the previous incorporation of *p*-hydroxynorephedrine into the noradrenaline-containing storage vesicles should protect animals against the noradrenergic effects of a subsequent amphetamine challenge.

An attempt has also been made to interpret certain of the findings in the tolerance experiments with the known features of amphetamine abuse in man.

Review of Amphetamine Metabolism

Metabolism is one of the most important factors governing the pharmacological and toxic properties of a drug in the animal body. For many drugs in common usage today, little information is available on their metabolism and indeed, where such metabolic data are provided, not only are studies frequently restricted to a single animal species, but only rarely are data available concerning the fate of the drug in man. Interspecies variations in drug metabolism are widespread, and for this reason alone extrapolation of data derived from experiments in animals to man is unreliable (Williams, 1964).

When, however, the metabolism of a series of related compound is compared in a variety of species it is often possible to correlate the chemical structure of the compound, the species and the metabolic route. Thus, predictions can be made regarding the fate of a new drug in certain species including man (Williams, 1964).

Continuing interest in the metabolism of amphetamine is stimulated by the well-established similarity between the amphetamine psychosis and certain forms of schizophrenia (Randrup & Munkvad, 1970) and it could be suggested that studies on the mode of action of amphetamine and its metabolites may well increase our knowledge of the biochemical basis for this crippling disease. In addition because of the increasing world problems of drug abuse, tolerance and addiction, research on the biochemical correlates of the tolerant state are required, particularly with reference to amphetamine which is known to be metabolised in certain species to a compound capable of acting as a false neurotransmitter, the formation of which has been suggested to be causally related to the development of tolerance.

Since the important paper of Axelrod (1954), there have been several studies on the metabolism of amphetamine and other β -phenyl isopropylamines. These have been compiled and reviewed by Smith and Dring (1970) and Williams, Caldwell and

Dring (1974) and may be summarised here.

The structures of these compounds, which are given in Fig. 1:1 suggest several routes of biotransformation (Fig. 1:2) namely:-

Aromatic hydroxylation to the corresponding parahydroxy derivative which may be excreted free or as a conjugate with glucuronic acid or sulphuric acid.

N-Dealkylation of β -phenyl-N-alkylisopropylamines (e.g. ephedrine) to the primary amine.

Aliphatic hydroxylation of the α and β carbon atoms to the carbinolamine and corresponding ephedrine respectively.

Oxidative deamination to the ketone which may be reduced to other compounds or undergo oxidation to the carboxylic acid.

N-Oxidation to form hydroxylamines or N-oxides.

Some β -phenyl isopropylamines are largely unmetabolised and excreted free in the urine, e.g. norephedrine in man. Faecal excretion is minimal, as would be expected, since few of these compounds attain the molecular weight threshold required for biliary excretion (Millburn, 1970).

The biotransformation of amphetamine has been studied in greater detail than most of its analogues, and illustrates the metabolic routes outlined above (Fig. 1:2).

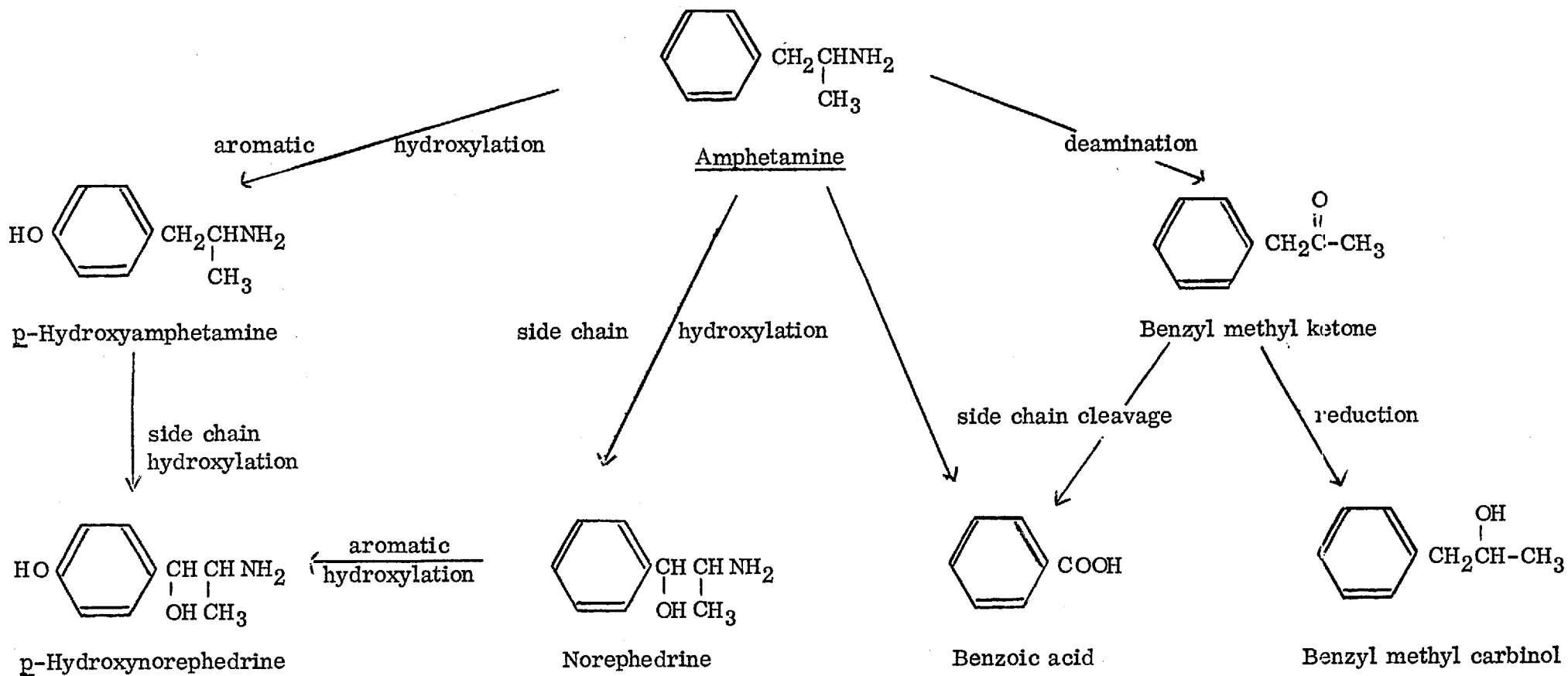
Aromatic Hydroxylation of Amphetamine

The exact nature of the enzyme system responsible for the ring hydroxylation of amphetamine is uncertain. Although in the isolated perfused liver the reaction proceeds as in the whole animal, amphetamine does not appear to be metabolised by

Figure 1:1 Structures of Amphetamine and Related Drugs

<u>Compound</u>	<u>Substituent</u>				
	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>	<u>R₄</u>	<u>R₅</u>
Amphetamine	H	H	H	H	H
Norephedrine	H	H	OH	H	H
Ephedrine	H	H	OH	H	CH ₃
p-Hydroxyamphetamine	OH	H	H	H	H
p-Hydroxynorephedrine	OH	H	OH	H	H
p-Methoxyamphetamine	CH ₃ OH	H	H	H	H
Fenfluramine	H	CF ₃	H	H	C ₂ H ₅
Methamphetamine	H	H	H	H	CH ₃
N-Ethylamphetamine	H	H	H	H	C ₂ H ₅
Mephentermine	H	H	H	CH ₃	CH ₃
Chlorphentermine	Cl	H	H	CH ₃	H
p-Chloroamphetamine	Cl	H	H	H	H
N-3-Chloropropyl-amphetamine	H	H	H	H	(CH ₂) ₃ Cl

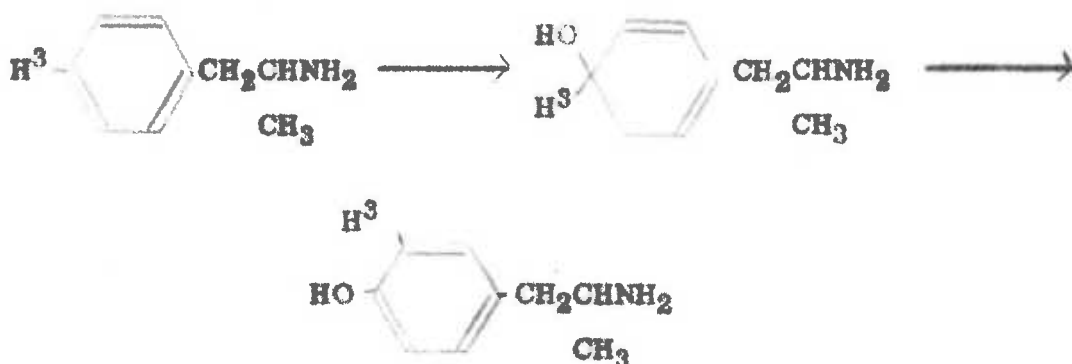
Figure 1:2 Metabolic Pathways of Amphetamine



All compounds in the free or conjugated form have been detected as urinary metabolites of amphetamine in various species.

the usual microsomal aryl hydroxylase enzyme system as shown by Daly *et al.* (1967) and Diagell and Bass (1969) who failed to demonstrate any significant biotransformation in rat liver microsomal preparations. Of interest is the finding of Creaven, Barbee and Roach (1970) who showed that although SKF 525 A inhibited the parahydroxylation reaction, this was not induced by either phenobarbital or 3,4-benzpyrene pretreatment. It has also been reported by Creaven and Barbee (1969) that ethanol inhibits this transformation *in vivo*. Other compounds which interfere with this route of metabolism are discussed in a later section of this chapter.

Despite the lack of information concerning the enzyme system responsible for aromatic hydroxylation, certain characteristics of this reaction have been determined. Daly *et al.* (1967) demonstrated that in common with other aromatic hydroxylation reactions, the hydroxylation of amphetamine involved the 'N. I. H. Shift' whereby, as a result of hydroxylation, there is an intramolecular migration of the atom displaced by the new hydroxyl group to the adjacent position on the ring.



Aliphatic Hydroxylation

Whereas recent studies report the hydroxylation of the α carbon atom of amphetamine (*vide infra*), hydroxylation occurring at the β -carbon position has been the subject of considerable interest, since the metabolite formed by both aromatic and β -hydroxylation (p-hydroxynorephedrine) has been suggested to be responsible for the development of tolerance to amphetamine.

Dopamine- β -hydroxylase, the enzyme responsible for the conversion of dopamine to noradrenaline, has been shown to lack substrate specificity (Levin & Kaufman, 1961) and several authors have confirmed that many phenylisopropylamines, including amphetamine, are substrates for this enzyme (Creveling *et al.*, 1962; Goldstein & Contrera, 1962), which is specific for the (+)-isomers of these optically active substrates. A fuller discussion of this pathway is given in Chapter 3.

Oxidative Deamination

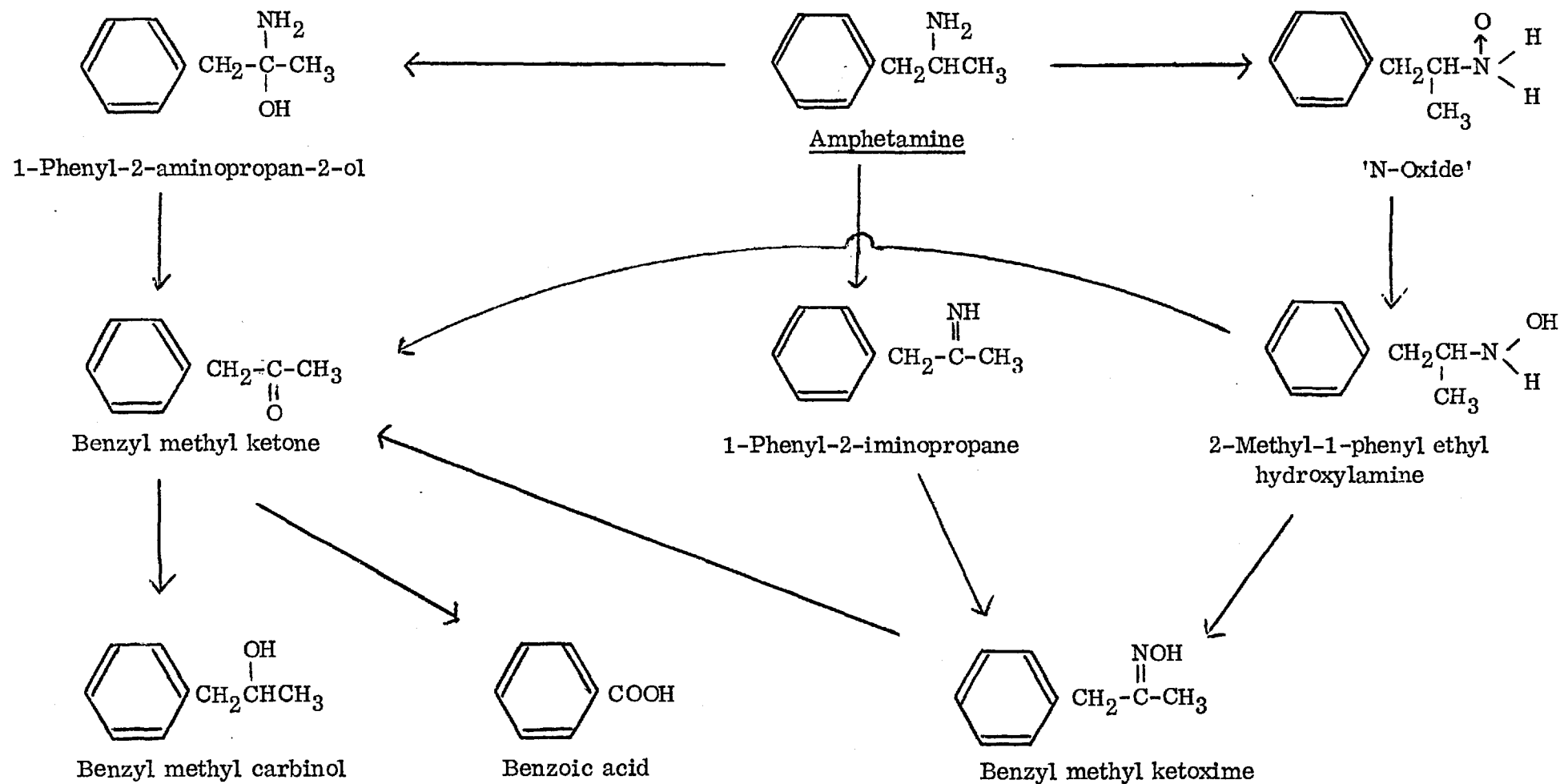
The proposed pathways for deamination of amphetamine (Fig. 1:3) include oxidative attack at two sites, namely the α -carbon and nitrogen atoms. However, the mechanism by which compounds of the amphetamine series undergo oxidative deamination is the subject of much controversy. Because of the presence of the methyl group on the carbon bearing nitrogen, these drugs are not substrates for monoamine oxidase.

Axelrod (1955) found that amphetamine was oxidised to benzyl methyl ketone by rabbit liver microsomes, and Brodie, Gillette and LaDu (1958) suggested that the oxidative attack occurred at the α -carbon position to form an unstable carbinolamine, which would then lose ammonia to give the ketone (Fig. 1:3). Smith and Dring (1970) suggested an alternative mechanism in which amphetamine was dehydrogenated to its imine, which would then undergo hydrolysis to the ketone.

Hucker, Michniewicz and Rhodes (1971) and Parli, Wang and McMahon (1971a) found that the chief metabolite of amphetamine in rabbit liver microsomal preparations was benzyl methyl ketoxime which can hydrolyse to the ketone (Caldwell, 1972).

Using the technique of ^{18}O labelling, Parli, Wang and McMahon (1971a)

Figure 1:3 Proposed Pathways for Deamination of Amphetamine



concluded that oxidative deamination resulted from an initial hydroxylation on the α -carbon atom giving the unstable carbinolamine which undergoes two fates, either dehydration to the imine or loss of ammonia to the ketone. These authors suggested that the imine could be oxidised to the oxime which was hydrolysed to benzyl methyl ketone. In a subsequent publication (Parli, Wang & McMahon, 1971b) evidence for this pathway was provided by the isolation of an enzyme from rat and rabbit livers capable of hydroxylating imines of this type. The incorporation of 95% ^{18}O into the oxime and 30% ^{18}O into the ketone suggested that most of the ketone was formed via the oxime (65%) and that the remainder was derived from the unstable carbinolamine.

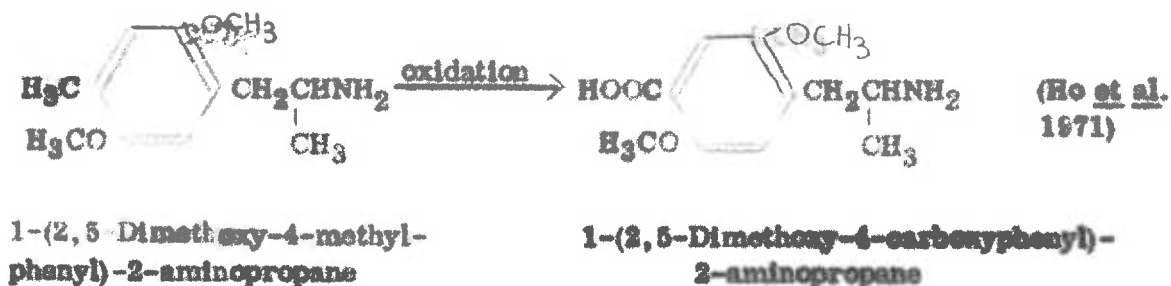
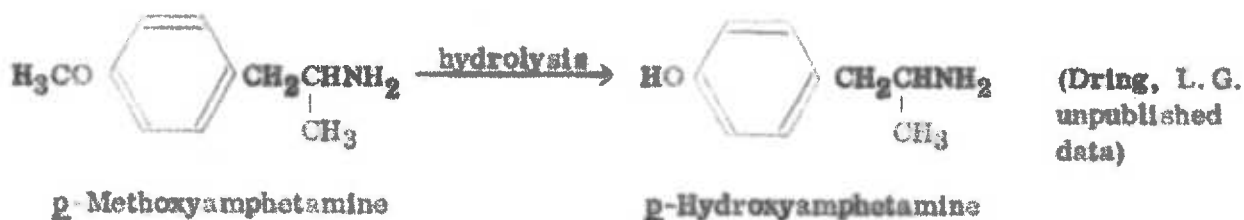
Beckett and Al-Sarraj (1972) proposed a further mechanism for the microsomal enzymatic attack on amphetamine. These authors suggested that in addition to α -carbon oxidation giving the ketone, oxidation occurred on the nitrogen atom to give the N-oxide which by proton transfer became the hydroxylamine. These hydroxylamines, which in alkaline and acidic solution and some organic solvents, notably ethyl acetate, break down readily to oximes, are formed in quantities dependent upon the particular species investigated. In rabbit liver microsomal preparations, the N-oxidation route was found to be equivalent to the $\alpha\text{C-}$ route, in guinea pig preparations the N- route was 10 times greater than the $\alpha\text{C-}$ route and in rats the $\alpha\text{C-}$ route was negligible and the N- route only about 1/100 activity of guinea pigs.

The fate of the ketone produced by oxidative deamination is the result of either further oxidation to benzoic acid, or stereospecific reduction of (+)-isomers to benzyl methyl carbinol (El Masry, Smith & Williams, 1956).

Substituted Amphetamines

Ring substituted amphetamines

Although many ring substituted amphetamines have been synthesised, such as *p*-hydroxyamphetamine, *p*-methoxyamphetamine, 2', 5'-dimethoxy-4'-methyl-amphetamine and fenfluramine, detailed metabolic studies are few. The fate of *p*-hydroxyamphetamine will be presented in Chapter 3. In general it may be stated that although substituent groups themselves may be metabolised, e.g. by hydrolysis or oxidation, further metabolic attack on unsubstituted groups has not been reported.



Nevertheless, these compounds are subject to side chain degradation as evidenced by, for example, the formation of *m*-trifluoromethylhippuric acid as the major urinary metabolite of fenfluramine in man (Bruce & Maynard, 1968).

N-Alkylated amphetamines

A detailed discussion of the metabolism of ephedrine is given in Chapter 5. Many other N-alkylated amphetamines have been the subject of metabolic studies,

e.g. N-methylamphetamine (Axelrod, 1954; Caldwell, Dring & Williams, 1972a), N-ethylamphetamine (Beckett, Brookes & Shenoy, 1969) and N-3-chloropropylamphetamine (Pondinil) (Long, 1970), and all species investigated N-dealkylate to varying extents. It is apparent that increasing the size of the alkyl group increases the extent of further metabolism by oxidative deamination of the side chain and aromatic hydroxylation.

In view of the importance and potential significance of interspecies variations in the biotransformation of the amphetamines, the patterns of metabolism of a number of amphetamines in several species will be reviewed briefly. The experiments to be presented in a later chapter were designed on the basis of evidence that certain species do not metabolise amphetamine by aromatic hydroxylation.

Metabolism in the Rat

The rat metabolises amphetamines principally by aromatic hydroxylation (Axelrod, 1954; Smith & Dring, 1970; Dring, Smith & Williams, 1970; Williams, Caldwell & Dring, 1974) (Table 1:1). Where N-substituent groups are present, N-dealkylation occurs to a moderate extent but deamination is minimal (Caldwell, Dring & Williams, 1972a; Axelrod, 1954). β -Hydroxylation of the side chain is a minor metabolic route with amphetamine but with methamphetamine this pathway is more significant (Caldwell, Dring & Williams, 1972b). The more polar compound, ephedrine, is excreted to a considerable extent unchanged (Bralet, Cohen & Valette, 1968), however, other compounds investigated such as Pondinil and mephentermine are almost totally metabolised and the amounts excreted unchanged in the urine are very small (Williams, Caldwell & Dring, 1974).

Metabolism in the Guinea Pig

The guinea pig contrasts strikingly with the rat in that the main metabolic

Table 1:1 Summary of Metabolic Pathways of Amphetamines in Laboratory Animals

Values are % dose excreted in the first 24 h after dose. For references see text.

	<u>Unchanged</u>	<u>As 1° amine</u>	<u>Total N-dealkylation</u>	<u>Aromatic hydroxylation</u>	<u>Benzolic acid</u>	<u>β-Hydroxylation</u>
<u>Rat</u>						
Amphetamine	14	X	X	48	2	0.6
Methamphetamine	11	3	28	53	4	16
Ephedrine	36	5	20	14	3	X
Pondinil	1	—	—	58	—	—
Mephentermine	0	13	45	32	—	—
<u>Guinea Pig</u>						
Amphetamine	22	X	X	0	62	—
Methamphetamine 10 mg/kg	0.5	4	79	0	63	1
45 mg/kg	3	13	64	0	31	19
Ephedrine	2	39	39	1	—	X
Pondinil	3	—	—	15	—	—
<u>Rabbit</u>						
Amphetamine	3	X	X	6	25	—
Methamphetamine	2	—	—	—	—	—
Ephedrine	0.5	2	93	12	62	X
Pondinil	18	—	—	6	—	—
Mephentermine	0	22	27	5	—	—
<u>Dog</u>						
Amphetamine	30	X	X	6	28	—
Methamphetamine	20	15	45	30	—	—
Ephedrine	6	58	59	2	—	X
Pondinil	17	—	—	22	—	—
Mephentermine	0	16	44	28	—	—

pathway in this species is oxidative deamination of the side chain to benzoic acid (Axelrod, 1954; Smith & Dring, 1970; Williams, Caldwell & Dring, 1974). With N-substituted amphetamines, N-dealkylation occurs (Caldwell, Dring & Williams, 1972a). Aromatic hydroxylation is a minor pathway (with the exception of Pondinil; Long, 1970), and probably does not occur with amphetamine and methamphetamine, and side chain hydroxylation on the β -carbon atom is also a minor reaction (Dring, Smith & Williams, 1970). Ephedrine is largely metabolised by N-demethylation to norephedrine (Axelrod, 1953) which by virtue of its polarity is excreted without further biotransformation. In general, in this species, metabolism is extensive and with the exception of amphetamine only small amounts are excreted in the urine unchanged.

Metabolism in the Rabbit

In the rabbit both aromatic hydroxylation and side chain degradation occur although the latter route is more significant. All compounds with the exception of Pondinil are extensively metabolised and N-dealkylation occurs where appropriate (Dring, Smith & Williams, 1970; Williams, Caldwell & Dring, 1974). In this species, in addition to benzoic acid, precursors of the deaminated ketone metabolites of both amphetamine and norephedrine are excreted in the urine and for amphetamine have been tentatively identified as the enol sulphate of the ketone (Dring, Smith & Williams, 1970).

Metabolism in the Dog

This animal, like the rabbit, is capable of both aromatic hydroxylation and oxidative deamination (Smith & Dring, 1970; Williams, Caldwell & Dring, 1974), the latter route being most evident with amphetamine, whereas with methamphetamine and Pondinil ring hydroxylation is more dominant. A significant fraction of the dose is excreted unchanged and, as with other species examined, N-dealkylation

of N-substituted amphetamines occurs.

Metabolism in Man

With the exception of methamphetamine (Caldwell, Dring & Williams, 1972a) and Pondinil (Long, 1970) (the more lipid soluble drugs), aromatic hydroxylation is a minor route in man (Williams, Caldwell & Dring, 1974) (Table 1:2). The increased polarity of the ephedrine, when compared with other amphetamines, appears to be reflected in the high levels of unchanged drug excreted in the urine (Wilkinson & Beckett, 1968; Sinshelmer, Dring & Williams, 1973). Interestingly, when ring hydroxylation is prevented by the presence of substituent groups (e. g. fenfluramine) metabolism occurs chiefly via the side chain degradative pathway (Bruce & Maynard, 1968).

It is evident thus, from the data presented that, when pharmacological observations are derived from experiments performed on laboratory animals, account must be taken of the metabolism of the drug under investigation in the particular species examined. This must be of vital importance when the biotransformation of the drug produces pharmacologically active metabolites, which because of widespread interspecies variations, may be formed in one species but not in another.

Other Factors influencing the Metabolism of Amphetamine

In common with many other drugs both the metabolism and pharmacokinetics of amphetamine may be influenced by a variety of factors. There are variable reports concerning the half life of amphetamine in young and old rats and according to Groppetti and Costa (1969) the rate of decline of amphetamine in young males is slower than in adults. However, the converse was found in females (Zeim et al. , 1970). A striking sex difference in the plasma $T_{1/2}$ was also reported by Groppetti and Costa (1969) and although this could result from an increased rate of metabolism

Table 1:2 Summary of Metabolic Pathways of Amphetamines in Man

Values are % dose excreted in the first 24 h after dose

<u>Compound</u>	<u>Unchanged</u>	<u>1° amine</u> <u>(N-dealkylation)</u>	<u>Ring</u> <u>hydroxylation</u>	<u>Benzoic</u> <u>acid</u>
Amphetamine	34	X	2	21
Methamphetamine	23	3	18	4
N-Ethylamphetamine	17	7	-	-
Pondinil	1	-	37	-
Norephedrine	90	X	low	low
Ephedrine	60-80	10-15	low	low
Fenfluramine	7	3	0	66-93

(For references see text)

in the male rats ($T_{1/2}$ - 76 mins.) compared with females ($T_{1/2}$ - 130 mins.), other variables, which include volume of distribution and renal clearance, could be responsible for the observed sex difference.

The significance of urinary pH on the excretion of amphetamine is more fully discussed in a later chapter. At this juncture, however, it should be noted that the urinary excretion of the weak base amphetamine is accelerated under conditions of acid urine formation, whereas excretion is retarded in alkaline urine because of reabsorption of amphetamine in the renal tubules by pH dependent nonionic diffusion. Anggard et al. (1973) showed that in association with an increased excretion of unchanged amphetamine in acid urines in man, there was a concomitant diminution in the excretion of metabolites.

Environmental factors appear to influence the disposition of amphetamine as evidenced by the increased brain concentrations of the drug and associated potentiation of amphetamine effects in starved rats (Campbell & Fibiger, 1971).

The influence of coadministered drugs on the disappearance rate and metabolism of amphetamine is reviewed by Lewander and Jonsson (1972) who showed that many tricyclic antidepressants, neuroleptics, sedatives, anticonvulsants, anorectic agents and many other drugs interfered with amphetamine metabolism. The importance of these observations was apparent when subjects were interviewed for participation in the metabolism study of amphetamine in man. The polypharmacy to which drug addicts expose themselves makes single drug analyses difficult.

Many compounds, notably the barbiturates, are able to increase their rate of metabolism with chronic exposure by stimulation of the hepatic cytochrome-P 450-dependent microsomal enzyme systems. No such changes have, however, been observed with amphetamine metabolism on repeated dosage (Ellison et al., 1971). Certain minor changes do occur in the metabolism and disposition of amphetamine in this situation, and these will be discussed in Chapter 4 and Chapter 6.

Structure-Activity Relationships of Amphetamines

Many amphetamine analogues have been synthesised and a great number possess stimulant and anorectic properties. Biel (1970) provides an excellent review of the structure-activity relationships of this class of compounds.

With the exception of N-methylamphetamine (Methedrine), alkylation of the side chain or amino group diminishes the CNS stimulant activity of amphetamine. Ring hydroxylation abolishes the stimulant effect and markedly diminishes the anorectic activity and side chain hydroxylation (resulting in the ephedrine series) also decreases both effects. Substitution of methoxy groups in the ring, however, gives the molecule hallucinogenic properties, the compounds thus produced resembling mescaline, a potent psychedelic agent. The structure-activity relationships of hallucinogenic amphetamines are discussed by Shulgin, Sargent and Naranjo (1969). A reduction of CNS stimulant action is effected also by methylation of the side chain carbon bearing the amino group (giving the phentermine series) and by N-alkylation with large groups, e.g. benzyl. Such compounds retain anorectic properties as do those with halogen substitution in the ring, such as fenfluramine and chlorphentermine. The activity of most amphetamine congeners rests principally in their various effects on the catecholamine containing nerve terminals in the periphery and CNS, as will be discussed later. However, a compound at present attracting much attention of p-chloroamphetamine, which is a short acting depletor of noradrenaline, but reduces brain 5HT levels in the rat for periods of up to 6 months, probably by inhibiting tryptophan hydroxylase irreversibly (Sanders-Bush & Sulser, 1970).

The Catecholamines

Introduction

In view of the structural similarities between amphetamines and the catecholamines and the wealth of evidence implicating catecholamine release in the mediation of many effects of amphetamine, it is essential at this point to consider summarily the biochemical and pharmacological disposition of catecholamines in the peripheral and central nervous systems. The explosive interest in this field is reflected by the vast numbers of publications during the past 10 years. The elegant investigations of many eminent scientists has led to our current understanding of the anatomy, physiology and biochemistry of the autonomic nervous system. These studies will not, however, be detailed here but outstanding contributions were made by von Euler. This author concluded that the principal neurotransmitter in the peripheral sympathetic nervous system was noradrenaline (von Euler, 1959).

The Localisation of Catecholamines in the Mammalian Body.

Although many studies have suggested that noradrenaline was distributed widely throughout the peripheral autonomic nervous system (e.g. von Euler, 1946), these investigations were greatly facilitated by the development of fluorescence-histochemical techniques (Falck et al., 1962). The direct correlation between the noradrenaline content of peripheral nerves and the abundance of sympathetic fibres that they carry is well established, the greatest content being found in the splenic nerve. The same correlation exists in peripheral tissues that are well supplied by adrenergic neurons, notably the vas deferens, ciliary body, salivary glands, the walls of arteries and veins and the atria of the heart (Iversen, 1967).

It is now known that the central nervous system stores noradrenaline, dopamine and serotonin, and evidence has accumulated that these substances function as neurotransmitters (Fuxe, 1965; Hillarp et al., 1966; Anden et al., 1966). The

distribution of these amines varies; noradrenaline concentrations are greatest in the hypothalamus and medulla oblongata, with small amounts in the striatum and cerebral cortex. Dopamine, on the other hand, is present in high concentrations in the striatum and caudate nucleus with negligible amounts found elsewhere in the brain (Iversen, 1967). In these areas it is probable that dopamine plays an essential role as a neurotransmitter substance (Carlsson, Falck & Hillarp, 1962). Dopaminergic neurons probably originate from nerve cells in the cranial-ventral part of the mesencephalon and can be traced in the retrolenticular part of the internal capsule ending in the neostriatum and limbic forebrain, notably the nucleus accumbens, tuberculum olfactorium and interstitial nucleus (Anden et al., 1965a,b; 1966; Hillarp, Fuxe & Dahlstrom, 1966).

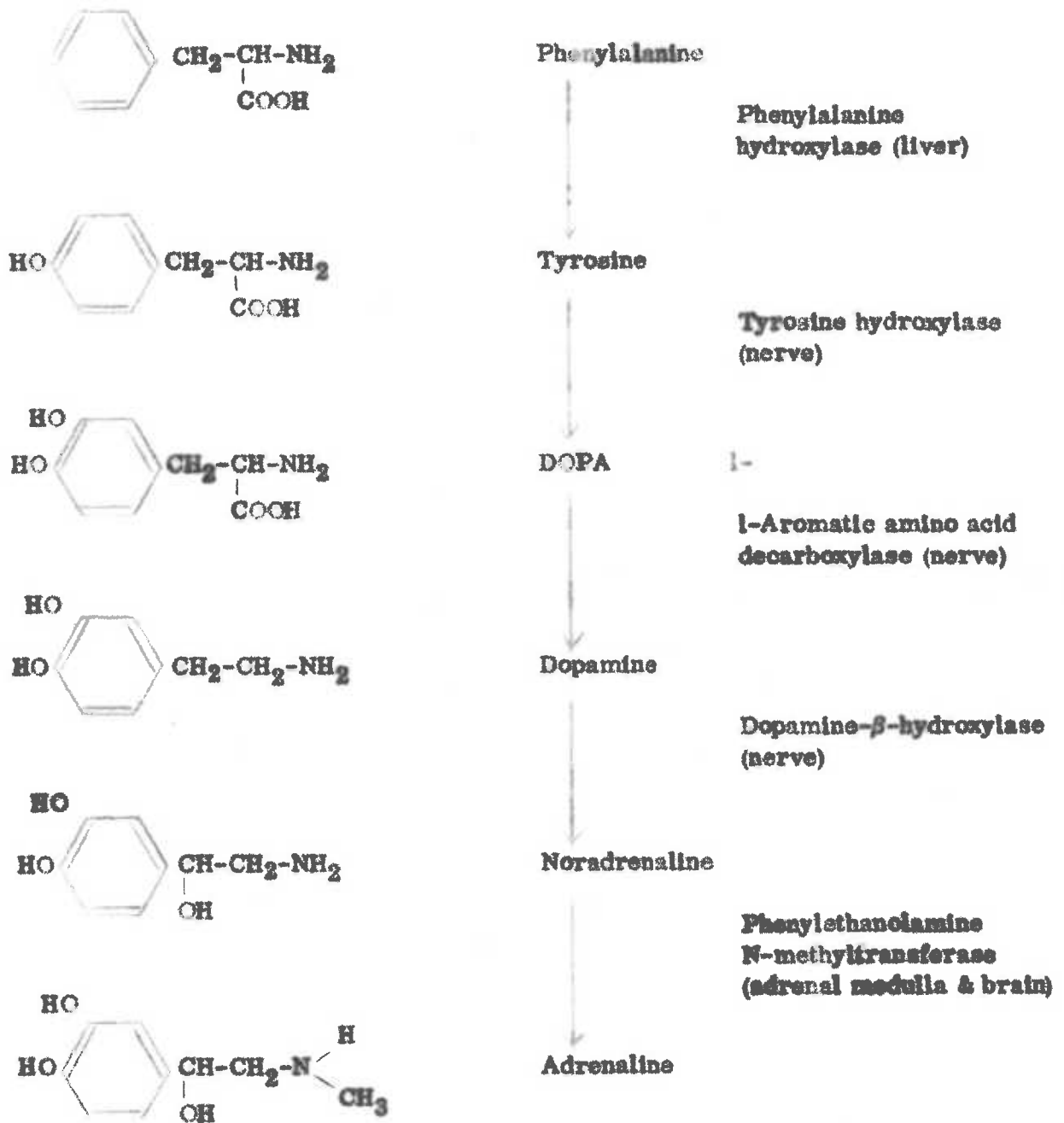
The cell bodies of the noradrenaline-containing neurons are found mainly in the pons and medulla (Anden et al., 1966), the largest group being within the locus coeruleus. Ascending noradrenergic axons pass through the dorsal part of the reticular formation and together with fibres from the pons ascend in the medial forebrain bundle to form noradrenergic synapses in the hypothalamus, limbic systems and frontal cortex (Anden et al., 1965b; Dahlstrom et al., 1964; Fuxe, 1965).

Metabolism of Catecholamines

Biosynthesis

The sequence of metabolic events leading to the synthesis of noradrenaline and adrenaline was proposed by Blaschko (1938) (Fig. 1:4) and subsequently confirmed by many investigators. With the availability of radioactively-labelled precursors, this pathway was originally demonstrated in adrenal medullary tissue (Demis, Blaschko & Welch, 1955) and later in sympathetic nerves and ganglia (Goodall & Kirshner, 1958) and in brain (Goldstein, Musacchio & Contreras, 1962).

Figure 1:4 The Biosynthesis of Noradrenaline and Adrenaline



From Goodman and Gillman, 1970.

The enzymes involved in the biosynthesis of catecholamines have been demonstrated in the tissues mentioned above. Tyrosine hydroxylase, which converts tyrosine to 3',4'-dihydroxyphenylalanine (DOPA), is associated with the particulate or synaptosomal fraction (McGeer, Bagchi & McGeer, 1965) and partially purified preparations of this enzyme require tetrahydropteridine cofactors and are stimulated by Fe^{2+} . It is now known that this enzymatic step which is specific for the 1-isomer of tyrosine is the rate limiting stage in the biosynthesis of catecholamines (Nagatsu, Levitt & Udenfriend, 1964; Levitt et al., 1965).

Aromatic 1-amino acid decarboxylase, which catalyses the decarboxylation of DOPA to 3',4'-dihydroxyphenylethylamine (dopamine) is chiefly found in the cytoplasm of cells, but may be loosely associated with intraneuronal storage vesicles in peripheral sympathetic terminals (Stjarne, 1966), and with synaptosomal fractions in brain (McGeer, Bagchi & McGeer, 1965). This enzyme has low substrate specificity and will decarboxylate many other aromatic 1-amino acids (Hagen, 1962). It requires pyridoxal phosphate as cofactor.

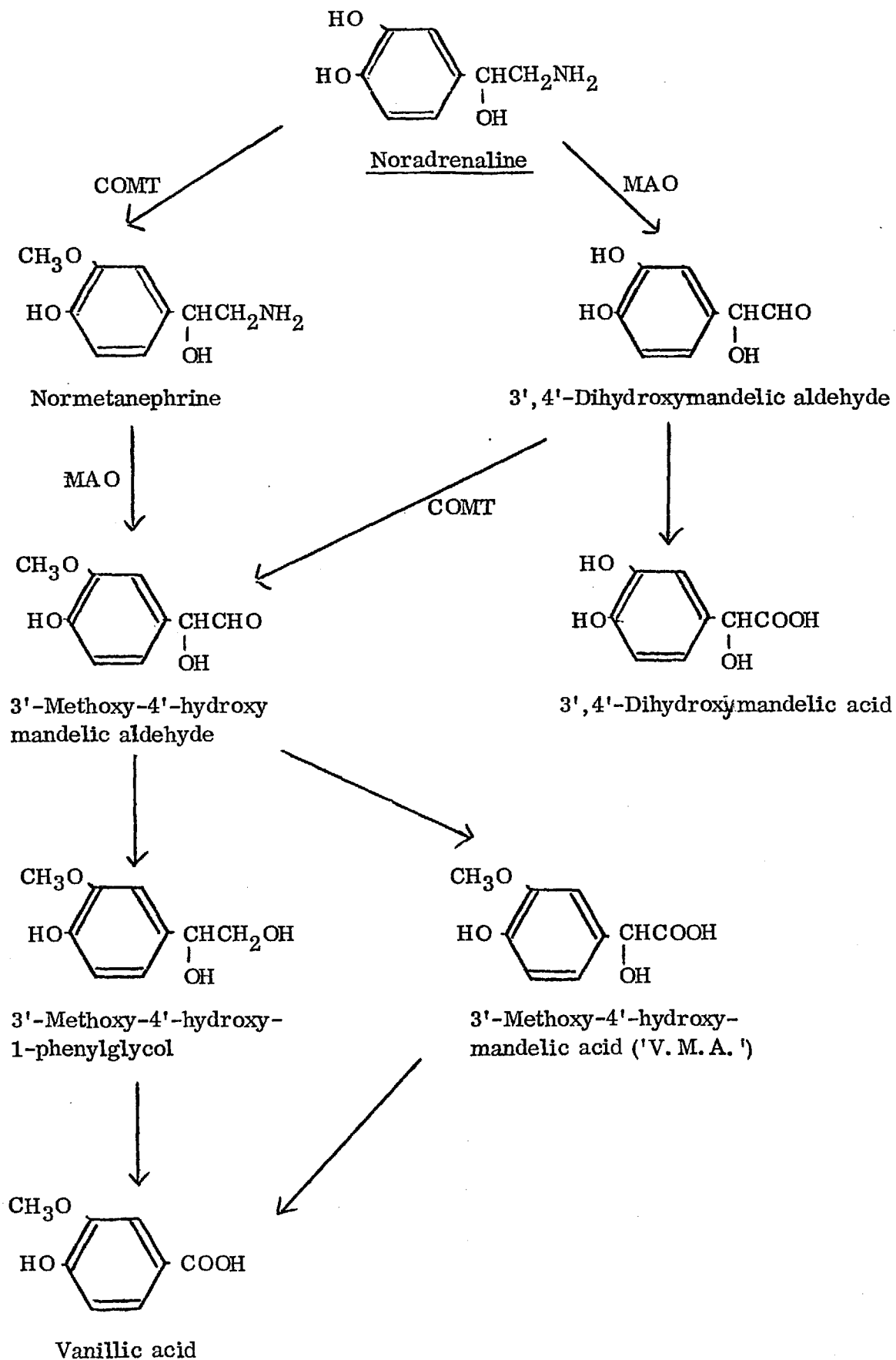
The final step in noradrenaline biosynthesis involves the copper containing enzyme dopamine- β -hydroxylase (Kaufman & Friedman, 1965). This enzyme is exclusively present in noradrenaline containing granules (Stjarne & Lishajko, 1967) and its distribution throughout the peripheral and central nervous system correlates well with that of noradrenaline (Udenfriend & Creveling, 1959). Other phenylethyl and phenylisoprylamines are substrates for this enzyme (see Chapter 3.)

Degradation

The metabolic pathways for noradrenaline degradation are shown in Fig. 1:5. It should be pointed out here and will be emphasised later that metabolism of noradrenaline is not the essential mechanism by which the released amine is inactivated.

The two enzymes responsible for the catabolism of noredrenaline are monoamine

Figure 1:5 Major Pathways of Noradrenaline Breakdown



oxidase (MAO) and catechol-O-methyl transferase (COMT). MAO converts many biogenic amines, including noradrenaline, adrenaline, dopamine and serotonin, by oxidative deamination to the corresponding aldehydes which are then further oxidised or reduced to the acid or glycol respectively (Blaschko, 1952). This mitochondrial enzyme is generally believed to be responsible for metabolising extragranular stores of noradrenaline, but it appears that extraneuronal and intragranular noradrenaline are protected from such attack. Although many biogenic amines are deaminated by MAO, amines with side chain substituent groups such as ~~β -hydroxyl or α -methyl~~ are not substrates for the enzyme (Blaschko, 1952).

The second enzyme involved in noradrenaline catabolism is catechol-O-methyl transferase. Unlike MAO, this enzyme is found in the cytosol and is widely distributed throughout the body (Axelrod & Tomchick, 1962), in association with the methyl donor S-adenosylmethionine. In the periphery the major end product of noradrenaline metabolism appears to be 3-methoxy-4-hydroxymandelic acid, whereas in brain 3-methoxy-4-hydroxy-phenylglycol is the main metabolite (Schanberg *et al.*, 1966).

This pathway for O-methylation of noradrenaline is largely responsible for the biodegradation of exogenously administered drug, and is probably responsible for removal of that fraction of endogenous noradrenaline released into the synaptic cleft which is not subsequently removed by reuptake into the nerve terminals. The overlap between these two pathways and their relative importance *in vivo* are discussed by Iversen (1967).

Uptake of Noradrenaline

There is much evidence that exogenous noradrenaline is taken up into postganglionic sympathetic neurons against a considerable concentration gradient and stored (Iversen, 1971). Iversen demonstrated two types of saturable uptake

processes for noradrenaline in the isolated perfused heart and it was possible to apply Michaelis-Menten kinetics to these active membrane transport carrier systems which he termed Uptake₁ and Uptake₂, the former having a lower K_m and V_{max} than the latter.

Uptake₁ is probably confined to peripheral sympathetic neurons and noradrenaline containing neurons within the central nervous system. (The dopamine uptake system in brain differs from this by lacking stereochemical specificity and furthermore it has a striking difference in drug sensitivity (Iversen, 1974). Peripherally the uptake is specific in favour of the naturally occurring (-)-isomer of noradrenaline and prefers noradrenaline to adrenaline. It is inhibited by a wide range of sympathomimetic amines which are structurally related to noradrenaline and which may or may not be taken up into the neurons themselves by the same transport mechanism, e.g. amphetamine inhibits uptake without being transported into the neuron, whereas p-hydroxyamphetamine both inhibits uptake and enters the neuron. Tables from which the following data have been extracted were presented by Iversen (1967) and illustrated the effects of various structural changes of the affinity of drugs for the noradrenaline uptake process.

<u>Drug</u>	<u>Concentration for 50% inhibition</u> <u>of noradrenaline uptake</u> <u>moles</u>	<u>Relative affinity</u> <u>for uptake site</u> (phenylethylamine = 100)
(+)-Amphetamine	1.8×10^{-7}	610
(+)-Amphetamine	4.6×10^{-7}	240
(-)-Amphetamine	37×10^{-7}	30
(+)-p-Hydroxyamphetamine	1.8×10^{-7}	610
(+)-Norephedrine	20×10^{-7}	55
(-)-Ephedrine	22×10^{-7}	50
Phenylethylamine	11×10^{-7}	100

Other drugs which inhibit catecholamine uptake (Uptake₁), but which will not be further discussed here include cocaine, imipramine, desipramine, the adrenergic blocking drugs and certain monoamine oxidase inhibitors (Iversen, 1967).

The likely physiological roles for Uptake₁ are: a) the removal of the transmitter substance from the extracellular space, so that it no longer acts on the postsynaptic receptors, and b) the reuse of noradrenaline by the nerve for further cycles of release and uptake.

Uptake₂ is a low affinity system which has maximum rates of uptake much greater than those associated with Uptake₁. The former is not stereospecific and has a higher affinity for adrenaline than noradrenaline. This membrane transport system takes up exogenous catecholamines into extraneuronal tissues (Iversen, 1971) and may be inhibited by the metanephrines, phenoxybenzamine, amphetamine and certain steroids (Iversen, 1971). The probable roles for Uptake₂ are a) the removal of transmitter from the extracellular space (less important in this respect than Uptake₁), b) the temporary uptake of noradrenaline into the cytosol of the postsynaptic cell, where it could act directly on intracellular processes before being metabolised, and c) the removal and inactivation of circulating catecholamines.

Most of the noradrenaline taken up into the nerve terminals is not metabolised, but is retained within the storage vesicles where it is protected from attack by MAO (Potter & Axelrod, 1963). This second process which transports noradrenaline into the storage vesicles is quite independent of Uptake₁ and may be inhibited by reserpine (Iversen, 1967).

Storage of Noradrenaline

Biochemical studies with sucrose-density-gradient centrifugation of a particulate fraction from the homogenates of sympathetic nerves have revealed a

bimodal distribution of noradrenaline suggesting two types of storage vesicle for this amine with different densities (Roth *et al.*, 1968). Ultrastructural and histochemical studies have suggested that these light and heavy vesicles correspond to the small and large dense cored vesicles respectively, the former being found only in nerve terminals, whereas the latter are found both in non-terminal axons and sympathetic nerve terminals (Bisby & Fillenz, 1971; Smith, 1972). A third form of vesicle has been identified (Tranzer *et al.*, 1969) which is electronlucent and has the ability to take up and store exogenous amines.

Several other components of the large dense cored vesicles have been identified (see review by Smith, 1972), namely dopamine- β -hydroxylase, a protein that cross-reacts with antisera to chromogranin A, ATP, adenosine triphosphatase, Mg^{++} and cytochrome b_{551} . It has been proposed that ATP may form high molecular-weight aggregates with noradrenaline. These have been demonstrated in vitro when the molar ratio of noradrenaline/ATP is 3.5:1 (Bernels, Platscher & Da Prada, 1970). In vivo, however, this molar ratio has been variously estimated to be between 4 and 12 (Helle, Lagercrantz & Stjarne, 1971; De Potter, Smith & De Schaepdryver, 1970).

Alternatively the vesicular membrane may be responsible for the binding of noradrenaline. This membrane probably contains the ATP-dependent active uptake process that counteracts the spontaneous loss of noradrenaline (von Euler & Lishajko, 1963).

In addition to the above mentioned granular storage compartments for noradrenaline, subcellular fractionation studies have indicated the presence of an extravesicular compartment containing 'free' noradrenaline (Hokfelt, 1974).

Release of Noradrenaline

Cell fractionation and ultrastructural studies on adrenergic nerves have shown that catecholamines are present in granules and also that some may be free in the cytosol. It is generally held that only granular stores of noradrenaline together with the protein constituents of the vesicle are released by the process of exocytosis (Smith, 1972; De Potter, 1974) and that noradrenaline free in the cytosol cannot be released by nerve stimulation.

Geffen and Rush (1974) reported that noradrenaline release was accompanied by release of dopamine- β -hydroxylase (DBH) and chromogranin, in addition to which, the finding that newly synthesised or recaptured transmitter is preferentially released suggests that "the small, easily releasable" pool of transmitter is contained in the young large DBH-rich vesicles, while the "large stable" pool is stored mainly in old, small, DBH-poor vesicles (Dahlstrom & Haggendal, 1974). In addition, these authors state that noradrenaline release occurs by partial rather than complete exocytosis (i.e. only a fraction of vesicular contents are released at any one time).

The lack of absolute specificity of the noradrenergic neuron with respect to Uptake₁ and the subsequent transport of noradrenaline into the vesicles enables many phenylethylamine derivatives to be similarly transported. Having entered the vesicle some of these compounds can be β -hydroxylated by DBH and stored prior to release as "false neurotransmitters" (Kopin, 1968). This subject will be discussed in relation to amphetamine metabolites in greater detail in subsequent chapters.

The Effect of Reserpine on Storage

Apart from the previously mentioned effect of reserpine on inhibiting the uptake of noradrenaline into granular stores, this drug is capable of producing

an almost complete depletion of catecholamines in tissues such as heart and spleen, following a dose of 0.1 mg/kg (Iversen, 1967). Maximal depletion occurs from 12-24 h after administering the drug and persists for several days. The mechanism of this depletion is not proven, but it is apparently neither due to inhibition of Uptake₁ nor to release of noradrenaline from the nerve terminals. It is speculated that newly synthesised noradrenaline, instead of being stored within the granules, leaks out of the particles and is rapidly destroyed by monoamine oxidase.

The Sympathomimetic Amines

A wide variety of amines structurally related to noradrenaline and adrenaline have been studied since Barger and Dale's classic work (1910). Fleckenstein and Stockle (1955) classified these drugs into three groups:-

- 1) Direct-acting amines, whose actions were potentiated by denervation and cocaine.
- 2) Indirect-acting amines, which were less effective after denervation and cocaine.
- 3) Mixed-acting amines, which are not appreciably affected by either procedure.

Burn and Rand (1958) proposed that the indirectly acting sympathomimetics produced their effect by releasing noradrenaline from tissue storage sites and this group, together with the direct and mixed acting groups were subsequently classified by Trendelenburg (1963). The structure-activity relationships for amines of the various groups have been noted by Iversen (1967) and whereas directly acting amines comprise the catecholamines and certain metahydroxylated monophenolic amines which have a β -hydroxyl group, the indirect-acting amines, which include amphetamine and certain analogues, are nonphenolic compounds or mono-

phenols without a p -hydroxyl group. The same author makes the point that the overall action (direct or indirect) of each amine may vary in different tissues and on different receptors. This fact may be of relevance in the general discussion for the mechanism of action of certain amphetamine metabolites.

The Pharmacology of Amphetamines

The amphetamine group of compounds possess a multiplicity of biological effects and modification of the molecular structure in a variety of ways has been popular in attempts to accentuate one desired effect over the others. Some idea of this will be gained from the brief outline of structure-activity relationships given earlier.

In common with other indirect acting sympathomimetics, amphetamine has peripheral α and β effects in addition to its powerful CNS stimulant action. Alles (1933) compared the pressor effects of amphetamine and p -hydroxyamphetamine with those of adrenaline and found that although the potency of this response was only 1/100 that of adrenaline, these effects of amphetamine and its hydroxylated analogue, p -hydroxyamphetamine were of much longer duration. Large doses may produce tachycardia and arrhythmias. Other smooth muscles respond to these drugs as with all sympathomimetics, for example bronchial relaxation occurs and the urinary bladder sphincter is constricted. Amphetamine is a potent anorectic and elevates plasma free fatty acids and body temperature (Goodman & Gilman, 1970).

More important, however, is the powerful CNS stimulant action, an action not shared with p -hydroxyamphetamine. Such stimulation affords these drugs their high abuse potential. The psychic effects depend upon dose and the mental

state and personality of the individual. Fatigue is suppressed, mood and confidence are elevated, and there is euphoria. Physical activity and some simple mental performance tasks are enhanced but with large doses stereotyped behavioural patterns and a toxic paranoid psychosis may occur (Van Rossum, 1972). In laboratory animals, locomotor activity is enhanced and stereotyped movements are evident.

Certain human subjects experience headache, palpitation, vasomotor disturbances, agitation, confusion and dysphoria (Goodman & Gilman, 1970), thus exemplifying the widespread interindividual variations in drug response.

With chronic administration there appears to be attenuation of the euphoriant effect in man. However, according to Kosman and Unna (1968) there is no such attenuation of fatigue suppression. Repeated intravenous administration of amphetamines is associated with an intensely pleasurable sensation immediately after each injection (which has been likened to an orgasm), over-activity which later becomes uncoordinated, and heightened sexual interest (Rylander, 1972).

Geerlings (1972) states that chronic amphetamine use causes marked mental changes in the majority of cases. About one third of his patients had experienced psychotic states with paranoid delusions, stereotyped behaviour, auditory and visual hallucinations and occasionally parasite delusions. In some cases aggressive behaviour was noted.

Discontinuation of amphetamines in a dependent subject without doubt produces profound symptoms of lethargy, depression and occasionally suicidal tendencies (Lloyd, 1973).

The desire to resume taking the drugs is thus motivated by a need to combat these symptoms (Kramer, Fischman & Littlefield, 1967). Management of patients with acute psychoses requires sedation with, for example, haloperidol,

promethazine or large doses of benzodiazepines. Rehydration is important, as fluid loss occurs as a result of hyperthermia. The studies of Anggard *et al.* (1970) suggest that acidification of the urine in psychotic subjects may be beneficial.

Mode of Action

It has proved very hard to pinpoint the precise locus of action of the amphetamines, and indications are that the observed effects are due to a number of contributing mechanisms. Although many actions of the amphetamines have been demonstrated, it is difficult to attribute the drug's action to any of these or even to assume that they are involved at all in the whole animal situation (Iversen, Axelrod & Glowinski, 1966).

A variety of theories for the mechanism of action of amphetamine have been put forward and four are currently regarded as of importance; (a) monoamine oxidase inhibition, (b) release of catecholamines from neuronal binding sites, (c) inhibition of catecholamine reuptake into neurons and (d) direct action on the noradrenergic receptor. All of these appear to contribute towards the net effect of amphetamine. Monoamine oxidase inhibition, although not correlated well with the activity of amphetamines, is almost certainly involved in the drug's action (Iversen, Axelrod & Glowinski, 1966; Rutledge, 1970).

The release of catecholamines appears to be the most important of the listed actions. Evidence for this is provided by the stimulant action of small doses of amphetamine which are without effect on catecholamine reuptake (Glowinski, 1970). Fuxe & Ungerstedt (1970), from combined histochemical and biochemical studies confirmed that amphetamine acted principally by releasing amines from both central dopamine and noradrenaline containing neurons. These authors provided evidence that this release occurred from extragranular stores of newly synthesised amines and in high doses of amphetamine it appeared that 5HT

was also released. However, most authors consider that amphetamine releases granular stores of catecholamines (Carlsson, 1970).

Functional studies have concentrated on the relative importance of both dopamine and noradrenaline in the action of amphetamine (Chapter 8).

Reserpine, which is able to deplete 80-90% of neuronal noradrenaline, has little effect on overall motor activity, however exploratory behaviour and central excitation may be diminished in contrast to stereotyped behaviour which remains unchanged (Fuxe & Ungerstedt, 1970).

Inhibition of the ongoing biosynthesis of catecholamines with α -methyl-p-tyrosine abolishes these effects of amphetamine. After such inhibition both effects can be restored by l-DOPA. On this evidence, it has been suggested that some of the central actions of amphetamine, particularly stereotyped behaviour, depend upon release of dopamine (Van Rossum, 1972). These conclusions are further substantiated by Creese and Iversen (1973) who showed abolition of stereotypy by local injection of 6-hydroxydopamine into the nigrostriatal dopaminergic pathway. Locomotor activity, on the other hand, may be more related to noradrenaline release as, according to Randrup and Scheel-Krüger (1966), diethyldithiocarbamate, which inhibits dopamine- β -hydroxylase, sedates animals with reduction in hypermotility, but no alteration of stereotypy.

Further illustration of the role of central noradrenergic neurons in the amphetamine syndrome was provided by Fuxe and Ungerstedt (1970) who showed that when clonidine (a central and peripheral noradrenaline-receptor agonist) and apomorphine (a central dopamine-receptor agonist) are given in combination, stereotyped behaviour predominates with high doses of apomorphine and low doses of clonidine, whereas the reverse is true with high doses of clonidine, in which case marked exploratory behaviour (gross locomotor activity) is dominant. Perhaps the

ratio of dopamine to noradrenaline receptor activity after amphetamine administration decides the resultant activity. High doses of amphetamine producing mainly stereotyped behaviour suggests that the ratio of dopamine to noradrenaline receptor activity is increased (see Chapter 8).

Amphetamine is also able to facilitate self-stimulation in rats implanted with electrodes in reward areas of the hypothalamus and mid-brain areas (Stein, 1964). This effect can also be blocked by dopamine- β -hydroxylase inhibitors, and is reversed by intraventricular administration of noradrenaline, but not dopamine (Wise & Stein, 1969), suggesting that noradrenaline plays an important role in certain aspects of amphetamine's central action.

The role of 5-hydroxytryptamine (5HT) in the mediation of amphetamine-induced CNS stimulation is at present a matter for speculation. As mentioned above, evidence has been provided for the release of 5HT in brain by large doses of amphetamine. Sparber and Tilson (1972) demonstrated that in rats the development of tolerance to the effects of amphetamine upon operant behaviour was accompanied by release of brain 5HT, in contrast to the drug naive state in which noradrenaline only was released.

Amphetamine has also been shown to increase the output of acetylcholine from the cerebral cortex in cats (Deffenu, Bartolini & Pepeu, 1970), however, the significance of this observation in relation to the known effects of the drug is uncertain.

So far, only the acute effects of amphetamines and their relation to the observed changes in catecholamine disposition have been discussed. After prolonged treatment with this drug other effects are apparent; tolerance develops to certain pharmacological effects, notably those mediated through noradrenaline release (see later chapters). Stereotyped behaviour in animals and psychoses in man may develop (Van Rossum, 1972). In discussing the mechanisms through which these changes develop, emphasis must be placed upon the possible roles of the

amphetamine metabolites, *p*-hydroxyamphetamine and *p*-hydroxynorephedrine.

There is little evidence that the acute effects of amphetamine are related to the formation of *p*-hydroxynorephedrine since the time course of accumulation of this drug in tissues does not correlate with the observed pharmacological effects of amphetamine. Also desmethylinipramine (DMI), which potentiates amphetamine-induced hyperthermia and activity, blocks the formation of this compound (Costa & Groppetti, 1970). Of importance, however, is the suggestion that the prolonged tissue depletion of noradrenaline, at least in the rat, is dependent upon the production of *p*-hydroxynorephedrine and possibly causally related to the development of tolerance. This subject will be discussed in greater detail. In addition to the peripheral cardiovascular effects of *p*-hydroxyamphetamine previously noted, this metabolite has been implicated in the central action of amphetamine. According to Costa and Groppetti (1970), DMI, which blocks the hydroxylation of dopamine to form noradrenaline, also stabilises the concentration of *p*-hydroxyamphetamine by preventing its conversion to *p*-hydroxynorephedrine, and, as stated above, the potentiation of amphetamine effect by pretreatment with DMI may be the result of enhanced levels of *p*-hydroxyamphetamine. Further support for the concept that *p*-hydroxyamphetamine mediates the central effects of amphetamine was provided by experiments in which reserpinisation of rats potentiated the motor stimulant activity following amphetamine, and this potentiation was associated with decreased concentrations of brain amphetamine. In addition, α -methyl-*p*-tyrosine which abolishes the CNS stimulation produced by amphetamine also decreases brain levels of *p*-hydroxyamphetamine, but in this situation the synthesis of both dopamine and noradrenaline is also interrupted.

It is proposed by Anggard *et al.* (1973) in the basis of metabolic studies in the human amphetamine psychosis that the intensity of this psychosis was positively

correlated with the renal excretion of ring hydroxylated metabolites of amphetamine but not to the amphetamine level in plasma, suggesting therefore that these metabolites were of importance in the development of the paranoid psychosis in chronic amphetamine abusers.

Taylor and Sulzer (1973) implicate both *p*-hydroxyamphetamine and *p*-hydroxynorephedrine in the pharmacological profile of amphetamine, on the basis of experiments in which these metabolites were injected into the cerebral ventricles. *p*-Hydroxyamphetamine elicited mainly locomotor stimulation, whereas *p*-hydroxynorephedrine caused predominantly an increase in stereotyped behaviour. Although extrapolation of data derived from such experiments to the situation in which amphetamine itself is administered systemically is speculative, nevertheless the possibility that these metabolites contribute to the overall CNS stimulation following amphetamine administration must be entertained.

CHAPTER TWO

MATERIALS AND METHODS

Chapter Two**Materials and Methods**

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Materials and Methods

Compounds

(+)-2-Amino-1-(4'-hydroxyphenyl)propane (p-hydroxyamphetamine) hydrobromide (Paredrine), m.p. 190-192°C, (+)-2-amino-1-phenylpropane (amphetamine) sulphate, m.p. 308°C and (+)-2-amino-1-phenylpropan-1-ol (norephedrine) hydrochloride, m.p. 190°C, were the gifts of Smith, Kline and French Laboratories, Philadelphia, Pennsylvania, U.S.A.; (+)-2-amino-1-(4'-hydroxyphenyl)propan-1-ol, (p-hydroxynorephedrine) hydrochloride, m.p. 190-192°C (Aldrich Chemical Company, Milwaukee, U.S.A.); (-)-2-methylamino-1-phenylpropan-1-ol (ephedrine) hydrochloride, m.p. 213°C (Sigma Chemical Company, St. Louis, Missouri, U.S.A.); (+)-2-methylamino-1-(4'-hydroxyphenyl)propan-1-ol (p-hydroxyephedrine) hydrochloride, m.p. 188-189°C (R. N. Emanuel Ltd., Wembley, U.K.). Other known compounds were purchased and purified as appropriate.

(+)-[1-¹⁴C]-Amphetamine sulphate of specific radioactivity 2.92 μ Ci/mg was a gift of Smith, Kline and French Laboratories. (+)-[¹⁴C]-p-Hydroxyamphetamine hydrochloride (specific activity 0.80 μ Ci/mg) and (-)-[¹⁴C]-ephedrine hydrochloride (specific activity 0.06 μ Ci/mg) were synthesised by Dr. L. G. Dring, of this Department. These syntheses are outlined in the Appendix to this thesis.

(+)-1,2-Dihydroxy-1-phenylpropane

Methyl styrene was prepared by the dehydration of 1-phenylpropanol (100 g) according to the method of Gauthier (1933), (Yield 58 g).

The methyl styrene was mixed with dry chloroform (50 ml), cooled in an ice bath, and a bromine solution (84 g in 50 ml chloroform) was added dropwise. Excess chloroform was evaporated and the solid residue recrystallised from aqueous ethanol to give 1,2-dibromo-1-phenylpropane of m.p. 73-74°C (Yield 50 g). This dibromo compound was subjected to mild hydrolysis (Zincke & Zahn, 1910) to give

(+)-1,2-dihydroxy-1-phenylpropane (m. p. 90-92°C, 7g) (Zincke & Zahn give 92-93°C).

Animals

Female Wistar albino rats, body weight, 220 ± 20 g; male Wistar albino rats, body weight 250 ± 20 g; and female Dunkin-Hartley guinea pigs, body weight 250 ± 20 g were used.

Human Volunteers

Eight informed human volunteers participated in the study, aged 25-45 years, body weight 55-75 kg. No subject gave a history of renal or hepatic disease.

Administration of Compounds

Compounds were administered in aqueous solution by gastric tube to rats, by intraperitoneal injection to guinea pigs and orally to human subjects. In a single study of a human volunteer, [^{14}C]-p-hydroxyamphetamine hydrochloride was made up in an isotonic saline solution and sterilisation was performed by ultra-filtration (Millipore filter) prior to intravenous injection.

Collection of Excreta

Animals were housed singly in metabolism cages and their urine and faeces were collected daily for 4 days. They were allowed free access to water, but food was withheld for the first 24 hours to avoid contamination of the urine.

Human volunteers were allowed a normal intake of fluids and food. Urine and faeces were collected daily for 4 days.

Storage of Excreta

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

Spectra

I. r. spectra were determined using a Perkin-Elmer Infracord Spectrophotometer (Model 204).

Radiochemical Techniques

¹⁴C in urine and faeces were determined according to Bridges, Smith and Williams (1967) using Packard Tri-Carb liquid scintillation spectrometers (models 3320 and 3375). Radiochromatogram scanning of paper and thin layer chromatograms was performed with a Packard radiochromatogram scanner (model 7200), identification of ¹⁴C peaks being made by comparison of R_F values with those of authentic compounds (see Table 2:1). The ¹⁴C content of peaks was estimated by cutting paper chromatograms into 1 cm segments and counting these in the scintillation counter. With thin layer plates, 0.5 cm sections of the adsorbent were scraped off and counted similarly.

Faeces were homogenised with 2 volumes of water in a Waring Blendor. The homogenate (20 ml) was made alkaline with 10M NaOH (2 ml) and an equal volume of H₂O₂ solution (100 vol. %) added with a few drops of iso-octanol to control foaming. The mixture was allowed to stand at room temperature for 3 days until decolourised, and aliquots then assayed for ¹⁴C by liquid scintillation spectrometry. In some cases after homogenising 24 hour collections of faeces, samples (5 ml) were freeze-dried and combusted in a Packard Tri-Carb sample oxidiser (Model 306). Products of combustion were collected in a 1:2 (v/v) solution of ethanolamine in 2-methoxyethanol. Carbon dioxide trapped in this solution was determined by counting measured samples (1 ml) of solvent in scintillator liquid (15 ml) composed of 5.5 g/litre of 2,5-diphenyloxazole in toluene:2-methoxyethanol (2:1, v/v) (Jeffay & Alvarez, 1961).

Isotope Dilution Procedures

Amphetamine, p-hydroxyamphetamine, p-hydroxybenzoic acid, hippuric acid and benzoic acid were assayed as described by Dring, Smith and Williams (1970). Norephedrine and p-hydroxynorephedrine were assayed according to the methods of

Caldwell, Dring and Williams (1972a). Approximately one-tenth of the daily urine output was used for each assay.

Ephedrine

The amine hydrochloride (1 g) was dissolved in urine (100 ml), which was adjusted to pH 14 by the addition of 10M NaOH, saturated with solid NaCl and extracted with ether (3 x 100 ml). The bulked extracts were dried over anhydrous Na_2SO_4 and reduced to a small volume (5 ml) by evaporation under reduced pressure. Dry HCl gas in ethanol (2.49N) was then added until the solution became acidic. After evaporation to dryness in vacuo, ephedrine hydrochloride was recrystallised from anhydrous ethanol to constant specific radioactivity (m. p. 213°C).

(+)-1,2-Dihydroxy-1-phenylpropane

The diol (1 g) was dissolved in urine, which had been hydrolysed with a sulphatase preparation (vide infra). After extraction with ether (3 x 100 ml) the bulked extracts were dried over anhydrous Na_2SO_4 and reduced to dryness in vacuo. The residue was treated with 10M NaOH (5 ml) and benzoyl chloride (5 ml). The mixture was shaken vigorously for 10 minutes and left to stand overnight. The dibenzoyl derivative was washed with 10M NaOH (60ml) and water (60ml) and recrystallised from dilute ethanol according to the method of Zincke and Zahn (1910). (m. p. 100°C; Zincke & Zahn give 101°C).

Chromatography

Table 2:1 gives R_F values of reference compounds used in this study, using the systems below. Descending paper chromatography was performed on either Whatman No. 1 or 3MM paper in solvents A, B, C and D. R_F values were identical on both papers. Systems E, F & G were used for thin layer chromatography (t. l. c.) using glass plates 5 x 20 cm, spread with Alumina G (Solvent E) or Silica Gel GF₂₅₄ (solvent F) (Merck A. G., Darmstadt, Germany) 0.25 mm thick, which

were heated at 120°C for 1 hour, before use.

System A. 2-methyl-butan-2-ol / 1,1-dimethylpropanol / water / formic acid (5:5:10:2 by volume). (Alleva, 1963).

System B. n-butanol saturated with M HCl

System C. iso-Propanol / ammonia / water (16:2:2 by volume)

System D. n-butanol saturated with 1.5M ammonia - ammonium carbonate buffer (Fewster & Hall, 1951).

System E. Methanol / chloroform (1:1 by volume) (Noirfalise, 1966).

System F. Chloroform / diethylamine / methanol (20:1:1) (Sinsheimer, Dring & Williams, 1973).

System G. Benzene / methanol / acetic acid (10:2:1) after chloroform / diethylamine / methanol (20:1:1) (Sinsheimer, Dring & Williams, 1973)

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

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

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

Diazotized *p*-nitroaniline. Chromatograms were sprayed with diazotized *p*-nitroaniline, as described by Wickström and Salvesen (1952). Phenols and amines gave colour reactions as shown in Table 2:1.

Hippuric Acid Detection Hippuric acid was seen as an orange spot after spraying with *p*-dimethylaminobenzaldehyde in acetic anhydride, and heating at 120°C for 2 mins. (El Masry, Smith & Williams, 1956).

Benzolic Acid Detection Benzolic acid was detected with a spray of bromophenol blue in ethanol, showing as a blue spot (Opienska-Blauth, Saklowska-Szymonowa & Kanski, 1951).

Ion Exchange Chromatography

Preparation of samples for ion exchange chromatography in *p*-hydroxy-amphetamine studies, involved preliminary hydrolysis of aliquots of urine (5-10 ml), to which *p*-hydroxyamphetamine (50 µg) and *p*-hydroxynorephedrine (50 µg) were added, with a sulphatase preparation (see Hydrolyses of Urine). These hydrolysates were reduced to a small volume (2 ml) by evaporation under reduced pressure and applied to the Dowex column (*vide infra*). In the case of urine samples (50 ml) from amphetamine metabolism studies in humans, after adjustment to pH 14 with 2M NaOH and extraction with toluene/isoamyl alcohol (84:16 by volume, 250 ml) to remove amphetamine and norephedrine, the aqueous phase was evaporated to dryness under reduced pressure, extracted with ethanol (3 x 10 ml) to remove salts, and the ethanol extract evaporated and taken up in distilled water (2 ml). All samples were then adjusted to pH 4.5 with 2M HCl and put on strong cation exchange resin columns (Dowex 50-W, mesh 200-400, Na⁺ form, 0.4 x 5 cm). The columns were then washed with redistilled water (5 ml), 1M sodium acetate buffer (5 ml) pH 6.0 containing 0.1% EDTA and again with redistilled water (10 ml). The amines were eluted with 1M HCl in 2 ml fractions. *p*-Hydroxynorephedrine and *p*-Hydroxy-amphetamine were determined by measuring each fraction with an Aminco-Bowman spectrophotofluorimeter (excitation 285 nm; emission, 375 nm) against standard solutions (1-5 µg/ml M HCl) of the two amines. Portions of each eluate were taken for the measurement of radioactivity by scintillation counting. Recoveries of *p*-hydroxynorephedrine and *p*-hydroxyamphetamine through this procedure varied from 80-90%.

Hydrolyses of Urine

Urines were subjected to either enzymic or acid hydrolysis prior to chromatographic separation of metabolites and reverse isotope dilution procedures.

Acid Hydrolysis

Equal volumes of urine and 10M HCl were refluxed together for 2 hours.

The resultant mixture was cooled and neutralized before chromatography. It was discovered, however, that when urine samples to which known amounts of p-hydroxynorephedrine had been added were subjected to this procedure, subsequent paper chromatography revealed that side chain cleavage had resulted in the formation of variable quantities of p-hydroxybenzaldehyde (Sinsheimer, Dring & Williams, 1973). The following method was therefore used for all hydrolyses of original urines, and for isotope dilution procedures for p-hydroxynorephedrine.

Enzymic Hydrolysis

A suitable volume of urine was adjusted to pH 5 with 2M acetic acid and incubated with either an equal volume of β -glucuronidase solution (Ketodase; Warner-Chilcott, Eastleigh, Hants., U.K.) or 0.2 vol. of an aryl-sulphatase preparation (Type H-2, Sigma Chemical Company, St. Louis, Miss. U.S.A. which also contains β -glucuronidase) and 0.5 vol. 0.2M sodium acetate/acetic acid buffer pH 5 for 24 hours at 37°C. Control samples were prepared with enzyme that had been denatured by boiling. The hydrolysed solutions were either applied directly to chromatograms, or were evaporated to dryness under reduced pressure at 40°C, extracted with ethanol (3 x 10 ml), re-evaporated and taken up in a small volume of methanol which was then chromatographed. With large volumes of urine from human experiments, samples were initially concentrated by freeze-drying.

Table 2:1. R_F Values and Colour Reactions of Amphetamine and Related Compounds

	R _F value in solvent:							
	Paper				Thin layer			
<u>Compound</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>	<u>G</u>	<u>Colour with diazotized</u> <u>p-nitroaniline</u>
Amphetamine	0.54	-	-	0.87	-	-	-	pink
p-Hydroxyamphetamine	0.32	0.50	0.83	0.78	-	-	-	purple
Ephedrine	0.52	-	-	0.85	0.52	-	0.73	pink
p-Hydroxyephedrine	0.23	-	-	0.79	0.15	-	-	pink
Norephedrine	0.39	-	-	0.80	0.30	-	0.70	pink
p-Hydroxynorephedrine	0.21	0.36	0.72	0.63	0.10	-	-	mauve
Benzoic acid	0.97	-	-	0.43	-	0.30	0.69	none
Hippuric acid	0.82	-	-	0.25	-	0.13	0.43	none
Benzoyl glucuronide	0.60	-	-	0.10	-	0.10	-	none
p-Hydroxybenzoic acid	0.85	0.86	-	0.01	-	0.10	-	mauve
p-Hydroxyhippuric acid	0.73	-	-	-	-	0.00	-	none
1,2-Dihydroxy-1-phenylpropane	0.81	-	-	-	0.70	0.63	0.85	none
1-Hydroxy-2-oxo-1-phenylpropane	-	-	-	-	-	0.99	-	none
1,2-Dioxo-1-phenylpropane	-	-	-	-	-	0.99	-	none

CHAPTER THREE

THE METABOLISM OF p-HYDROXYAMPHETAMINE IN RAT,
GUINEA PIG AND MAN

Chapter Three **The Metabolism of p-Hydroxyamphetamine in Rat, Guinea Pig**
and Man

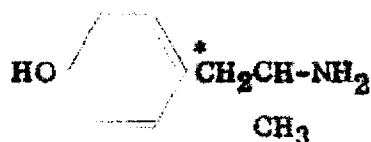
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The Metabolism of p-Hydroxyamphetamine In Rat, Guinea Pig and Man

Introduction

Comprehensive studies of drug biotransformation were greatly facilitated by the introduction of radiochemical techniques some twenty years ago. The disposition of a drug following its administration to laboratory animals can be determined with a high degree of sensitivity by utilising radioactive isotopes, the choice of which is dependent upon the chemical structure of the compound and the positions within the molecule where substitution of a label allows detection of the greatest number of potential metabolites.

In general for metabolic studies ^{14}C is preferable to ^3H and in this case the ^{14}C isotope was inserted in the β -position of the side chain, adjacent to the ring, thus enabling metabolism both in the aromatic ring and in the side chain to be detected.



Identification of Metabolites

Rat urine

Urine samples (0.1 ml) were chromatographed on paper in solvent A, and chromatograms scanned for ^{14}C . A major peak (R_F 0.00) and a lesser peak (R_F 0.32) were found in urines from female animals (Fig. 3:1). However, when samples from male animals were chromatographed a single major peak occurred at R_F 0.32 (Fig. 3:2). Hydrolyses of urines from the female rats with β -glucuronidase caused the peak of R_F 0.00 to disappear with a corresponding increase in the height of peak R_F 0.32. On some chromatograms, after hydrolysis of urines from both sexes a small peak of R_F 0.85 was seen.

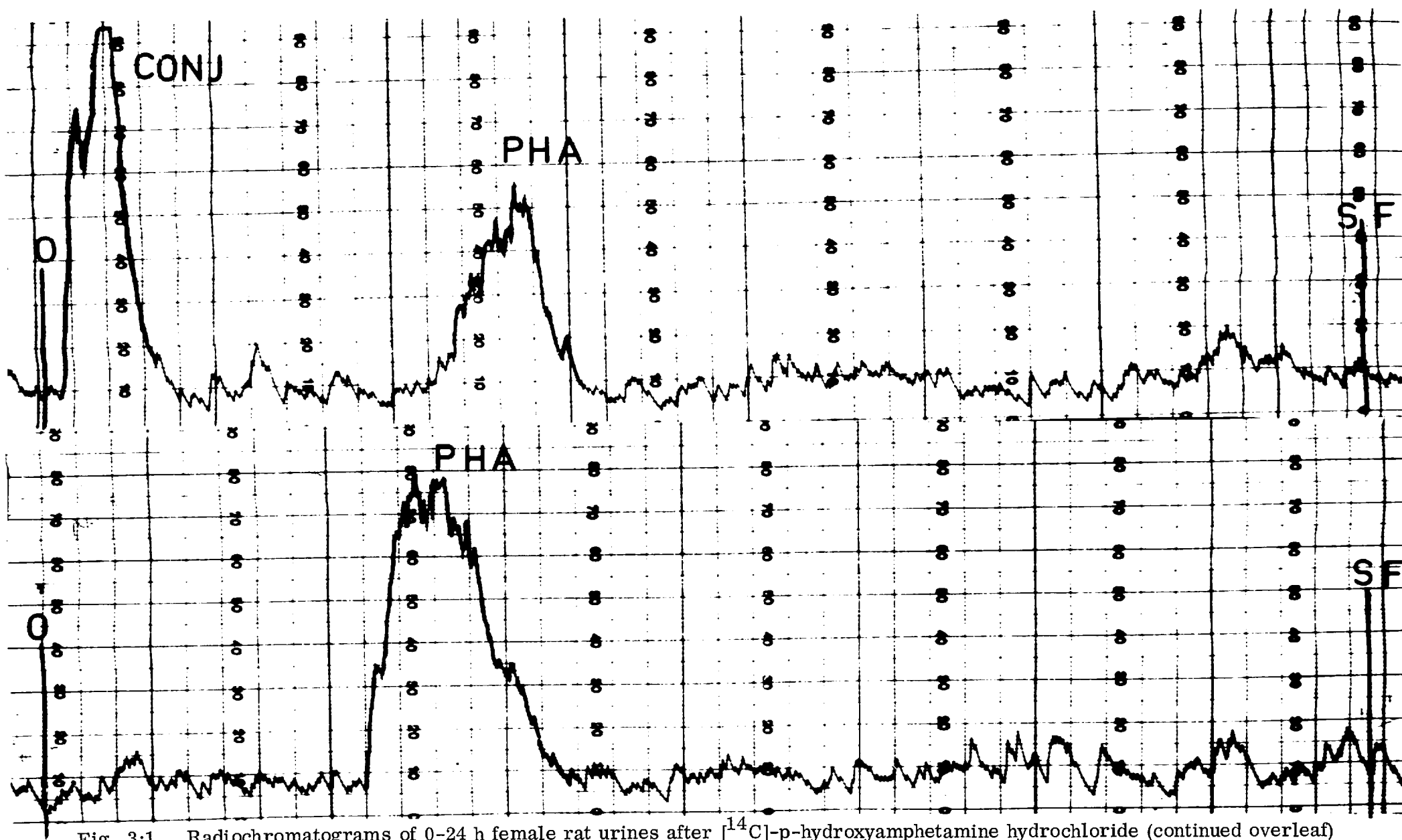


Fig. 3:1. Radiochromatograms of 0-24 h female rat urines after [^{14}C]-p-hydroxyamphetamine hydrochloride (continued overleaf)

Fig. 3:1

Radiochromatograms of 0-24 h female rat urines after [^{14}C]-p-hydroxy-
amphetamine hydrochloride (15 mg/kg; 9 μCi /kg) orally, in solvent A.

Upper chromatogram - prior to hydrolysis

Lower chromatogram - after hydrolysis

O = Origin
SF = Solvent front
CONJ = Metabolites conjugated with glucuronic acid
PHA = p-Hydroxyamphetamine.

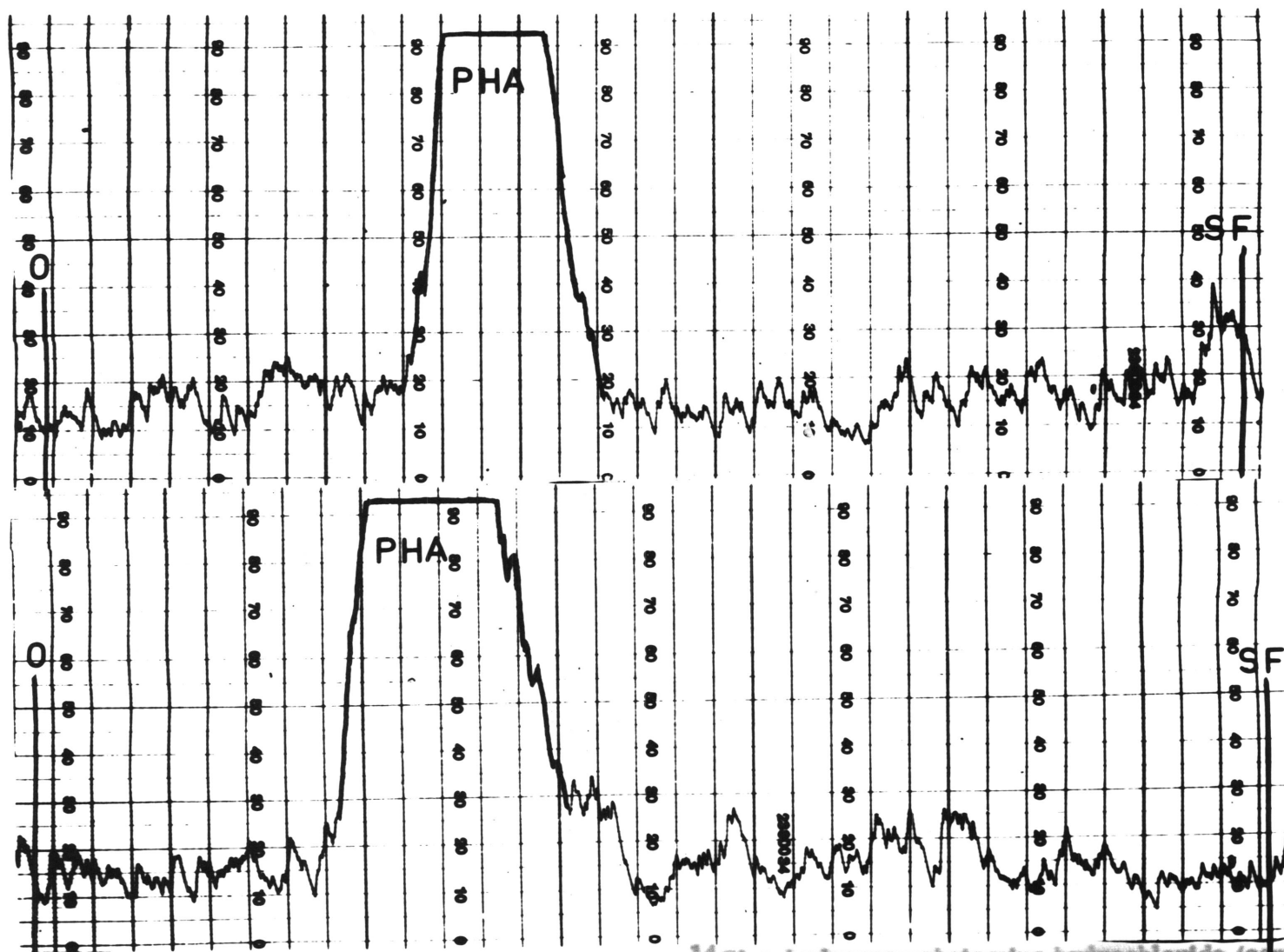


Fig. 3:2. Radiochromatograms of 0-24 h male rat urines after $[^{14}\text{C}]$ -p-hydroxyamphetamine hydrochloride (continued overleaf)

Fig. 3:2.

Radiochromatograms of 0-24 h male rat urines after [^{14}C]-p-hydroxyamphetamine hydrochloride (15 mg/kg; 9 $\mu\text{Ci/kg}$) orally, in solvent A.

Upper chromatogram - prior to hydrolysis

Lower chromatogram - after hydrolysis

O = Origin
SF = Solvent Front
PHA = p-hydroxyamphetamine

After hydrolysis of male and female urines with an aryl sulphatase/
glucuronidase preparation and subsequent chromatography in solvent B, two peaks
were identified of R_F 0.36 and 0.50 corresponding to *p*-hydroxynorephedrine and
p-hydroxyamphetamine respectively (Fig. 3:3).

Separation of acidic and basic metabolites was performed by adjusting urine
samples (10 ml) to pH 2 with 2M HCl and extracting with ether (3 x 30 ml). The
aqueous phase, containing amines, was readjusted to pH 6 with 2M NaOH and an
aliquot chromatographed in solvent A. Two peaks were again found, R_F 0.00
and R_F 0.32. The peak at the origin in female urines was eluted with water,
hydrolysed with β -glucuronidase and chromatographed in solvent B to yield
peaks of R_F 0.36 and 0.50, corresponding to *p*-hydroxynorephedrine and *p*-hydroxy-
amphetamine respectively.

The ethereal phase, containing acidic metabolites, was extracted with 5%
NaHCO₃, and the aqueous phase acidified to pH 2 with 2M HCl, saturated with
NaCl, and re-extracted with ether. This final ethereal extract was dried over
anhydrous sodium sulphate, evaporated to dryness and redissolved in methanol
(1 ml). This solution was chromatographed in solvents A and B, and yielded
one peak corresponding to authentic *p*-hydroxybenzoic acid (R_F 0.85 solvent A,
0.86 solvent B).

The formation of *p*-hydroxynorephedrine was confirmed by ion exchange
chromatography, and by reverse isotope dilution procedures.

Guinea Pig Urine

Urine samples were subjected to paper chromatography essentially as
described above, in solvents A and B. In the former solvent a major peak
occurred at the origin (R_F 0.00) and a minor peak of R_F 0.32. Hydrolysis with
 β -glucuronidase caused disappearance of the peak at the origin with a consequent
increase in the size of the peak at R_F 0.32. In solvent B, after hydrolysis of

Fig. 3:3. Histograms prepared from radiochromatograms of 0-24 h urines from 3 species after [^{14}C]-p-hydroxyamphetamine hydrochloride, after hydrolysis, in solvent B

Female rat (15 mg/kg; $9\mu\text{Ci/kg}$) orally. Female guinea pig (15 mg/kg; $9\mu\text{Ci/kg}$) i. p.
Man (0.4 mg/kg; $0.07\mu\text{Ci/kg}$) orally

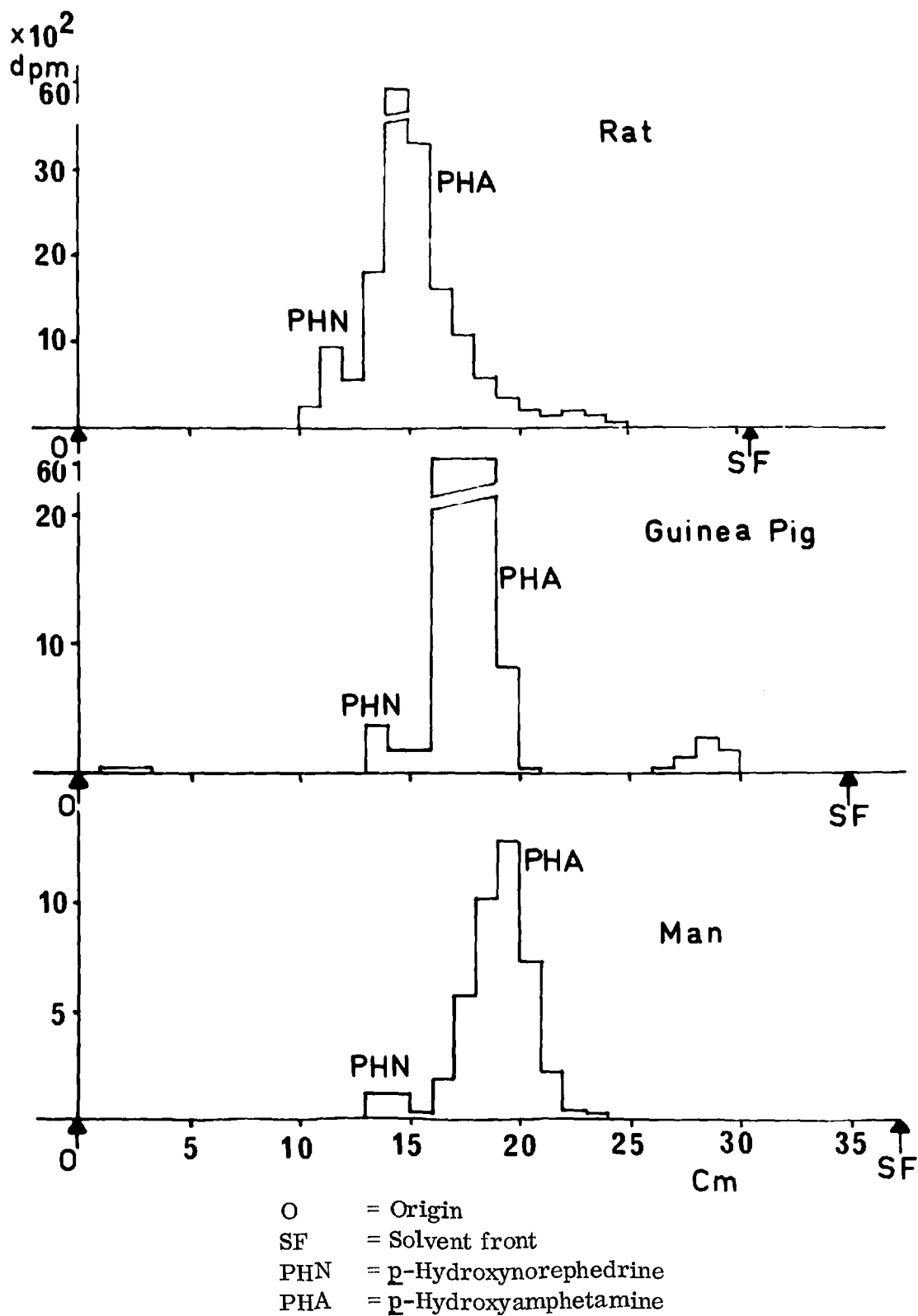


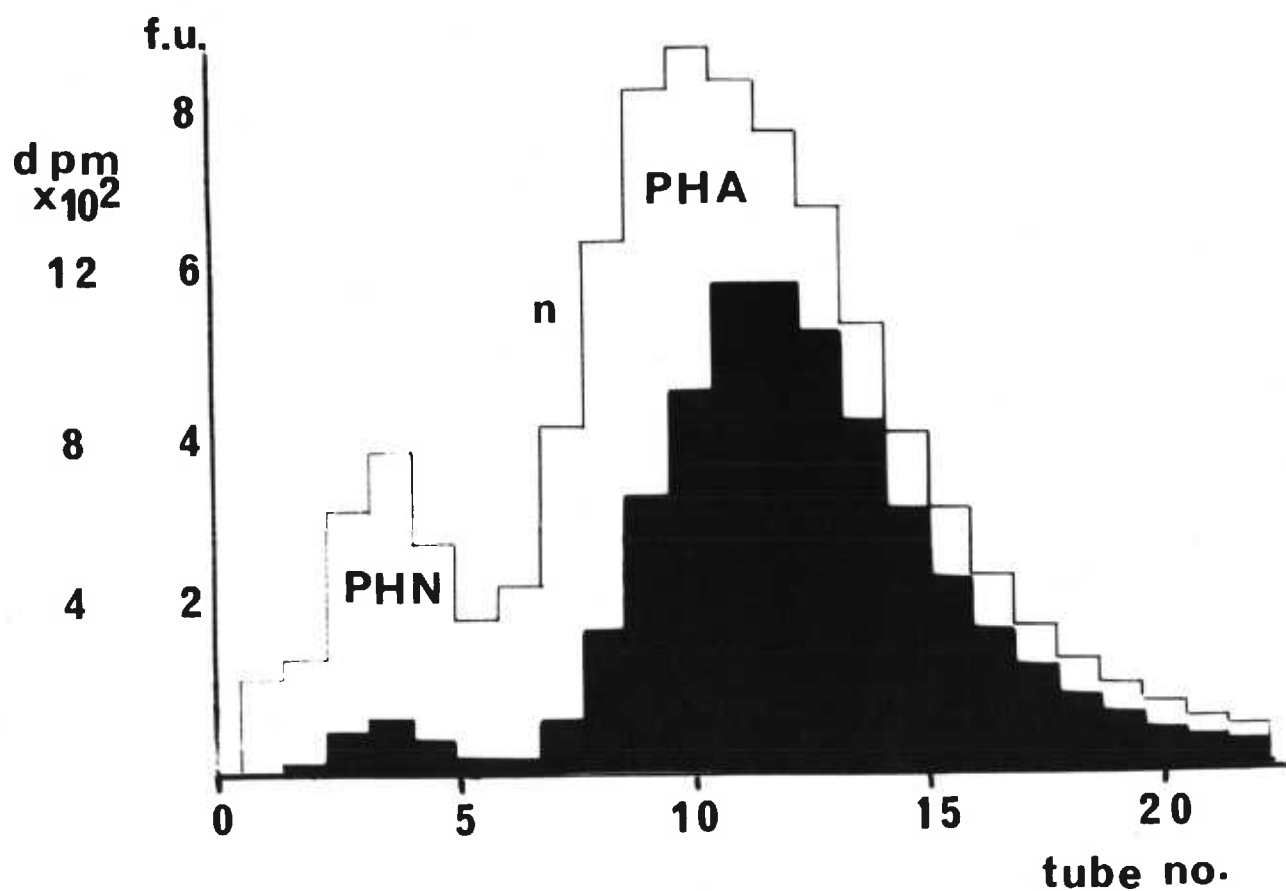
Fig. 3:4. Ion exchange chromatography of 0-24 h human urine after $[^{14}\text{C}]$ -p-hydroxyamphetamine hydrochloride (0.4 mg/kg; 0.07 $\mu\text{Ci/kg}$) orally.

Unshaded - native fluorescence (286/338 nm) in fluorescence units (f.u.)

Shaded - radioactivity in dpm

PHN = p-Hydroxynorephedrine

PHA = p-Hydroxyamphetamine



urine, three peaks were detected, a major peak R_F 0.50 corresponding to *p*-hydroxyamphetamine, and two minor peaks, R_F 0.36 and 0.86, corresponding to *p*-hydroxynorephedrine and *p*-hydroxybenzoic acid respectively (Fig. 3:3).

Human urine

Preliminary chromatograms of urine (0.3-1 ml) in solvent A showed a major peak at the origin and a minor peak of R_F 0.32. In solvent B again two peaks were found, one of R_F 0.31 and a second minor peak R_F 0.51.

Urine samples (10 ml) were hydrolysed with either sulphotase or β -glucuronidase preparations and after evaporation to dryness in vacuo, residues were extracted with ethanol, the salts filtered out and the supernatant re-evaporated. The residue was taken up in a small volume of ethanol (ca. 1 ml) and chromatographed in solvents B and C. In solvent B (Fig. 3:3) after hydrolysis with sulphotase, but not glucuronidase, the major peak (R_F 0.31) seen prior to hydrolysis had disappeared to give an increase in the peak (R_F 0.51) corresponding to *p*-hydroxyamphetamine. In addition, a small peak (R_F 0.36 in solvent B, R_F 0.72 in solvent C) appeared corresponding to *p*-hydroxynorephedrine.

Confirmation and quantitation of *p*-hydroxynorephedrine and *p*-hydroxyamphetamine was performed by ion exchange chromatography (Fig. 3:4) and isotope dilution analyses.

For the detection of acidic metabolites, urine samples (100 ml) were adjusted to pH 2 with 2M HCl and subjected to continuous ether extraction for a total period of 72 hours. The organic phase was further extracted with 5% NaHCO_3 and the aqueous extract acidified to pH 2, saturated with NaCl and re-extracted with ether. After drying the final ethereal extract over anhydrous Na_2SO_4 , 1 ml aliquots were counted in the scintillation spectrometer.

Results

Rat

The excretion of ^{14}C after the administration of [^{14}C]-p-hydroxyamphetamine orally to male and female rats is shown in Table 3:1. The total recovery is approximately 80% in both sexes, almost exclusively from the urine. Most of the [^{14}C]-p-hydroxyamphetamine is excreted unchanged (Table 3:2). In female rats, two of the three animals studied excreted large amounts (40-45%) of the glucuronide conjugate of this compound; however in one female and the three male animals, most of the p-hydroxyamphetamine was excreted free in the urine.

Two other minor metabolites were excreted in the urines of both sexes. Side chain β -hydroxylation gave rise to p-hydroxynorephedrine (4% in females, 1% in males), and oxidative deamination occurred, resulting in p-hydroxybenzoic acid (1% in females, 2% in males).

Guinea Pig

Table 3:3 shows the excretion of ^{14}C after the administration of [^{14}C]-p-hydroxyamphetamine by intraperitoneal injection to three guinea pigs. The total recovery is high (93%), largely confined to the first 24 h urine (90%). The bulk of the radioactivity is excreted as the glucuronide conjugate of p-hydroxyamphetamine (84%), with a small amount unchanged (8%) (Table 3:4).

Minor metabolites resulting from side chain β -hydroxylation giving rise to p-hydroxynorephedrine (3%) and oxidative deamination giving rise of p-hydroxybenzoic acid (2%) were also identified.

Human subjects

Following oral administration of [^{14}C]-p-hydroxyamphetamine to man, 97% of the dose was recovered in the urine within three days, 92% in the first 24 h after dose (Table 3:5). When the drug was administered by the intravenous route,

Table 3:1 The Elimination of ^{14}C by Male and Female Rats after Administration of ^{14}C -p-Hydroxyamphetamine Hydrochloride

^{14}C -p-Hydroxyamphetamine hydrochloride (15 mg/kg; 9 $\mu\text{Ci/kg}$) was administered to rats orally. Values given are % of ^{14}C dose in excreta expressed as the mean of 3 animals. Individual figures are in parentheses.

		<u>% of ^{14}C dose</u>	
		<u>Female</u>	<u>Male</u>
Urine	Day 1	87.7 (84.7, 100.0, 78.5)	84.0 (85.3, 81.0, 85.8)
	2	1.5 (3.0, 0.8, 0.8)	1.9 (1.6, 1.3, 2.8)
	3	0.3 (0.3, 0.1, 0.4)	0.3 (0.3, 0.3, 0.3)
	4	0.2 (0.3, 0.1, 0.3)	
	Total	89.7 (88.3, 101.0, 80.0)	86.2 (87.2, 82.6, 88.9)
Feces	Day 1	0.1 (0.0, 0.1, 0.2)	-
	2	0.2 (0.2, 0.1, 0.2)	-
	3	0.1 (0.2, 0.0, 0.0)	-
	4	0.0 (0.0, 0.0, 0.0)	-
	Total	0.4 (0.4, 0.2, 0.4)	-
Cage washings		0.2 (0.3, 0.0, 0.2)	3.3 (4.1, 3.0, 2.7)
Total ^{14}C Excreted		91.3 (89.0, 101.4, 80.6)	89.5 (91.3, 85.6, 91.6)

Table 3:2 The Metabolites of [^{14}C]-p-Hydroxyamphetamine in Rat Urine

Dose - see Table 3:1. The urine analysed was that excreted in the first 24 h after dosing. Values given are the mean of 3 animals, expressed as a % of the dose. Individual values are in parentheses.

<u>Metabolite</u>		<u>% of ^{14}C dose</u>	
		<u>Female</u>	<u>Male</u>
p-Hydroxyamphetamine	free	52 (35, 91, 30)	80 (80, 77, 82)
	total	82 (80, 91, 74)	81 (81, 78, 82)
p-Hydroxynorephedrine	total	4 (3, 5, 4)	1 (1, 1, 1)
p-Hydroxybenzoic acid	total	1 (1, 1, 1)	2 (3, 2, 2)
Total metabolites		87 (84, 97, 79)	84 (85, 81, 86)
Total ^{14}C in urine		88 (85, 100, 79)	84 (85, 81, 86)

Table 3:3 The Elimination of ^{14}C by Female Guinea Pigs after Administration of ^{14}C -p-Hydroxyamphetamine Hydrochloride

^{14}C -p-Hydroxyamphetamine hydrochloride (15 mg/kg; 8 $\mu\text{Ci/kg}$) was administered to guinea pigs i. p. Values given are % of ^{14}C dose excreted in urine expressed as the mean of 3 animals, individual figures are in parentheses.

			<u>% of ^{14}C dose</u>
Urine	Day	1	89.5 (91.9, 84.4, 92.3)
		2	2.3 (2.3, 2.4, 2.1)
		3	1.1 (1.0, 1.8, 0.3)
Total ^{14}C			92.9 (95.2, 88.7, 94.7)

Table 3:4 The Metabolites of [^{14}C]-p-Hydroxyamphetamine in Guinea Pig Urine

Dose - see Table 3:3. The urine analysed was that excreted in the first 24 h after dosing. Values given are the mean of 3 animals, expressed as a % of the dose. Individual values are in parentheses.

<u>Metabolite</u>		<u>% of ^{14}C dose</u>
p-Hydroxyamphetamine	free	8 (7, 14, 4)
	total	84 (88, 79, 86)
p-Hydroxynorephedrine	free	2 (1, 1, 3)
	total	3 (2, 3, 3)
p-Hydroxybenzoic acid	free	<1 (<1, <1, <1)
	total	2 (1, 1, 2)
Total metabolites		89 (91, 83, 91)
Total ^{14}C in urine		90 (92, 84, 92)

Table 3:5 The Elimination of ^{14}C by Human Subjects after Administration of
 ^{14}C -p-Hydroxyamphetamine Hydrochloride

Two male subjects received ^{14}C -p-hydroxyamphetamine hydrochloride (30 mg, 5 μCi) orally, and a single subject received 2 mg, 2 μCi intravenously. Values given are the % of ^{14}C dose excreted.

		<u>% of ^{14}C dose</u>		
		<u>Oral</u>		<u>i. v.</u>
Subject		A	B	C
Urine	Day 1	61.6	93.1	63.8
	2	3.7	2.6	5.5
	3	1.4	1.0	3.1
	4	-	-	1.9
	5	-	-	0.7
	Total	66.7	96.7	75.0
Faeces	Day 1	-	-	-
	2	-	-	0.4
	3	-	-	0.3
	4	-	-	-
	5	-	-	-
	Total	-	-	0.7
Total ^{14}C		66.7	96.7	75.7

Table 3:6 The Metabolites of [^{14}C]-p-Hydroxyamphetamine in Human Urine

Dose - see Table 3:5. The urine analysed was that excreted in the first 24 h after dosing. Values given are expressed as a % of the dose.

<u>Metabolite</u>		<u>% of ^{14}C dose</u>		
		<u>Oral</u>		<u>i. v.</u>
	<u>Subject...</u>	<u>A</u>	<u>B</u>	<u>C</u>
p-Hydroxyamphetamine	free	10	13	23
	total	85	84	56
p-Hydroxynorephedrine	free	2	3	3
	total	4	4	6
p-Hydroxybenzoic acid	total	n. d.	n. d.	n. d.
Total Metabolites		89	88	62
Total ^{14}C in urine		92	93	64

although the total recovery of ^{14}C from the urine was only 75%, the bulk of this appeared in the 0-24 h urine, smaller amounts being excreted on days 2-5. Little faecal excretion occurred after either route of administration.

Identification and quantitation of metabolites showed that after oral administration this compound is excreted largely as its sulphate conjugate (75%) with 10% being excreted free (Table 3:6). Following intravenous injection approximately half the [^{14}C]-p-hydroxyamphetamine was conjugated (again with sulphuric acid) and half eliminated in the urine unchanged.

p-Hydroxynorephedrine was identified in both studies as a minor metabolite, 4% and 6% after oral and intravenous routes of administration respectively. No acidic metabolites were identified.

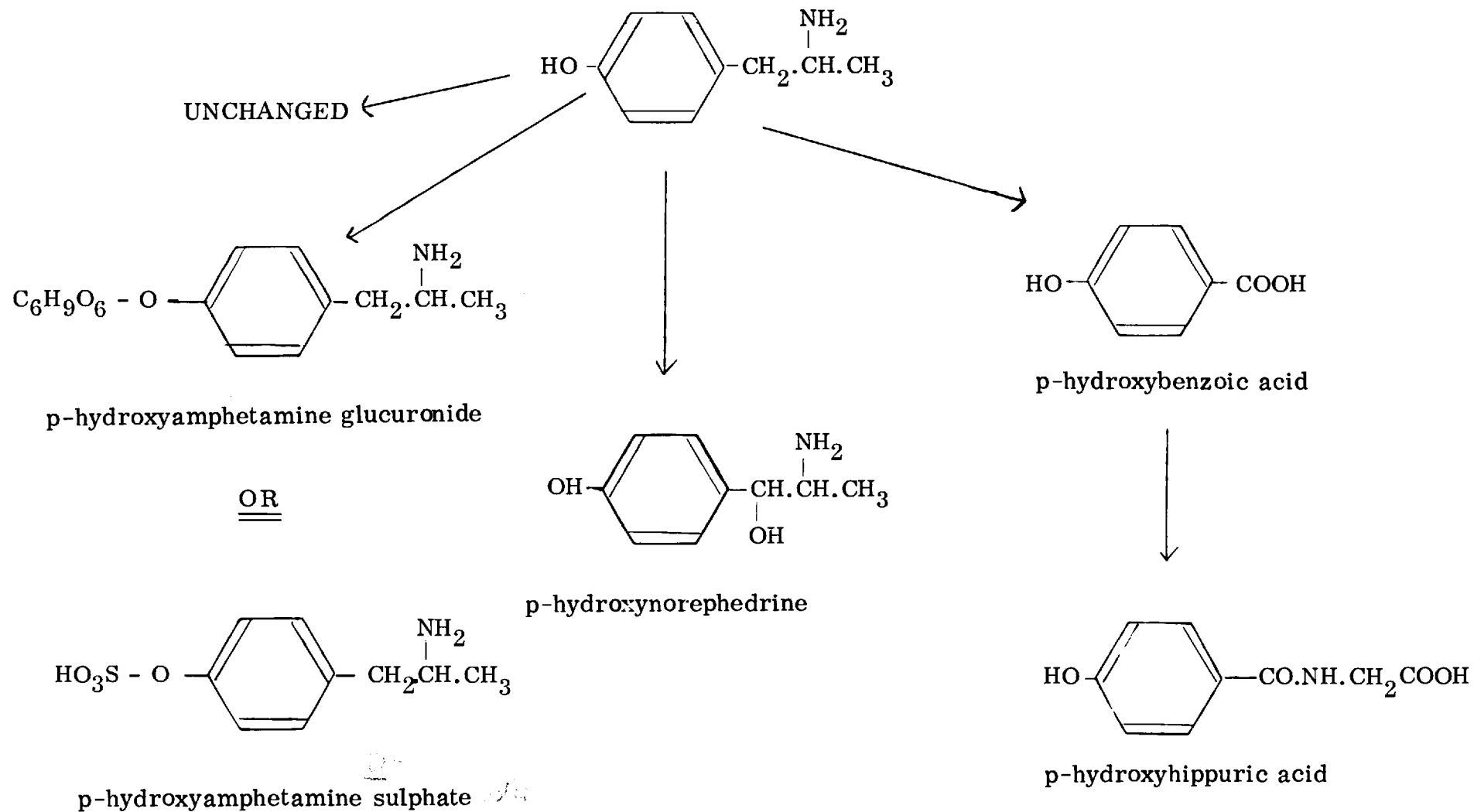
Discussion

In contrast to the considerable species variations in the metabolism of amphetamine (Dring, Smith & Williams, 1970), p-hydroxyamphetamine was found to undergo little biotransformation in the three species examined. A summary of the metabolic pathways of this drug is given in Fig. 3:5. Amphetamine is open to metabolic attack on the aromatic ring and on the side-chain, and it is in the relative extent of metabolism at these two sites that species differences are most apparent. In the case of p-hydroxyamphetamine, however, the presence of the hydroxyl group in the 4'-position of the benzene ring appears to prevent, with the exception of conjugation, further biotransformation occurring at this site, in addition to which the OH moiety, by rendering the compound more polar will tend to promote rapid urinary excretion of the drug. Urine radioactivity showed that 50% of the dose was recovered within 12 h.

The guinea pig and female rat form glucuronic acid conjugates of p-hydroxyamphetamine, the former to a greater extent, which agrees with the in vitro data

Fig. 3:5

Metabolic Pathways of p-Hydroxyamphetamine



of Dingell *et al.* (1974). Man, however, conjugates the drug with sulphate, and this occurs to a greater extent after oral administration than after i.v. dosage, which raises the question as to the site of this conjugation reaction. From the current data it is not possible to state whether sulphate conjugation of p-hydroxyamphetamine occurs predominantly in the liver, as opposed to the bowel wall where sulphate conjugation may occur (Dollery, Davies & Conolly, 1971).

One remarkable feature of the present study was the apparent sex difference in p-hydroxyamphetamine conjugation seen in rats. Female animals conjugated the compound to a significant extent with glucuronic acid, whereas in males little conjugation occurred. Dingell *et al.* (1974) reported a sex difference in the ability of liver microsomal preparations from Wistar rats to conjugate p-hydroxyamphetamine with glucuronic acid, in that female animals conjugated the compound more effectively than males. This is unusual, as when sex differences in glucuronide formation occur, males show higher activity than females (Dutton, 1971). Indeed in general, where sex differences in metabolism occur in the rat, the male metabolises to a greater extent than the female (Dutton, 1971) which contrasts with the observations presented here.

Side chain hydroxylation

All species under investigation β -hydroxylated p-hydroxyamphetamine resulting in the formation of p-hydroxynorephedrine, but only to a minor extent. This reaction has been shown to occur both in vitro and in vivo and involves dopamine- β -hydroxylase, a copper-containing enzyme responsible for the formation of noradrenaline from dopamine which is not normally involved in the biotransformation of drugs.

Levin and Kaufman (1961) demonstrated that dopamine- β -hydroxylase was not specific for the conversion of dopamine to noradrenaline, and like these authors,

both Goldstein and Contrera (1962) and Creveling et al. (1962) confirmed that many substituted phenylethyl and phenylisopropyl amines were substrates for this enzyme when tested in vitro.

Goldstein, McKereghan and Lauber (1964) showed that enzymic β -hydroxylation was stereospecific, in that only the (+)-isomers of optically active substrates were hydroxylated. The in vivo demonstration of the formation of p-hydroxynorephedrine from p-hydroxyamphetamine has been reported in rat heart (Goldstein & Anagnoste, 1965) and brain (Lewander, 1971b). Further in vivo studies confirm that this reaction also occurs in man (Sjoerdsma & von Studnitz, 1963; Cavanaugh, Griffith & Oates, 1970).

The subcellular localisation of dopamine- β -hydroxylase appears to be restricted to the catecholamine storage particles of the adrenal medulla and sympathetic nerves (Kirschner, 1957; Stjärne, 1966). Immunofluorescence studies of Goldstein et al. (1974) confirm the widespread distribution of dopamine- β -hydroxylase in brain, the highest concentration being found in the cell bodies of the locus ceruleus.

The finding of this enzyme in plasma has been confirmed and quantitated by both chemical (Nagatsu & Udenfriend, 1972) and radioimmunoassay techniques (Rush & Geffen, 1972) and should prove a useful measure of peripheral sympathetic activity, since dopamine- β -hydroxylase is released from the neuronal vesicles into the extraneuronal space together with noradrenaline. Preliminary reports suggest that in some disease states, for example essential hypertension and hypertension secondary to pheochromocytoma, levels of dopamine- β -hydroxylase in plasma are raised, perhaps a reflection of increased sympathetic activity (Schanberg et al. 1974).

Evidence has accumulated that *p*-hydroxynorephedrine is formed from *p*-hydroxyamphetamine within the noradrenergic storage granules. The former compound can be taken up by sympathetic nerve endings (Carlsson & Waldeck, 1964) and released on nerve stimulation, according to Thoenen *et al.* (1966), who gave amphetamine to cats and demonstrated the release of *p*-hydroxynorephedrine from the spleen on electrical stimulation of the sympathetic nerves to this organ. This compound, therefore, fulfills the criteria proposed by Kopin (1968) for false transmitter function, and the production of this metabolite from both amphetamine (Dring, Smith & Williams, 1970; Caldwell, Dring & Williams, 1972b) and *p*-hydroxyamphetamine may be related to the tolerance seen with repeated exposure to these drugs (see later chapters).

The pharmacological effects of *p*-hydroxyamphetamine as a sympathomimetic amine, with potential therapeutic value as a pressor agent, were reported by Abbott and Henry, 1937; Altschule and Iglauer, 1940; Iglauer and Molle, 1943; Korns and Randall, 1938, and others. The pharmacological actions of the phenylethyl and phenylisopropyl amines are dependent upon an aromatic nucleus separated by two carbon atoms from an amino group. Hydroxylation of the ring in the 3 or 4 position appears to coincide with loss of CNS stimulant action, but enhancement of the peripheral sympathomimetic effects (Barger & Dale, 1910). It was presumably for this reason that *p*-hydroxyamphetamine was first investigated. However, Ohler and Wackerlin (1953), noting Wastl's observations ten years previously, commented upon the possible therapeutic action of this compound as a hypotensive agent, following their work on experimental renal hypertension in dogs. Gill, Mason and Bartter (1967) administered *p*-hydroxyamphetamine over a prolonged period to normotensive human subjects and observed impairment of sympathetic nerve function as evidenced by postural hypotension. These authors implicated *p*-hydroxy-

norephedrine as a cause of the sympathetic blockade. In support of this, Rangno et al. (1973) have reported the antihypertensive effect of *p*-hydroxynorephedrine in human subjects.

It is postulated therefore that *p*-hydroxynorephedrine could accumulate in tissues, as it is not a substrate for monoamine oxidase or catechol-O-methyl transferase, would be released on nerve stimulation and, since this compound is a less effective neurotransmitter than noradrenaline (Brodie, Cho & Gessa, 1970), would impair noradrenergic function. Indeed these authors demonstrated that the administration of amphetamine led to the formation of *p*-hydroxynorephedrine, in tissues, in quantities that were stoichiometrically related to the noradrenaline deficit.

Although in the current studies presented here less than 5% of the administered *p*-hydroxyamphetamine is recovered from urine as *p*-hydroxynorephedrine in the first 24 h after dosage, these values may not truly represent the tissue situation. For the reasons outlined above *p*-hydroxynorephedrine would only be released slowly from the noradrenergic granules into the extraneuronal space as a consequence of nerve stimulation. Lewander (1971b) reported that the half life ($T_{1/2}$) of *p*-hydroxynorephedrine in brain following *p*-hydroxyamphetamine administration was 22 hours. This observation taken in conjunction with those of Rangno et al. (1973), who showed that the urinary excretion of this compound after oral administration was biexponential, with first and second phase half lives of 13 and 55 h respectively, suggest that tissue stores of *p*-hydroxynorephedrine may be considerably greater than one might infer from the percentage excretion of this metabolite in urine.

Oxidative deamination of sidechain

Very small quantities of acid metabolites were identified in the urines of rat and guinea pigs and in man none were detected. A discussion of the current controversy concerning the mechanism by which amphetamines are deaminated is provided in Chapter 1, and there would appear to be little reason to doubt that *p*-hydroxyamphetamine undergoes oxidative degradation of the side chain to a very limited extent by the same routes.

CHAPTER FOUR

THE METABOLISM OF AMPHETAMINE IN AMPHETAMINE
TOLERANT HUMAN SUBJECTS AND CHRONICALLY DOSED
GUINEA PIGS

**Chapter Four The Metabolism of Amphetamine in Amphetamine Tolerant Human
Subjects and Chronically Dosed Guinea Pigs**

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The Metabolism of Amphetamine in Amphetamine Tolerant Human Subjects and
Chronically Dosed Guinea Pigs

A) The Metabolism of Amphetamine in Tolerant Human Subjects

Introduction

The metabolism of amphetamine in drug naive subjects has been previously reported (Dring, Smith & Williams, 1970; Caldwell, Dring & Williams, 1972b). The purpose of the current study was to determine whether, on prolonged and repeated dosage of amphetamines, there was any alteration in the biotransformation of this drug as determined from the urinary excretion of free drug and its metabolites. Since it appears that p-hydroxynorephedrine can act as a false neurotransmitter at noradrenergic nerve endings in the peripheral sympathetic nervous system and brain (Gill, Mason & Bartter, 1967; Cavanaugh, Griffith & Oates, 1970) and thus may be implicated in the development of tolerance to certain effects of amphetamine, it was of interest to determine the urinary output of norephedrines in amphetamine dependent subjects.

It was thought initially, that obtaining volunteers for such a study would present no great problem, but contrary to expectations, considerable difficulty was encountered. Firstly, drug abusers tend to be unreliable subjects and their full cooperation with investigators is frequently lacking. Secondly, the pattern of drug abuse in the U.K. is changing, in all probability as a result of the current drive against the prescription of amphetamines by medical practitioners, and few drug dependency centres treat amphetamine 'addicts'. Finally, the polypharmacy to which drug abusers subject themselves makes single drug studies more difficult.

The two subjects who volunteered for this study were housewives aged 46 and 51 years. They had been taking (+)-amphetamine regularly for at least 10 years and for the year prior to this study had been taking between 75 and 100 mg of

Dexedrine ((+)-amphetamine sulphate; Smith, Kline & French) daily. Both subjects denied abuse of any other drugs and an examination of their urine (Guy's Hospital Poison Centre) showed only amphetamine and no other drug to be present.

The patients were admitted to a hospital ward for 48 hours during which time they were under constant observation. A routine medical examination, hepatic and renal function tests were found to be within normal limits.

Each subject was given 2 mg of (+)-[^{14}C]-amphetamine sulphate (5 μCi) orally in a small volume of water together with their regular daily dose (90 mg in divided doses) of unlabelled Dexedrine, which was administered orally when requested.

The ^{14}C -label in this study was sited on the β -carbon atom of the side chain enabling identification of metabolites formed either by substitution in the aromatic ring or by side chain degradation. Urine was collected for 48 hours.

Identification of Metabolites

Radiochromatograms of the urine (1 ml) in solvent A showed two major peaks of R_F 0.54 and 0.82 which correspond to amphetamine and hippuric acid, respectively, and two minor peaks of R_F 0.21-0.35 and 0.39 which corresponded in order to *p*-hydroxynorephedrine and *p*-hydroxyamphetamine (not resolved) and norephedrine (Fig. 4:1). A small peak at the origin was seen on some chromatograms and was considered to be conjugates of the phenolic metabolites. This very small peak was not found after the urine had been incubated with the arylsulphatase preparation, but persisted after treatment with β -glucuronidase (Ketodase), suggesting that this peak contained sulphate esters. The other peaks were not affected by these enzyme preparations. The identity of the major urinary metabolites was confirmed using solvent D, in which two peaks of R_F 0.24 and 0.87 were found, corresponding to hippuric acid and amphetamine respectively. The minor peaks observed with solvent A were not apparent in solvent D.

Confirmation and quantitation of metabolites by reverse isotope dilution was

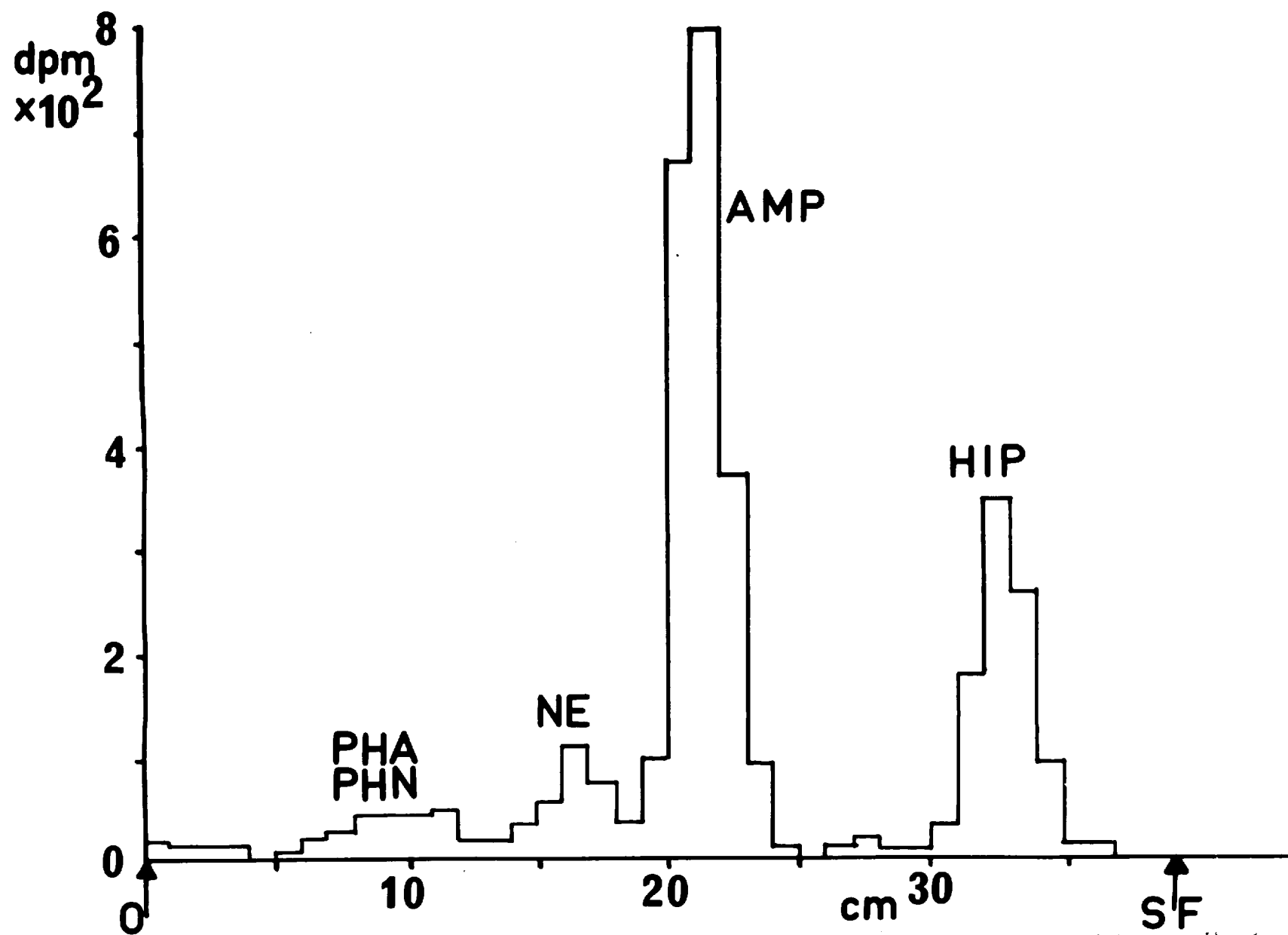


Fig. 4-3. Radiochromatogram from an/autoradiogram of 0-2% human urine after oral (150 mg) amphetamine sulphate orally, in solvent 2.

(continued)

Fig. 4:1

Histogram prepared from radiochromatogram of 0-24 h human urine after
(+)-[¹⁴C]-amphetamine sulphate (2 mg; 5 μ Cl) orally, in Solvent A.

O = Origin
SF = Solvent front
PHN = p-Hydroxynorephedrine
PHA = p-Hydroxyamphetamine
NE = Norephedrine
AMP = Amphetamine
HIP = Hippuric acid

performed for amphetamine, hippuric acid, norephedrine, *p*-hydroxyamphetamine and *p*-hydroxynorephedrine.

Results

Quantitative data for the two tolerant subjects are shown in Table 4:1. The urinary excretion of ^{14}C , 85% of the dose in subject R.F. and 74% in E.L. in two days, is similar to that found for the naive subjects C and D, i.e. 81 and 85% (taken from Caldwell, Dring & Williams, 1972b). Regarding the metabolites, the two tolerant subjects excreted in 24 hours rather more unchanged amphetamine (30 and 28%) than the naive subjects (18 and 20%) and correspondingly less hippuric acid, that is 16 and 13% compared with 28% and 27%. The norephedrine output (4.1 and 2.9%) in the dependent subjects was slightly more than that of the naive subjects (2.2 and 2.6%) and this appeared to be also the case with *p*-hydroxynorephedrine (1.6 and 1.1% compared with 0.3 and 0.4%). The output of *p*-hydroxyamphetamine was essentially the same in the tolerant subjects (1.1 and 2.4%) as in the naive subjects (2.4 and 4.4%).

Discussion

In comparing the quantitative results for the dependent subjects with the naive subjects, it must be borne in mind that the former were receiving the (+)-isomer of amphetamine at about five times the dose level that the latter were receiving (±)-amphetamine. Any differences between the two types of subjects could therefore be due to differences in the dose level and the isomer administered. The fact that the dependent subjects were women and the naive subjects were men is assumed to be of no significance. In general, it can be said that the gross pattern of metabolism of amphetamine is quantitatively and qualitatively similar in the tolerant and naive subjects.

The pH of the 0-24 h urines for the subjects under study is recorded in Table 4:1. Drug naive subjects were producing urine of slightly greater acidity (pH 6.5

Table 4:1. The Urinary Excretion of [¹⁴C]-Amphetamine Metabolites

The dependent subjects R. F. and E. L. received orally 2 mg of [¹⁴C]-(+)-amphetamine (5 μ Cl) together with the daily dose of 90 mg of Dexedrine as described in the text. The metabolites were determined by radiochromatogram scanning and for Day 1 also by reverse isotope dilution. The values for naive subjects C and D given 20 mg of [¹⁴C]-(-)-amphetamine orally are quoted from Caldwell, Dring and Williams (1972b) for comparison.

% of dose excreted as metabolites

	<u>Dependent subjects</u>						<u>Naive subjects</u>					
	<u>R. F.</u>			<u>E. L.</u>			<u>C</u>			<u>D</u>		
	<u>Day...1</u>	<u>2</u>	<u>Total</u>	<u>1</u>	<u>2</u>	<u>Total</u>	<u>1</u>	<u>2</u>	<u>Total</u>	<u>1</u>	<u>2</u>	<u>Total</u>
Amphetamine	29.6	14.1	43.7	28.3	11.6	39.9	18.0	-	-	20.0	-	-
Norephedrine	4.1	1.8	5.9	2.9	1.5	4.4	2.2	-	-	2.6	-	-
Hippuric acid	16.2	9.4	25.6	13.0	7.5	20.5	28.2	-	-	27.1	-	-
p-Hydroxyamphetamine (total)	1.1	*	1.1	2.4	*	2.4	2.4	-	-	4.4	-	-
p-Hydroxynorephedrine (total)	1.6	*	1.6	1.1	*	1.1	0.3	-	-	0.4	-	-
Benzyl methyl ketone	-	-	-	-	-	-	0.9	-	-	1.7	-	-
Total of above metabolites	52.6	25.3	77.9	47.7	20.6	68.3	52.0	-	-	56.2	-	-
¹⁴ C in urine	60.5	24.8	85.3	51.1	22.7	73.8	56.9	24.5	81.4	63.6	21.1	84.7

* = not detectable by radiochromatogram scanning. - = not determined

The pH of the urine (Day 1) for the naive subjects C and D was 6.5 and 6.7 respectively, and for the dependent subjects R. F. and E. L. was 7.5 and 7.0 respectively.

and 6.7) than the dependent subjects (pH 7.5 and 7.0). The importance of minimising fluctuations in urine pH has been stressed by Beckett and Rowland (1965). These authors point out that under normal conditions, the ratio of metabolite to unchanged drug in the urine varies greatly with the diurnal changes in urine pH, and that under conditions of acidic urine production, greater proportions of the free unmetabolised drug are excreted. Such variations are most evident when the excretion of N-substituted amphetamines is compared with their dealkylated metabolite. However, Anggard *et al.* (1973) report that following the administration of amphetamine to human subjects, when the urine is rendered acidic by giving ammonium chloride orally, the excretion of unchanged amphetamine increases, with a concomitant diminution in the excretion of metabolites.

In the current study there is a slight difference in the pH of the urine excreted by the four subjects. However, since the dependent subjects are excreting more unchanged drug, and it is in these two patients that the urines are more alkaline, it is not possible to account for the alteration in the pattern of excretion of amphetamine and metabolites on the basis of variations in urine pH.

Both types of subjects are excreting small quantities of norephedrine and it would appear that the tolerant subjects are excreting in 24 hours more norephedrine (3-4%) and p-hydroxynorephedrine (1-1.6%) than the naive subjects (2.2-2.6 and 0.3-0.4% respectively). However, this could be explained by the fact that, according to Goldstein and Anagnoste (1965), only (+)-amphetamine is a substrate for dopamine- β -hydroxylase and since the naive subjects were given (+)-amphetamine one might expect less β -hydroxylation.

It has been stated that tolerance to amphetamines may occur through the replacement of noradrenaline at sympathetic nerve endings by p-hydroxy-

norephedrine which could act as a false neurotransmitter. With chronic administration of amphetamine, *p*-hydroxynorephedrine formed metabolically could be responsible for the persistent noradrenaline depletion in both the central and peripheral nervous systems (Brodie, Cho & Gessa, 1970; Lewander, 1971a). In Chapter 3 evidence was quoted for *p*-hydroxynorephedrine being taken up by sympathetic nerve endings (Carlsson & Waldeck, 1964) and released on nerve stimulation (Thoenen *et al.*, 1966) thus fulfilling the criteria for a false neurotransmitter.

In view of the biological implications of false transmitters in tolerance, the possibility that these compounds might be formed in increased quantities in amphetamine tolerant subjects was investigated. The results obtained here appear to confirm the work of Gunne *et al.*, (1972) in that the excretion of unchanged amphetamine is greater in the tolerant state in man. Mitchard *et al.*, (1971) have stated that the kinetic parameters of amphetamine in a tolerant man were essentially the same as those found in naive subjects and our results for total metabolism appear to support this.

The amounts of metabolites in the urine may not be truly representative of their tissue levels. Although *p*-hydroxynorephedrine has been found only in small amounts (about 1% of the dose) in the urine of both dependent and naive subjects, the former, receiving 100 mg/day of amphetamine, are excreting much more of this metabolite in absolute terms than the latter who received one dose of 20 mg. The formation of greater amounts of *p*-hydroxynorephedrine in these subjects may thus result in its accumulation, leading to the production of tolerance in the way outlined above.

B) Metabolism of Amphetamine in the Guinea Pig Chronically Dosed with

(+)-Amphetamine

According to Dring, Smith and Williams (1970) and Lewander (1971c), (+)-amphetamine is not metabolised by ring hydroxylation in the guinea pig. In this species the major metabolic pathway is side chain degradation to benzoic acid, which is excreted in the urine both free and as its glycine and glucuronic acid conjugates (total 62%). A lesser fraction of the dose is excreted as unchanged drug (22%). The figures given are those reported by Dring, Smith and Williams (1970) and were determined by radiochromatogram scanning. They represent the fraction of a dose of [^{14}C]-(+)-amphetamine sulphate (5 mg/kg) as each metabolite excreted in the urine from 0-24 h after dosing.

With this information, and acting upon the premise that tolerance to amphetamines is related to the production of *p*-hydroxynorephedrine, it was suggested that tolerance should not develop in the guinea pig, if this species was chronically dosed with amphetamine. A full discussion of this work is presented in Chapter 8, but it was deemed necessary at the conclusion of the experiment to determine whether or not any alteration in metabolism had occurred with repeated dosage, and more specifically, whether aromatic hydroxylation had been induced with the subsequent formation of *p*-hydroxynorephedrine.

Identification of Metabolites

Urine samples (2 ml) were evaporated to dryness in vacuo at 40°C, extracted with ethanol (3 x 10 ml) to remove salts, and the ethanol removed on the rotary evaporator. The residue was taken up in a small volume of methanol (0.5 ml) and chromatographed in solvents A and D. Additional samples of urine (2 ml) were first subjected to enzymic hydrolysis with β -glucuronidase and then treated in the manner described above. On all chromatograms in solvent A two major

peaks (R_F 0.55 and 0.93) were present, corresponding to amphetamine and benzoic acid respectively. On some chromatograms the major peak running near the solvent front could be split into two components (R_F 0.84, 0.87) suggesting that this peak represented both hippuric and benzoic acids (Fig. 4:2). Prior to hydrolysis minor peaks (R_F 0.41 and 0.60) were identified, the former corresponding to norephedrine and the latter, which disappeared on hydrolysis, to benzoyl glucuronide. In solvent D, three major peaks were present (R_F 0.88, 0.43 and 0.27) corresponding to amphetamine, benzoic acid and hippuric acid respectively. Smaller peaks (R_F 0.80 and 0.0-0.2) were present on some chromatograms, and corresponded to norephedrine and benzoyl glucuronide respectively. The latter peak was not identified when hydrolysed urine samples were chromatographed.

Reverse isotope dilution procedures were carried out for the hydroxylated metabolites, norephedrine and p-hydroxynorephedrine.

Results

The excretion of ^{14}C in the 0-24 h urine following dosage is shown for drug 'tolerant' and naive animals in Table 4:2. The former group excreted slightly less (55%) than the latter group (66%) during this period. Quantitative data for the excretion of metabolites in the guinea pigs chronically dosed with the drug show no appreciable difference when compared with the saline pretreated animals. The tolerant and naive animals excrete amphetamine (26 and 30% respectively) and benzoic acid (17 and 21% respectively). Both groups of animals excrete small amounts of norephedrine (1-2%) and p-hydroxynorephedrine (1%).

Discussion

Comparison of the total urine radioactivity excreted by the animals in the present study (55-66%) with that reported by Dring, Smith and Williams (1970), who

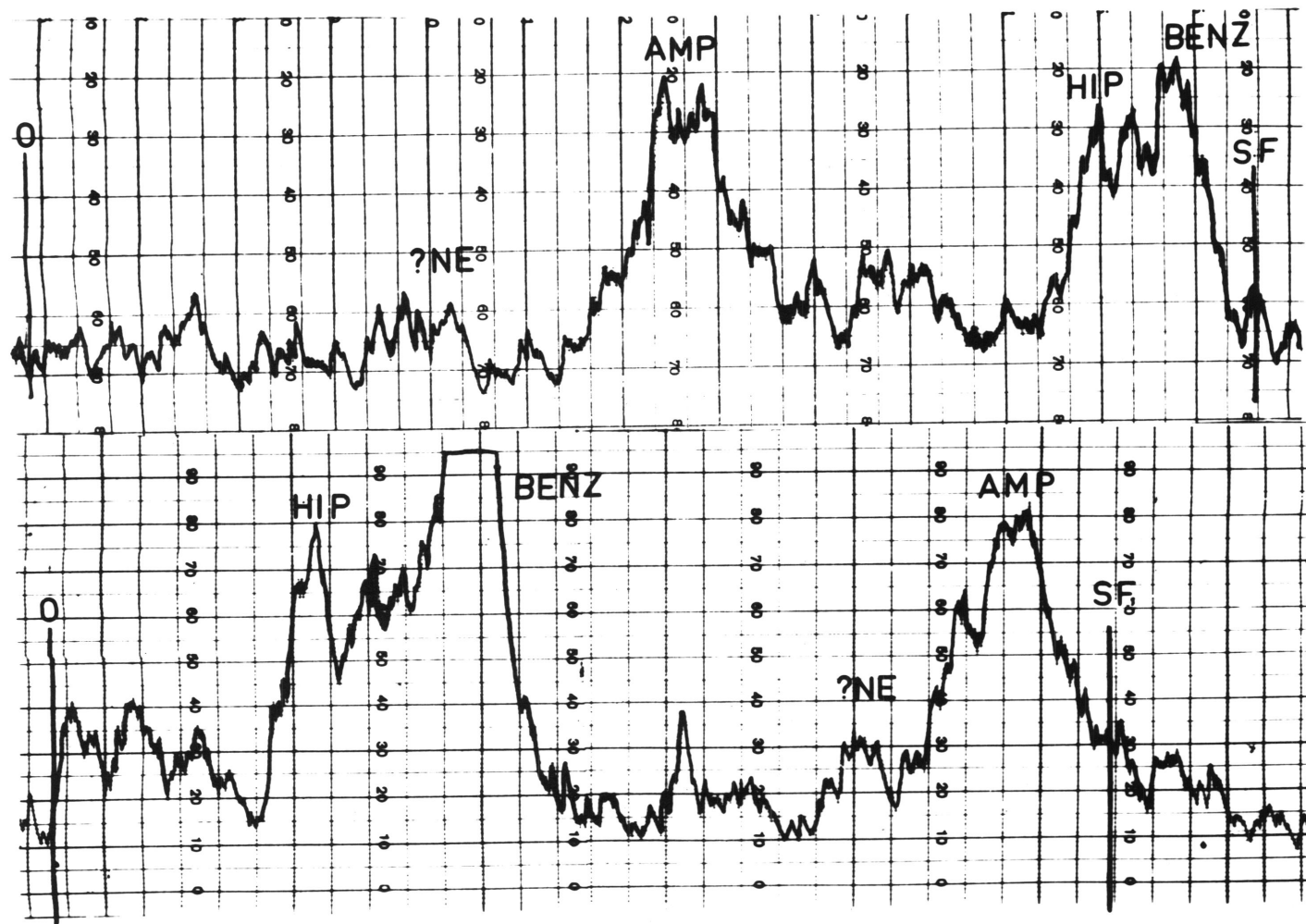


Fig. 4:2. Radiochromatograms of hydrolysed guinea pig urine (0-24 h) after (+)-[¹⁴C]-amphetamine sulphate (20 mg/kg; 3 μ Cl) i.p.
(continued overleaf)

Fig. 4:2

Radiochromatograms of hydrolysed guinea pig urine (0-24 h) after (+)-[¹⁴C]-
amphetamine sulphate (20 mg/kg; 3 μ Ci) i. p. Animals had received unlabelled
(+)-amphetamine sulphate (20 mg/kg i. p. twice daily) for 9 days prior to this
experiment.

Upper chromatogram - solvent A

Lower chromatogram - solvent D.

O = Origin
SF = Solvent front
NE = Norephedrine
AMP = Amphetamine
HIP = Hippuric acid
BENZ = Benzoic acid

Table 4:2 The Elimination of ^{14}C and Metabolites of [^{14}C]-(+)-Amphetamine**Sulphate in Guinea Pig Urine**

[^{14}C]-(+)-Amphetamine sulphate (20 mg/kg, 3 μCi) was administered to guinea pigs, i. p. Group A had received unlabelled (+)-amphetamine sulphate (20 mg/kg i. p. twice daily) for 9 days prior to this study. Group C had received saline (0.5 ml i. p. twice daily) for a similar period.

Mean values given are % of ^{14}C dose in 0-24 h urine and the % of dose excreted as metabolites in this period. Individual values are given in parentheses.

	<u>% of ^{14}C dose</u>	
	<u>Group A</u>	<u>Group C</u>
Amphetamine	26 (16, 27, 35)	30 (23, 39, 28)
Benzoic acid (total)	17 (17, 19, 16)	21 (26, 11, 27)
*Norephedrine	1.5 (1.1, 1.0, 2.4)	2.0 (2.2, 1.7, 2.2)
*p-Hydroxynorephedrine (total)	0.3 (0.5, 0.5, 0.4)	0.7 (0.8, 0.3, 1.1)
Total metabolites	45 (35, 48, 54)	54 (52, 52, 58)
Total ^{14}C in urine	55 (46, 55, 63)	66 (70, 59, 67)
Urine pH	9.0 (9.2, 9.0, 8.9)	9.0 (9.0, 9.1, 9.0)

* Determined by reverse isotope dilution

recovered 83% of a dose of 5 mg/kg i.p. of [^{14}C]-(+)-amphetamine sulphate in the urine within 24 hours, shows a discrepancy which requires explanation. Lewander (1971g) recovered 46% of a dose of [^{14}C]-(+)-amphetamine sulphate (20 mg/kg i.p.) from the urine of guinea pigs in 16 hours after dosing and these results are in greater accord with those presented here. There are two possible explanations for the lower recoveries in the current experiment, namely that either these result from an alteration in the excretion rate with change in dose, or alternatively, as discussed previously, differences in urine pH between the groups of animals may contribute to variations in the renal elimination rate of amphetamine (Beckett & Rowland, 1965). In the study of Dring, Smith and Williams, (1970) the urine of a group of three animals had a pH of 6, but by contrast, in Lewander's study, the urine pH varied from 7.8-8.5, and in the present report, urine pH varied from 8.6-9.2. It would appear, therefore, that under these conditions of alkaline urine pH, the excretion of amphetamine and metabolites is diminished. Assuming this to be so, why should there be such variation in urine pH within a species treated with the same drug? The more alkaline urine pH values are recorded from animals receiving a higher dose (20 mg/kg) of amphetamine, and at this dose considerable elevations in body temperature occur (ca. 2°C) compared with ca. 1°C) at a dose of 5 mg/kg (See Chapter 8). At the higher dose (and higher temperature) the stimulus to respiration is considerable, and it could be suggested that such hyperventilation produces a respiratory alkalosis. As a result of this, a renal compensatory mechanism would tend to diminish the tubular excretion of H^{+} ions with a concomitant increase in urine pH.

The formation of the hydroxylated metabolites norephedrine and p-hydroxy-norephedrine from (+)-amphetamine in the guinea pig has not previously been reported. According to Dring, Smith and Williams (1970) and Lewander (1971g)

amphetamine is metabolised in this species exclusively by side chain oxidative deamination. Of the amphetamine analogues only Pondinil (N-3-chloropropyl amphetamine) is metabolised by ring hydroxylation in the guinea pig (see Williams, Caldwell & Dring, 1974). It should be noted however, that chromatographic methods are not generally sufficiently sensitive to allow reliable quantitation of these metabolites, which represent a very small percentage of the dose, and indeed such methods were used by the above authors, whereas the currently reported values for norephedrine and p-hydroxynorephedrine were determined by reverse isotope dilution, a much more sensitive procedure.

The original premise upon which the current experiment was designed, that is to compare and contrast the development of tolerance in two species, one of which produces the false transmitter metabolite of amphetamine and the other which does not, was to a certain extent nullified by the identification of aromatic and side chain hydroxylated metabolites of amphetamine in the urine of the guinea pig after both single and repeated doses.

Lewander's study (1971d) on the effects of chronic amphetamine intoxication on the tissue concentration of noradrenaline shows that in rats the reduction in brain and heart noradrenaline by 40-70% seemed to be due partly to the presence of amphetamine in the tissues and partly to the formation and incorporation of p-hydroxynorephedrine into noradrenaline storage sites - the latter perhaps accounting for the prolonged depletion (72-96 hours) observed in this species.

In the guinea pig, however, the same author (1971c) reported that although the initial depletion of noradrenaline in the tissues (60%) after similar doses of amphetamine was of the same order as in rats, noradrenaline levels returned almost to normal within 24 hours of the last dose of amphetamine, and was related in time to the disappearance of amphetamine from the tissues. It is inferred,

therefore, that the presence of *p*-hydroxynorephedrine formed metabolically in the rat is responsible for the prolonged depletion of noradrenaline in this species (Costa & Groppetti, 1970).

In conclusion, it must be considered that either the identification of the metabolite of amphetamine, *p*-hydroxynorephedrine, in the urine of both species, does not necessarily reflect the tissue situation, since Lewander (1971a,c) identified this metabolite only in the brain of rats and not in guinea pigs, or that the formation of the false transmitter metabolically is unrelated to the prolonged tissue depletion of noradrenaline seen in rats.

CHAPTER FIVE

THE METABOLISM OF EPHEDRINE IN MAN

Chapter FiveThe Metabolism of (-)-Ephedrine in Man

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The Metabolism of (-)-Ephedrine in Man

Introduction

The metabolic fate of ephedrine has been determined in several species including rat (Axelrod, 1953; Bralet, Cohen & Valette, 1968; Nagase, Baba & Matsuda, 1967), rabbit (Axelrod, 1953; Matsuda, Baba & Nagase, 1971), dog and guinea pig (Axelrod, 1953) and pony (Nicholson, 1971). According to Axelrod the biotransformation of (-)-ephedrine can occur via two metabolic pathways, namely N-demethylation and ring hydroxylation. More recent studies suggest that in certain species oxidative deamination of the side chain occurs to a significant extent, as shown by the identification of 1,2-dihydroxy-1-phenylpropane as a urinary metabolite of both ephedrine in rabbit and pony (Matsuda, Baba & Nagase, 1971; Nicholson, 1971) and norephedrine in the rabbit (Sinshelmer, Dring & Williams, 1973). The extent of metabolism of ephedrine by these pathways shows considerable species variation (Williams, Caldwell & Dring, 1974). Thus N-demethylation is the major pathway in the dog and guinea pig, but in the rat ring hydroxylation is the main metabolic route (Table 1:1), which follows the same species pattern seen in the metabolism of other substituted 1-phenyl-2-amino-propanes.

The presence of the β -hydroxyl group in the ephedrine molecule gives the compound increased polarity, and this could account for the greater excretion of unchanged drug when compared with its deoxy analogue, N-methylamphetamine.

Metabolism of ephedrine appears to be stereospecific, at least in the rat, as reported by Nagase, Baba and Matsuda (1967) who showed that only the (-)-antipode underwent N-demethylation and ring hydroxylation. Bralet, Cohen and Valette (1968), however, who administered ephedrine as the racemate to rats provided similar figures to those quoted by Nagase for ring hydroxylation.

Few reports detail the metabolism of ephedrine in man. Beckett and Wilkinson (1965) using g.l.c. showed that, in a single male volunteer, 75% of an oral dose of (-)-ephedrine was excreted unchanged in the urine within 24 hours and that 4% of the N-demethylated metabolite, norephedrine, was produced. These authors in a later communication (Wilkinson & Beckett, 1968) demonstrated that the urinary excretion of (-)-ephedrine was pH dependent and that the 24 hour urinary excretion of unchanged drug showed a two to five fold increase under conditions of acid urine production when compared with alkaline urine formation, which again emphasises the important role that urinary pH plays in the kinetics of excretion and metabolism of these basic compounds.

The current study was designed to investigate in further detail the biotransformation of a drug to which on repeated administration tolerance or tachyphylaxis develops and which could be metabolised to the false transmitter metabolite, p-hydroxynorephedrine. The drug was administered orally to three human volunteers. No attempts were made to control the urinary pH throughout the experiment.

Identification of urinary metabolites

Urine samples (50 ml) were concentrated by freeze-drying to approximately 25% of their original volume and 1 ml was chromatographed in solvents A and D. In both solvents a single major peak was found (R_F 0.4-0.5 and R_F 0.85 respectively) corresponding to but failing to separate the amine metabolites ephedrine and norephedrine (Fig. 5:1). In solvent A a small peak R_F 0.75 suggested the presence of acidic metabolites, however, positive identification in this solvent was not possible.

For further chromatographic identification of metabolites, urine samples (10-50 ml) were subjected to extraction procedures under alkaline or acidic conditions. Thus, urine (10 ml) was adjusted to pH 12 with 2M NaOH, saturated

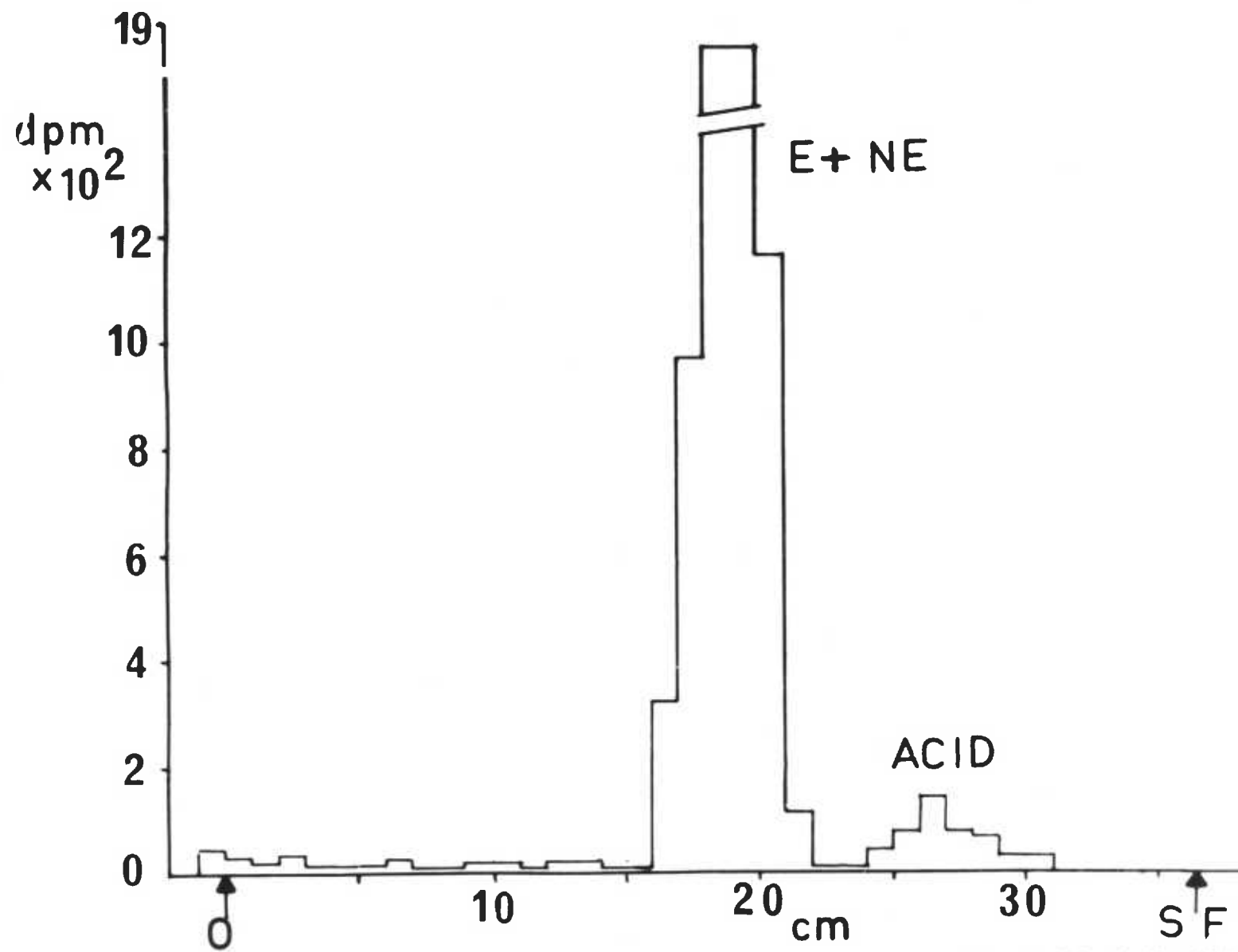


Fig. 5:1. Histogram prepared from radiochromatogram of human 0-24 h urine after (-)-(¹⁴C)-ephedrine hydrochloride orally, in solvent A.
(continued overleaf)

Fig. 5:1

Histogram prepared from radiochromatogram of human 0-24 h urine after

(-)-[¹⁴C]-ephedrine hydrochloride (0.35 mg/kg; 1.6 μ Ci) orally, in solvent A.

OD	=	Origin
SF	=	Solvent front
E	=	Ephedrine
NE	=	Norephedrine
ACID	=	Acid metabolites

with NaCl and extracted with ether (3 x 15 ml). The ethereal phase was dried over Na_2SO_4 and re-extracted with 2M HCl (2 ml). The aqueous phase was evaporated to dryness in vacuo at 40°C and the residue taken up in 0.5 ml ethanol, a fraction of which (0.3 ml) was chromatographed in solvent E (Fig. 5:2). In this solvent two major peaks were identified having R_F 0.30 and 0.52, corresponding to norephedrine and ephedrine respectively. On some chromatograms a small peak (R_F 0.70) was identified, which disappeared when the above procedure was carried out following preliminary extraction of urine at acid pH, and which corresponded to the reference compound 1,2-dihydroxy-1-phenylpropane.

Samples of urine (50 ml) were initially hydrolysed with an aryl sulphatase preparation (containing β -glucuronidase), adjusted to pH 2 with 2M HCl, saturated with NaCl, and extracted with ethyl acetate (3 x 40 ml). After evaporation of the organic phase to dryness in vacuo at 40°C, the residue was taken up in methanol (0.5 ml) and an aliquot (0.1-0.2 ml) chromatographed in solvents F and G. In solvent system G, a peak (R_F 0.85) corresponded to authentic 1,2-dihydroxy-1-phenylpropane, and a second broader peak (R_F 0.47-0.68) probably represented both hippuric and benzoic acids. (Fig. 5:3).

Reverse isotope dilution procedures were carried out in certain cases for ephedrine, norephedrine, benzoic acid, hippuric acid, 1,2-dihydroxy-1-phenylpropane and p-hydroxynorephedrine.

Results

The excretion of ^{14}C following the administration of (-)-[^{14}C]-ephedrine hydrochloride orally to 3 male subjects is shown in Table 5:1. Of the dose, 96-98% was excreted in the urine within 48 hours and most of this appeared in the first 24 hours (84-91%). The bulk of the radioactivity consisted of unchanged ephedrine (53-74%) and its N-demethylated metabolite, norephedrine (8-20%) (Table 5:2). Oxidative deamination of the side chain occurred to a variable

Figs. 5:2 & 5:3. Histograms prepared from radiochromatograms of extracts of human 0-24 h urine after (-)-¹⁴C-ephedrine hydrochloride (0.35 mg/kg; 1.0 μ CI orally.

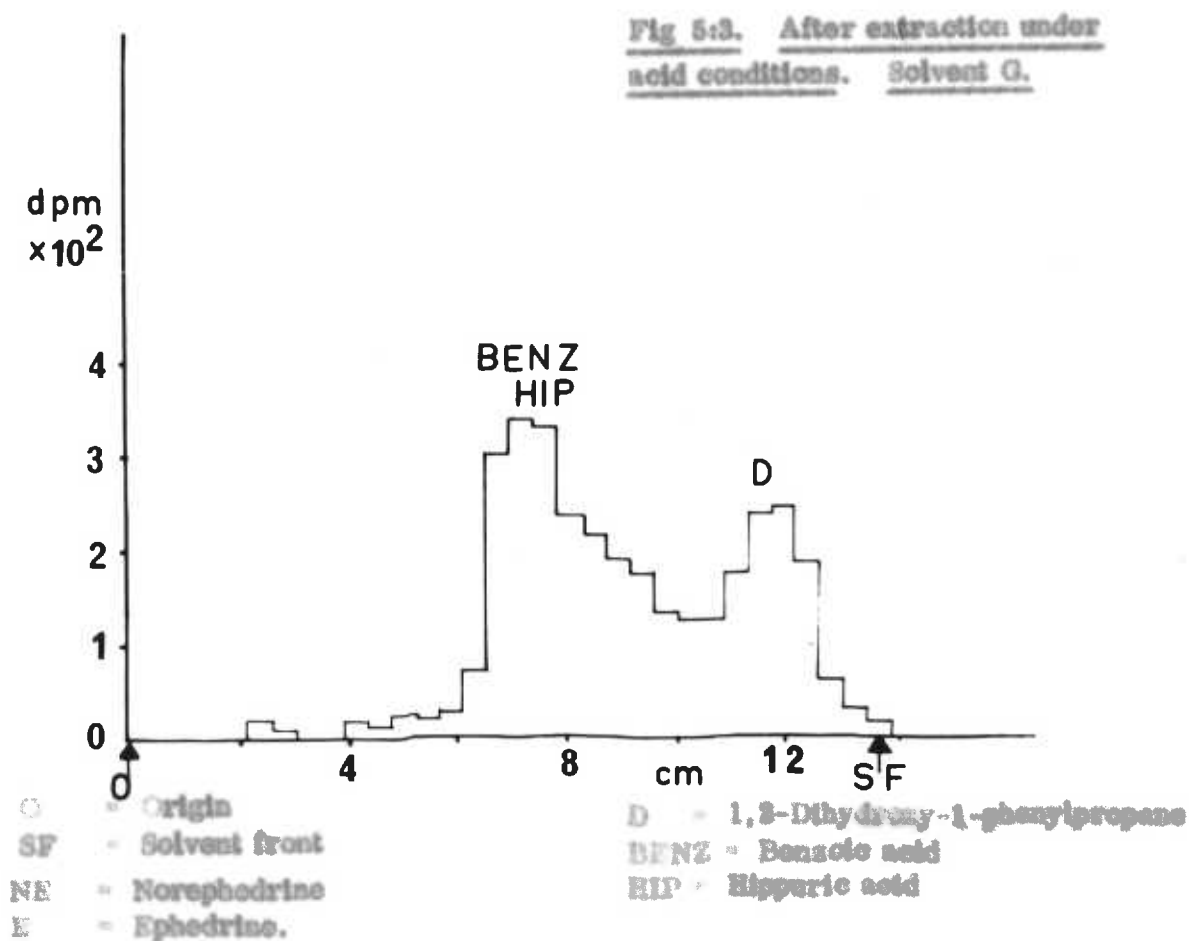
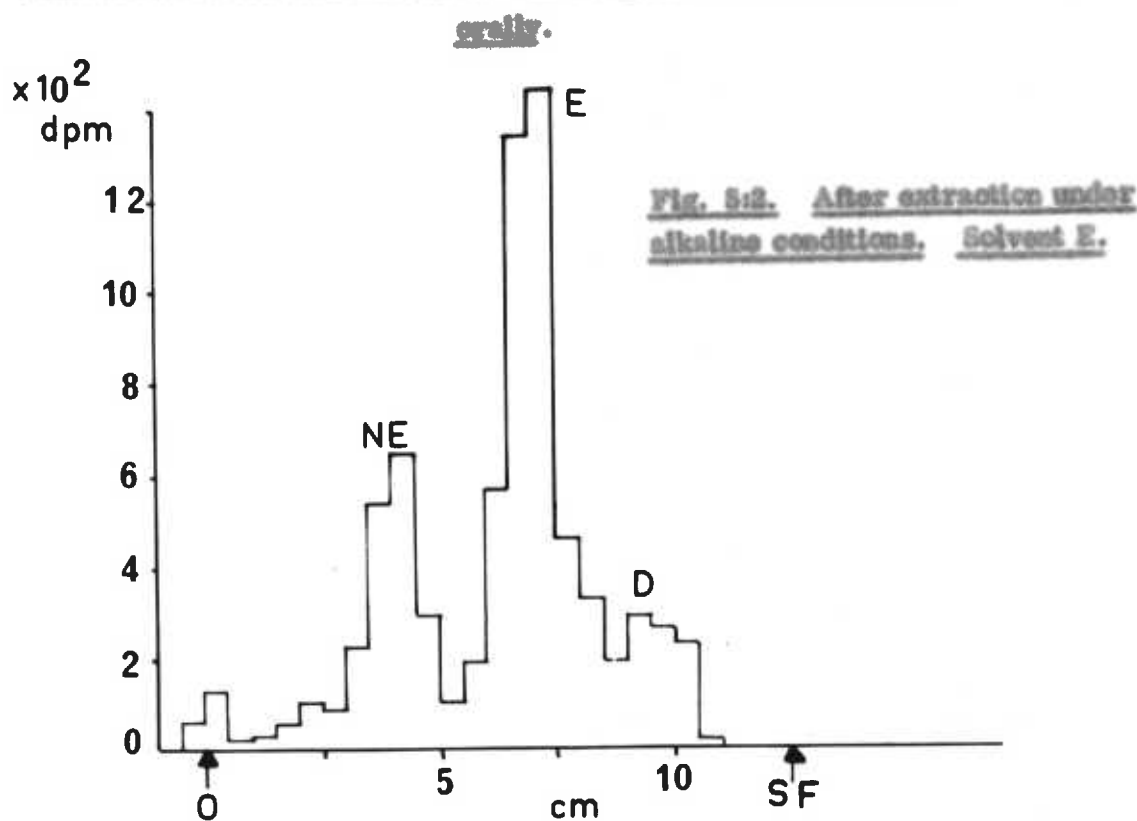


Table 5:1 The Elimination of ^{14}C by Human Subjects after Administration of
 $(-)-[^{14}\text{C}]\text{-Ephedrine Hydrochloride}$

$(-)-[^{14}\text{C}]\text{-Ephedrine}$ hydrochloride (0.35 mg/kg; 1.6 μCi per subject) was dissolved in a small volume of water and administered to human subjects orally. Values given are % of ^{14}C dose in urine expressed as the mean of 3 subjects. Individual figures in parentheses.

	<u>% of ^{14}C dose</u>
Urine Day 1	88.0 (88.9, 83.8, 91.3)
Urine Day 2	8.7 (7.5, 12.3, 6.3)
Total ^{14}C excreted	96.7 (96.4, 96.1, 97.6)

Table 5:2 The Metabolites of (-)-[¹⁴C]-Ephedrine Hydrochloride in Human Urine

Dose see Table 5:1. The urine analysed was that excreted in the first 24 hours after dosing. Values given are expressed as a % of the dose and are the mean of 3 subjects, with individual values in parentheses.

<u>Metabolite</u>	<u>% of ¹⁴C dose</u>
Ephedrine	61.3 (74.2, 56.4, 53.3)
Norephedrine	13.2 (7.7, 11.8, 20.1)
Ring hydroxylated metabolites	- (n.d., n.d., n.d.)
Hippuric acid	4.6 (2.8, 6.1, 4.8)
Benzoic acid	4.9)
1,2-Dihydroxy-1-phenylpropane	8.9 (4.2 9.5 1.2)
Other acidic metabolites	7.0)
Total metabolites	83.4 (86.1, 77.2, 86.5)
Total ¹⁴ C in urine	88.0 (88.9, 83.8, 91.3)

n.d. = not detected.

extent (4-13%) and excretory products identified were hippuric acid, benzoic acid and 1,2-dihydroxy-1-phenylpropane. Other products of deamination, including 1-hydroxy-2-oxo-1-phenylpropane and 1,2-dioxo-1-phenylpropane were not detected. In addition, ring hydroxylated metabolites of ephedrine were not detected by chromatography and an isotope dilution procedure for p-hydroxynorephedrine in one subject confirmed the absence of this compound in the urine.

The urine pH recorded for the 0-24 hour urine showed no variation between the 3 subjects (pH 6.5-6.8).

Discussion

The definitive fate of ephedrine in man has not been previously reported. The analytical method of Richter (1938) did not differentiate between ephedrine and norephedrine, and in the study of Wilkinson and Beckett (1968) the only metabolite determined was norephedrine. The extent of N-demethylation quoted by these authors appeared to be dependent upon urine pH, as in their study the three subjects with alkaline urines (pH c. 8) excreted 11-24% of the dose of (-)-ephedrine as the metabolite norephedrine, which contrasted with the same subjects, under conditions of acid urine production (pH c. 5.0), in whom N-demethylation only occurred to the extent of 3-10%. An observation not noted by these authors, but based upon their figures was the twofold variation in the extent of N-demethylation, at the same urine pH, between the different subjects. This observation is confirmed in the current study in which it is seen that at similar urine pH values (6.5-6.8) for the three subjects, the extent of N-demethylation varies from 8 to 21% of the dose. It should be pointed out that on no occasion had any of the volunteers in this study received ephedrine previously, eliminating therefore any possibility of increased drug metabolism resulting from repeated therapy. By implication, these observations might account for interindividual variations in the pharmacological effects of ephedrine when this drug is administered to man,

since the N-demethylated metabolite, norephedrine, has no central stimulant effects (Goodman & Gilman, 1968).

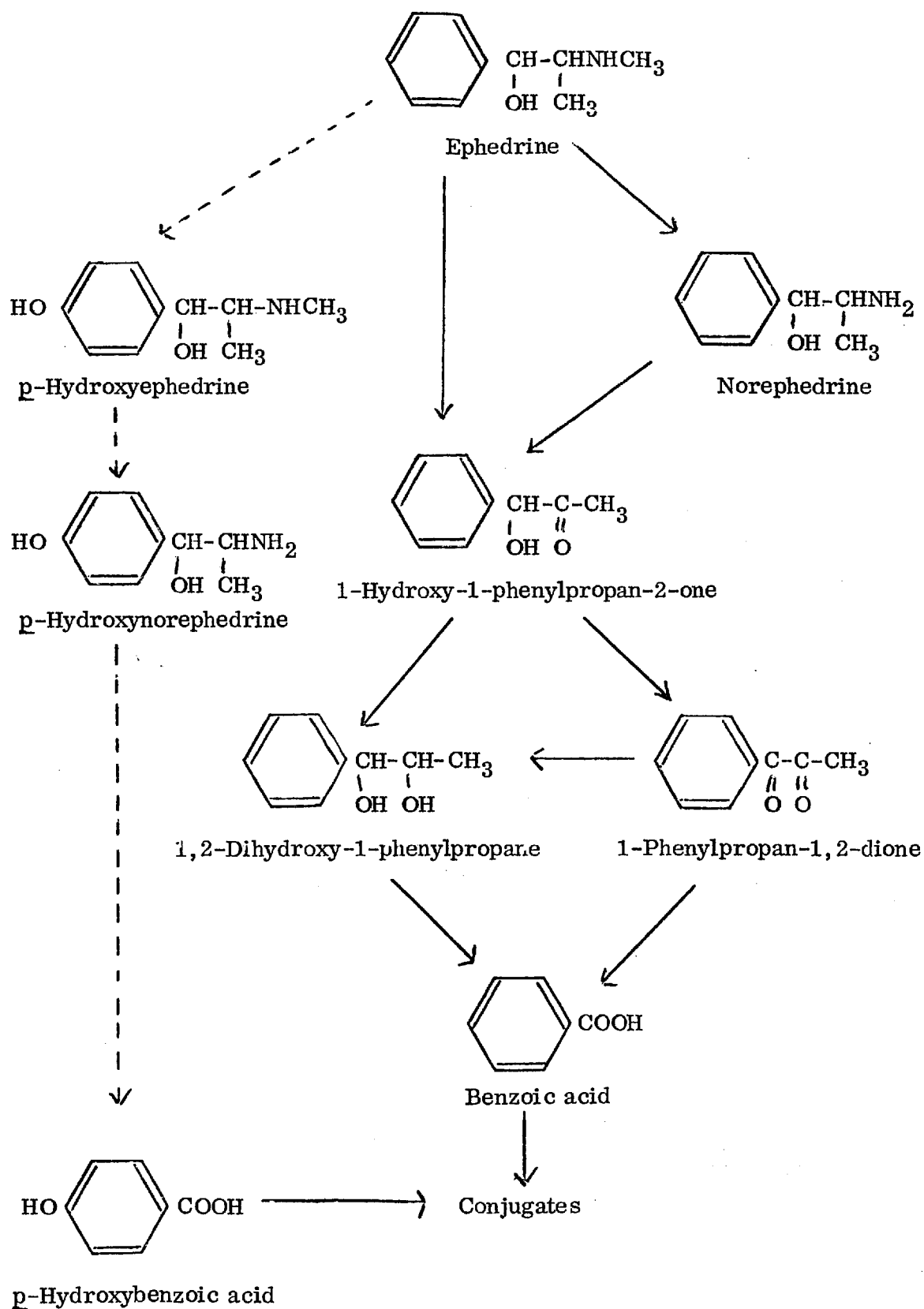
Oxidative deamination of the side chain (4-13%) is greater than that found with norephedrine (Sinsheimer, Dring & Williams, 1973) and probably reflects the increased lipid solubility of the ephedrine molecule. The probable route of this metabolic pathway is an extension of that suggested by Sinsheimer, Dring and Williams (1973) and is shown in Fig. 5:4, and the identification, in the present study, of the diol (1,2-dihydroxy-1-phenylpropane) as a urinary metabolite of ephedrine is in support of this proposal. However, other products of deamination, namely the ketol (1-hydroxy-2-oxo-1-phenylpropane) and the dione (1,2-dioxo-1-phenylpropane), which, together with the diol, were found as metabolites of norephedrine in the rabbit (Sinsheimer, Dring & Williams, 1973), were not identified in this experiment.

The failure to identify phenolic metabolites by chromatographic methods and more specifically the failure to identify p-hydroxynorephedrine by reverse isotope dilution procedures, is in striking contrast to methamphetamine, the deoxy analogue of ephedrine which in man undergoes ring hydroxylation to the extent of 18% and ring and side chain hydroxylation to form p-hydroxynorephedrine which is excreted in the urine (2% of dose) (Caldwell, Dring & Williams, 1972a). It is probable that since methamphetamine is eighty times more lipid soluble than ephedrine the former is more susceptible to tissue uptake and metabolism (Williams, Caldwell & Dring, 1974).

Ephedrine is a mixed-acting sympathomimetic amine (Trendelenburg, 1963) and exerts many of its effects by releasing noradrenaline from peripheral sympathetic neurons, and, like amphetamine, it has a central stimulant action but less pronounced (Goodman & Gilman, 1970).

In contrast to amphetamine, ephedrine has a direct stimulant effect at α and

Fig. 5:4. Metabolism of (-)-Ephedrine



(Based on material from references quoted in the text).

β receptors and for this reason it is a useful therapeutic agent as a bronchodilator and nasal decongestant (Goodman & Gilman, 1970).

Acute tolerance or tachyphylaxis develops rapidly to the peripheral actions of ephedrine on repeated dosage, possibly as a result of the depletion of noradrenaline stores (Goldstein, Aranow & Kalman, 1968). It seemed possible that p-hydroxynorephedrine could be formed metabolically from ephedrine and be responsible not only for the depletion of noradrenaline, but also for the development of tolerance. The failure to identify p-hydroxynorephedrine in this study would suggest that this compound is not involved as a false transmitter in the acquisition of tolerance to ephedrine.

PART II

CHAPTER SIX

INTRODUCTION

Chapter SixIntroduction

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Introduction

Drug Tolerance

The original term addiction has been largely superseded by the terms 'tolerance' and 'dependence'. The importance of these two phenomena has been stressed in relation to the biological effects of many psychoactive drugs, certain of which may induce both tolerance and dependence, whereas others apparently induce tolerance alone.

It is well established that regular drinkers are able to tolerate larger amounts of alcohol on repeated exposure, or alternatively, that the heavy drinker requires progressively larger quantities of alcohol in order to produce the change in feelings and behaviour which has previously been experienced with a lower dose. Dependence, however, is the production of dramatic withdrawal signs and symptoms on cessation of drinking.

It is generally held that alcohol, barbiturates and opiates induce dependence, but there is certain conflict as to whether amphetamines can produce this phenomenon, although tolerance indisputably develops to some of their pharmacological effects.

The different connotations of the term 'tolerance' are defined by Kalant, Le Blanc and Gibbins, (1971).

Initial or innate tolerance is reflected by that amount of drug which a subject requires on first exposure to it, to produce a designated degree of effect. This reflects a diversity of hereditary and environmental factors which contribute to the marked interindividual variations in drug response that may depend upon sex, species, age and dietary habits.

Acquired tolerance within the same individual results from repeated exposure to a drug such that an increased amount of drug is required to produce the same degree of effect. This may vary from one effect of a given drug to another.

Acquired tolerance, to which the term tolerance used in this thesis refers, may be further subdivided into:

Dispositional tolerance which develops as a result of an alteration in the absorption, distribution, metabolism or excretion of a drug, and which leads to a reduction in the intensity and duration of contact between the drug and the target tissue.

Functional tolerance or Tissue tolerance which refers to the possible changes or adaptation in the properties and functions of the target tissue which render it less sensitive to the same degree of exposure to the drug.

Distinction is made in the literature between acute and chronic tolerance. Undoubtedly 'acute' tolerance can develop within the duration of a single exposure to a drug (vide infra) and can be temporally separated from 'chronic' tolerance which gradually develops with prolonged and repeated exposure. Mechanistically such a distinction may be misleading and will be referred to later in this chapter.

The following discussion outlines selected psychoactive (and abused) drugs and the various mechanisms that have been proposed to explain tolerance.

Dispositional Tolerance

Studies on the absorption, distribution and excretion of many psychoactive compounds including alcohol, amphetamines, barbiturates and opiates have failed to show any gross alteration with chronic exposure. In the case of alcohol there is even a suggestion that a slight enhancement of absorption occurs with repeated dosage, and probably results from an altered gastrointestinal motility preventing the normal delay in gastric emptying, but there is no overall significant difference in distribution or excretion (Kalant, Le Blanc & Gibbins, 1971).

However, many drugs, most notably the barbiturates, have the ability to increase their own metabolism on chronic exposure. The rate of ethanol metabolism has been shown to increase with repeated administration, but the mechanism of this has been the subject of much conjecture. Although hypertrophy of the smooth endoplasmic

reticulum occurs, associated with an increase in several microsomal enzyme activities (Kato, 1966) and an increase in cytochrome P₄₅₀ levels, the evidence that a microsomal enzyme system participates in the metabolism of ethanol is equivocal.

Other mechanisms, however, must be involved in the acquisition of tolerance to ethanol, for even assuming that enzyme induction increases the metabolic rate threefold it is probable that this could only account for a slight reduction in the maximal pharmacological effect, although the duration of action of the drug would, undoubtedly, be shortened (Kalant, Le Blanc & Gibbins, 1971).

Virtually all the barbiturates are enzyme inducers and of these phenobarbitone is the archetype and the increased rate of metabolism seen on prolonged administration is of importance in explaining tolerance to these drugs (Conney, 1967). However, according to Remmer (1969), whereas tolerance to the shorter acting barbiturates appears to be mediated almost exclusively by enzyme induction, as the length of action of the barbiturate increases, the extent of CNS adaptation becomes progressively greater.

In this context it must be noted that the metabolism of a wide range of psychoactive agents, including other hypnotics, and sedatives, major and minor tranquillisers and antihistamines, is substantially increased by enzyme induction (Kalant, Le Blanc & Gibbins, 1971).

Alterations in drug disposition do not appear to contribute significantly to opiate tolerance, nor to the tolerance that develops to amphetamines. Ellison et al. (1971) found that the half life of amphetamine is increased in tolerant cats, and in this experiment the total excretion of ³H amphetamine and metabolites excreted over a five day period were essentially the same in tolerant and non tolerant animals. These authors stated, however, that the excretion of unchanged amphetamine was slightly

increased in the tolerant animals. Tissue distribution studies showed that although brain distribution of radioactivity was similar in both groups, the rate of decline in the level of free amphetamine in the cerebrum was five-fold greater in the non tolerant cats in the four hour period after dosing. In other organs differences were not significant. Studies in humans have revealed similarly that the half-life of amphetamine is increased in drug dependent individuals when compared with naive controls (Gunne *et al.*, 1972). Mitchard *et al.* (1971) found that this drug's absorption and distribution kinetics in a single tolerant man were essentially the same as in drug naive subjects. A detailed metabolic study in the amphetamine tolerant state was therefore indicated, the results of which are ~~not~~ presented in Chapter 4.

Functional Tolerance

An alternative mechanism for the development of tolerance to many drugs has already been suggested in relation to ethanol and the barbiturates. Further evidence that a process of CNS adaptation forms the basis for tolerance that develops to these drugs has resulted from cross tolerance experiments. Kalant, Le Blanc and Gibbins (1971) and Khanna, Kalant and Lin (1972) concluded that changes in barbiturate sleeping time of rats made tolerant to ethanol were the result of alteration in the sensitivity of the CNS and not modification of drug disposition. Similar findings in animals and man with the general anaesthetics (Han, 1969; Lee & Atkinson, 1968) would appear to support this as their elimination is not primarily the result of hepatic metabolism. Similarly the failure of phenobarbitone pretreatment to change the metabolism of ethanol (Khanna, Kalant & Lin, 1972) suggests that the resistance of barbiturate addicts to ethanol is not due to increased oxidation.

The main indications which can be gained from the literature are (i) that

alcohol tolerance is due principally to CNS adaptation, not metabolic alteration, (ii) that while tolerance to shorter acting barbiturates can be explained by induction of metabolising enzymes, as the length of action of the drug increases, CNS adaptation becomes more important in tolerance, and (iii) that cross tolerance between alcohol and the barbiturates is principally due to alteration of CNS sensitivity.

The exact nature of the CNS adaptation involved in tolerance is, however, uncertain. It does appear that this is essentially the same for both groups of drugs. Some aspects of this have been studied, most notably with respect to the rapid appearance of the adaptation (the so-called acute tolerance). This so-called acute tolerance, which develops within a single exposure to a drug may be shown in two ways: either as (i) a difference between the concentrations of the drug at the appearance and subsequent disappearance of symptoms of CNS depression, or (ii) as a positive correlation between the plasma concentration of the drug at the disappearance of signs of intoxication and the size of the dose administered.

This phenomenon was first demonstrated by Mellanby (1919) with ethanol when he showed that, for a given plasma concentration of ethanol, the degree of functional impairment was greater while the concentration was rising than when falling. Similarly, Mirsky et al. (1941) abolished ethanol metabolism in rabbits by hepatectomy and found that when a constant plasma level of ethanol was maintained, tolerance developed to the depressant effects of the drug.

With the barbiturates, acute tolerance was first noted in man by Brodie et al. (1951) who found that the plasma level of thiopentone after recovery from anaesthesia, rather than being constant, was directly proportional to the dose. In a fuller study of this phenomenon in dogs, Maynert and Klingman (1960) have shown that the curves of plasma level on awakening versus size of dose reach a plateau probably indicating

maximal tolerance. In this paper Maynert and Klingman have described acute tolerance to a range of CNS depressants, including trichloroethanol and paraldehyde in addition to ethanol and two barbiturates.

It would appear then, that as a wide range of chemically dissimilar drugs give rise to acute tolerance, the tolerance is produced by the CNS depression produced by the drug rather than by the presence of the drug molecule. It does appear (Maynert & Klingman, 1960) that the extent of acute tolerance is related to the depth of CNS depression, but not to its length. This suggestion would appear to be supported by the finding of acute cross-tolerance between ethanol and hexobarbitone by Wahlström and Widerlöv (1971).

The development of tolerance to ethanol may be intensified by behavioural loading. Thus, animals and humans made to carry out tasks while intoxicated become tolerant much more rapidly than if allowed to remain passive. Kalant (1972) opines that this supports the concept that tolerance is evoked by CNS depression due to the drug, rather than by the drug itself, but this reasoning is hard to follow. When behavioural loading is applied, the net depression due to a given dose of drug is less, yet tolerance is developed faster. It appears that, at the present, these arguments cannot be reconciled.

The relationship of acute tolerance to chronic tolerance is as yet uncertain. There are indications that chronic tolerance may consist of the summation of a series of acute tolerances, because in experiments involving chronic dosage with ethanol, as the treatment proceeded, the acute tolerance from each dose became more marked and appeared earlier (Kalant, 1972). Tolerance to CNS depressants appears to be fixed as a long-lasting trace which facilitates quicker development of tolerance on further exposure to the drug (Kalant, 1972).

This schema suggests an analogy with learning and memory, in which acute

tolerance can be equated with short term learning and chronic tolerance with long term laying down of memory. Such an analogy is strengthened by the ability of the protein synthesis inhibitor cycloheximide and bilateral frontal cortical ablations to prevent the development of tolerance to ethanol (Kalant, 1972). These treatments are also able to impair the processes of learning and memory very profoundly.

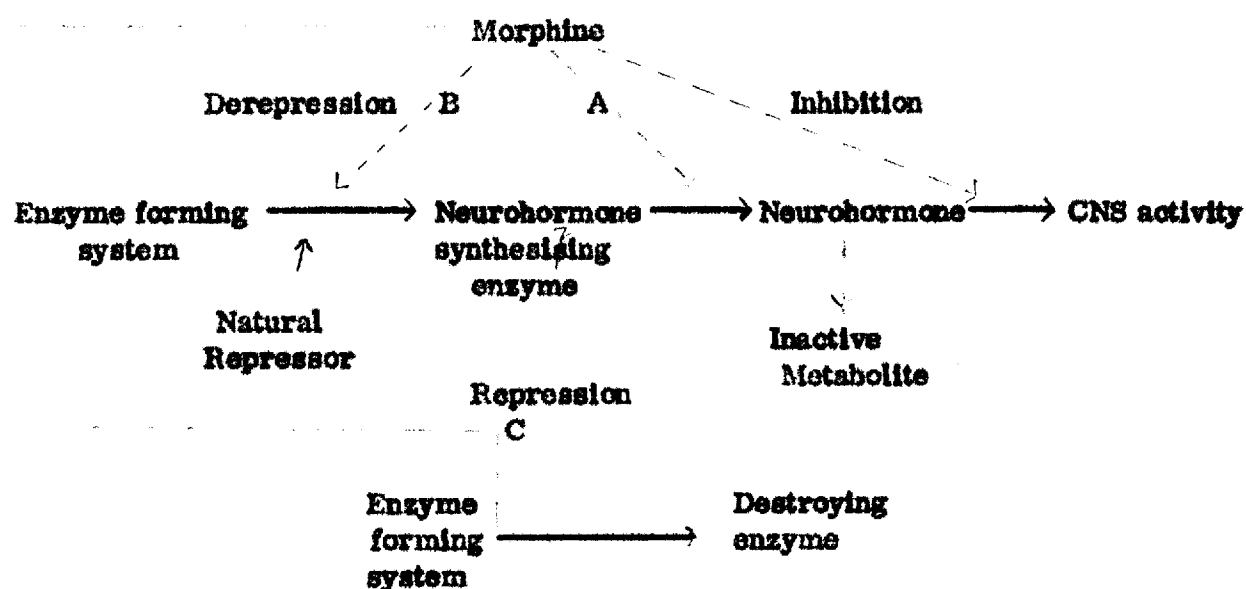
It is thus seen that the production of tolerance to CNS depressants may well be due to a basic cellular adaptation to the effects of the drug, rather than to the drug itself.

The opiates, being the archetypal drugs of dependence, have long been studied with respect to the mechanisms giving rise to the development of tolerance, and many hypotheses for its genesis have emerged.

Schmidt and Livingston (1933) suggested that morphine combines with receptors in the brain and that tolerance is due to occupation and saturation of these receptors. In this case more drug would be required to overcome the blockade as dosage proceeded. This theory adequately explains cross tolerance between opiates and antagonism by related molecules such as nalorphine, but fails to account either for the persistence of tolerance long after the drug has left the body or for withdrawal phenomena. However, the findings of Misra and Mule (1972) that a metabolite of methadone is found firmly bound to brain proteolipids at very low levels for up to 3 weeks after a single dose may have significance and lead to a re-evaluation of the receptor occupation hypothesis.

Axelrod (1956) formulated a theory for the development of tolerance to opiates on the basis of the striking decrease in N-demethylase activity in the liver microsomes of rats made tolerant to morphine. He proposed that the receptor sites in the CNS and in the liver enzyme systems were similar, and that continuous interaction of narcotics with receptors served to inactivate them.

According to Shuster (1961) these observations represented a possible drug induced enzyme repression which could explain the features of tolerance to, and dependence upon, narcotics. This author presented the following schema to explain the acquisition of tolerance to morphine.



From Shuster (1961).

Morphine could act at site A by inhibiting the action or synthesis of a neurohormone required for normal function of the CNS, (thus producing depression, analgesia, etc.). With repeated dosage morphine could act at site B causing derepression of the enzyme required for synthesis of the neurohormone, thereby tending to overcome the blockade. Tolerance would develop because more morphine would be required to depress the CNS. Alternatively, the same result could be achieved by repression at site C.

Collier (1965) proposed that the development of tolerance to and dependence upon drugs could be explained on the basis of the concept of receptors. His hypothesis required the following assumptions:

- 1) that receptors were of two types - active and silent;
- 2) that the number of receptors of either type for a substance can increase or decrease at the same rate as tolerance or dependence develops;
- 3) that a drug causing dependence acts by increasing or decreasing the amount of an endogenous substance available to receptors;
- 4) that when excess or deficiency of a chemical substance distorts a living system, the numbers of receptors of either type for that substance change in the direction lessening the distortion;
- 5) that the ease with which a substance excites a tissue depends upon the number of accessible receptors for that substance.

This and other theories for the genesis of tolerance imply that CNS neurotransmitters are involved. Many investigators have concentrated upon the effects of tolerance producing drugs on brain levels and turnover of neurotransmitter substances. Collier, Francis and Schneider (1972) tested the action of a number of drugs on several aspects of the morphine withdrawal syndrome. *p*-Chlorophenyl-alanine, atropine and indomethacin all modified the withdrawal. They therefore implicated serotonin, acetyl choline and prostaglandins in morphine dependence. However, although neurotransmitter changes are of importance in understanding the problems of drug tolerance, they do not themselves explain adaptation to the drug.

Martin (1970) outlined the principles of pharmacological redundancy and postulated that the depression of certain functional pathways in the CNS produced by large doses of a neurohumoral antagonist, e.g. morphine, could be surmounted by increasing the stimulus strength. This could lead to a 'hypertrophy' of a redundant pathway which resists the inhibitory effect of morphine. Negative feedback pathways could be responsible for initiation of this pathway.

negative feedback

morphine
insensitive

morphine
sensitive

Effector
Cell

From Martin (1970)

This theory is one of many 'homeostatic adjustment' hypotheses that attempt to explain tolerance and dependence. Seevers and Woods (1953) proposed a 'dual action' theory based on the fact that morphine and its congeners are both CNS depressants and excitants and would act at two different sites on the same neuron. Drug-receptor interaction on the surface of the cell could be responsible for the depressant activity, and as more receptors were occupied, tolerance resulted. Drug-receptor interaction within the cell on the other hand could induce excitation which was masked by the depressant activity, but unmasked upon drug withdrawal.

It has often been suggested that the development of tolerance to morphine and similar drugs is in some way connected with protein synthesis and that cellular adaptation to the drug is under genetic control. Cohen et al. (1965) found that an RNA polymerase inhibitor, actinomycin D, prevented the development of tolerance to morphine in rats and mice. In addition, Cox and Osman. (1970) concluded that the development of tolerance to the analgesic effect of narcotics, including morphine, heroin and pethidine, in rats was dependent on DNA directed RNA synthesis leading to the synthesis of new protein. However, although protein synthesis inhibitors prevent the development of tolerance to the analgesic effect of the narcotics, cycloheximide has no effect on tolerance to the increased locomotor activity seen with morphine (Karbowski, 1973).

If indeed CNS protein synthesis is involved in the genesis of tolerance and

dependence it is interesting to speculate on the nature of the 'Tolerance protein' being produced. Cox and Ginsburg (1969) suggest that possible roles for this new protein could be in the production of a drug uptake receptor, a neurotransmission receptor or a transmittable tolerance factor. It is appropriate to note here the work of Ungar and Cohen (1966) who found that the brain of morphine-tolerant animals contains a substance which confers morphine tolerance to non-tolerant animals when injected into them. They suggested that this substance is a small molecule, possibly a hexapeptide. Other workers (Cox & Ginsburg, 1969) have been unable to repeat this, although they do not rule out the possibility of such a transmittable tolerance factor occurring. It may be that the protein synthesised during tolerance development can be transmitted from animal to animal and that the findings of Cox and Osman (1970) and Ungar and Cohen (1966) are linked in such a way. This still does not elucidate a mode of action for this 'tolerance protein' but here it is possible to see a striking analogy with work on learning and memory. The ability of animals to learn simple tasks may be impaired by the administration of protein synthesis inhibitors, and it has been demonstrated that extracts from the brains of rats trained to perform a given task, when administered to untrained rats, convey the ability to perform this task (Ungar, Desiderio & Parr, 1972; Brand & Brand, 1972). The similarity of these findings with the work described on morphine tolerance would suggest that the basic mechanism of tolerance development is similar to that of memory; that the brains of the animals in some way 'learn' to 'ignore' the presence of the drug.

As an alternative to these neurochemical explanations, Cochin (1971) and Berkowitz and Spector (1972) have suggested that the long persistence of tolerance, its apparent transferability and the involvement of protein synthesis indicate a possible participation of the immune mechanisms of the body. Berkowitz and

Spector (1972) have shown that mice can be actively immunised against morphine with a conjugate of 3-carboxymethylmorphine with bovine serum albumin. Sera from these mice showed a greatly increased (thirty fold) ability to bind dihydromorphine, and the plasma half-life of the drug in immunised mice is much prolonged. These mice showed tolerance to morphine, but not dependence, as nalorphine was without effect. These findings do not, of course, explain tolerance to the opiates, still less dependence, but it must be considered that active or passive immunity may influence tolerance. Proteins able to bind heroin have been isolated from the plasma of heroin dependent subjects (Ryan, Parker & Williams, 1972) indicating the potential involvement of the immune mechanisms in man.

In conclusion then, it is seen that the answer to the problem of tolerance and dependence remains very obscure. Most studies have concentrated on opiate tolerance and new approaches have yielded fascinating possibilities as it does seem that tolerance and dependence rather than being a single discrete phenomenon, represent the net result of several distinct mechanisms.

Tachyphylaxis

In the previous discussion of tolerance developing to barbiturates and ethanol, reference was made to the phenomenon of acute tolerance. This term is often used synonymously, but perhaps incorrectly, with tachyphylaxis, which, strictly speaking, implies the rapid attenuation of a pharmacological response with repeated dosage (Goldstein, Aronow & Kalman, 1968). Tachyphylaxis has been demonstrated with successive administrations of many sympathomimetic agents and was originally considered to result from the occupancy or saturation of drug receptors (Winder, Anderson & Parke, 1948). However, certain substituted phenylethylamines, notably ephedrine and amphetamine, produce tachyphylaxis more readily than others, e.g. tyramine (Day & Rand, 1963), and when it was

established that many of these drugs acted indirectly by liberating noradrenaline and did not have a direct effect (see Chapter 1), the receptor occupation theory seemed less likely. It was subsequently postulated that these indirectly-acting amines released noradrenaline and that the development of tachyphylaxis was associated with depletion of noradrenaline stores, such that further challenge with the drug would release increasingly small amounts of noradrenaline (Axelrod *et al.*, 1962). However, considerable amounts of noradrenaline were found within the nerve terminals in the tachyphylactic state, and could be released normally on nerve stimulation, with a normal pharmacological response (Potter, Axelrod & Kopin, 1962).

Later evidence showed that certain indirectly-acting amines including amphetamine would only liberate newly synthesised noradrenaline (Glowinski, 1970). This evidence taken in conjunction with the hypothesis that certain metabolites of these drugs replace (and release) noradrenaline from storage sites, and are themselves released on subsequent drug challenge as false neurotransmitter, could account for the diminished noradrenaline release with repeated drug challenge, and therefore tachyphylaxis.

The False Transmitter Hypothesis

At this stage in the discussion of drug tolerance and the possible underlying mechanisms, it is apparent that most theories of tolerance and dependence have concentrated upon alterations in drug disposition or 'target organ' adaptation. However, it has been stated by Kalant, Le Blanc and Gibbins (1971) that tolerance to amphetamines may occur by means of a unique mechanism involving the replacement of noradrenaline at sympathetic nerve endings by the metabolite, p-hydroxynorephedrine, which could act as a false neurotransmitter.

According to Kopin (1968) the criteria for a false neurotransmitter are:

1) that the compound must be stored in the nerve endings at the same site as the normal physiological transmitter, and 2) that it must be released on physiological nerve stimulation. (There is no requirement, however, that the released substance be as active as the physiological transmitter).

As will be appreciated from the foregoing discussions of the biosynthesis of noradrenaline, the relative nonspecificity of dopa decarboxylase and dopamine- β -hydroxylase allows many phenylethylamine derivatives (both endogenous and exogenous) to be converted to their β -hydroxylated metabolites in vivo, for example:

α -methyl dopa $\longrightarrow$ α -methylnoradrenaline

α -methyl-m-tyramine $\longrightarrow$ metaraminol

(Carlsson and Lindqvist, 1962)

tyramine $\longrightarrow$ octopamine

(Carlsson and Waldeck, 1964; Fischer, Horst & Kopin, 1965), and other drugs (see Kopin, 1968).

The formation of p-hydroxynorephedrine from amphetamine by aromatic and β -hydroxylation has been considered previously. This metabolite has been shown to be taken up and stored in sympathetic nerve endings (Carlsson and Waldeck, 1964) and released on nerve stimulation (Thoenen et al., 1966), thus fulfilling the accepted criteria for false transmitter function.

What effect then could the false neurotransmitter have upon the noradrenergic neuron? Firstly the replacement of noradrenaline by false transmitter at the release site, would be expected to limit the amount of noradrenaline liberated on nerve stimulation and indeed this was demonstrated by Muscholl and Maitre (1963). Secondly, with release of false transmitter (and some noradrenaline) the pharmacological response would depend upon the agonist activity of the false

transmitter itself. For *p*-hydroxynorephedrine, its pressor activity is only 1/500 that of noradrenaline (Brodie, Cho & Gessa, 1970). Thirdly, according to Kopin, (1968), false transmitters may, by displacing granular noradrenaline, liberate free noradrenaline into the neuronal cytoplasm which would antagonise further synthesis by feedback inhibition.

With repeated administration of amphetamine, it is proposed that the false neurotransmitter, *p*-hydroxynorephedrine accumulates in the vesicular stores and is responsible for tolerance to those effects of amphetamine mediated by noradrenaline (Brodie, Cho & Gessa, 1970; Davanaugh, Griffith & Oates, 1970; Lewander, 1971d). In favour of this hypothesis is that *p*-hydroxynorephedrine depletes neuronal noradrenaline, has only weak sympathomimetic activity and is released preferentially with respect to noradrenaline by (+)-amphetamine (Brodie, Cho & Gessa, 1970).

The aims of the present thesis therefore were to correlate on the basis of metabolic investigations and studies of drug tolerance, the metabolic production of *p*-hydroxynorephedrine and the development of tolerance to amphetamines.

In the first part of the thesis, a number of potential precursors of *p*-hydroxynorephedrine were studied to determine whether or not this compound was metabolically produced. In addition, human tolerant subjects were investigated in order to quantitate the formation of *p*-hydroxynorephedrine in the tolerant state. In the second part of the thesis, the false transmitter hypothesis was tested in two ways:-

- 1) by pretreating animals with precursors of the false transmitter and subsequently challenging them with amphetamine since the response would be expected to diminish with such pretreatment if the hypothesis were true,
- 2) by comparing and contrasting the development of tolerance in two species, one of which is known to produce the false transmitter metabolically, and the other which does not. Theoretically the latter species should not become tolerant to

amphetamines.

CHAPTER SEVEN

MATERIALS AND METHODS

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Materials and Methods

Compounds

(+)-Amphetamine sulphate; (±)-norephedrine hydrochloride; (±)-p-hydroxyamphetamine hydrobromide; (±)-p-hydroxynorephedrine hydrochloride (see Chapter 2); (±)-amphetamine sulphate (Smith, Kline and French Laboratories). α -Methyl-p-tyrosine methyl ester hydrochloride (Sigma Chemical Company, St. Louis, Missouri, U.S.A.), 6-hydroxydopamine hydrobromide (Aldrich Chemical Company, Inc., Milwaukee, U.S.A.), desmethylinipramine (Geigy Pharmaceuticals, Macclesfield, Cheshire), p-chloroamphetamine (Dr. R.E. McMahon, Eli Lilly Research Laboratories, Indianapolis, Inc., U.S.A.), tetraethylammonium bromide (Hopkin and Williams, Chadwell Heath, Essex).

Animals

Female Wistar albino rats, body weight 220 ± 20 g, male Wistar albino rats, body weight 220 ± 20 g, female and male Sprague-Dawley rats, body weight 220 ± 20 g and female Dunkin-Hartley guinea pigs, body weight 250 ± 25 g, were used.

Housing and Food.

Animals were housed in groups of 5 or 6 in plastic cages (45 x 32 x 15 cm) at constant ambient temperature ($20^{\circ}\text{C} \pm 1^{\circ}$) and with free access to food (rats - Oxoid No. 41b pellets; guinea pigs - Oxoid No. SG1 pellets with fresh cabbage) and water. Both animals and food were weighed daily during repeated dosage experiments.

Drug Administration

All compounds with the exception of 6-hydroxydopamine were dissolved in distilled water or normal saline and administered by intraperitoneal injection. 6-Hydroxydopamine was dissolved in distilled water containing 10 mg/ml ascorbic acid and injected into the tail vein of rats.

In the chronic dosage experiments amphetamine solutions (2.5 mg/ml) and

10 mg/ml in normal saline) were sterilised by ultra-filtration (Millipore).

Temperature recordings

Colonic temperatures were taken with a Yellow Springs Instruments Model 46 Thermistor Thermometer using a No. 402 probe inserted into the colon, 5 cm from the anus. Control and test groups were compared simultaneously at the same times each day, to minimise diurnal variations in temperature.

Animal Activity Monitoring

Motor activity (gross locomotor and stereotyped activity) was measured on a two level electronic activity meter (Stoelting No. 30416, Stoelting Instrument Co., Chicago). The meter was adjusted in order to distinguish between total motor activity (high sensitivity channel) and gross motor movements (low sensitivity channel). Stereotyped behaviour (fine movements) was calculated as the difference between the counts registered on each channel. Motor activity was recorded for animals housed singly in plastic cages (45 x 32 x 15 cm). Behavioural effects of amphetamine in some tolerance experiments in the rat were scored with a scale modified from that of Quinton and Halliwell (1968) which was expanded to score gross locomotor activity in addition to stereotyped movements (Table 7:1).

After drug administration a period of acclimation (30 minutes) was allowed following which animals were monitored. Whenever possible activity was measured at the same time each day.

Table 7:1 Visual Rating Scales for Animal Activity

Animals observed for 30 secs. and scored every 10 mins. for the period 30-60 mins. after amphetamine administration.

Gross Locomotor Activity**Score**

- | | |
|---|--|
| 0 | No movement |
| 1 | Short bursts of activity, running less than $\frac{1}{2}$ the length of the cage |
| 2 | Bursts of activity, running $\frac{1}{2}$ -1 length of the cage |
| 3 | Activity, running > 1 length of the cage |

Stereotyped behaviour***Score**

- | | |
|---|--|
| 0 | Asleep |
| 1 | Alert but not moving |
| 2 | Gross movement (running) |
| 3 | Sniffing and licking paws |
| 4 | Persistent sniffing and licking bars of cage |
| 5 | Mock biting of bars of cage |
| 6 | Gnawing bars of cage |

* Modified from Quinton and Halliwell (1963)

CHAPTER EIGHT

THE EFFECTS ON RATS AND GUINEA PIGS OF CHRONIC
ADMINISTRATION OF AMPHETAMINE

Chapter Eight The Effects on Rats and Guinea Pigs of Chronic Administration
of Amphetamine

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The Effects on Rats and Guinea Pigs of Chronic Administration of Amphetamine

Introduction

In Chapter 6 one possible mechanism for the development of tolerance to amphetamine was discussed. In order to test this false transmitter hypothesis, the series of experiments described here was designed making the assumption that animal species which formed the false transmitter, *p*-hydroxynorephedrine from amphetamine, should develop tolerance on repeated exposure to the drug, whereas in species that were metabolically incapable of producing *p*-hydroxynorephedrine tolerance should not develop.

The rat and guinea pig were chosen to compare and contrast the development of tolerance, for, according to Dring, Smith and Williams (1970), who determined the urinary metabolites of amphetamine in these species, only the former produced the false transmitter. Corroboratory data from Costa and Groppetti (1970) and Lewander (1971a) in the rat, and Lewander (1971c) in the guinea pig show that after (+)-amphetamine, *p*-hydroxynorephedrine can only be identified in the brain and peripheral tissues of the rat and that (*vide supra*) this metabolite is responsible for the prolonged tissue depletion of noradrenaline observed in this species.

Some pharmacological effects of amphetamine are shown in Table 8:1 and of these, four were chosen for monitoring in the present study. It is seen that effects representative of each of the three main groups have been taken, namely peripheral and central effects mediated through release of noradrenaline and a central effect reputedly mediated through dopamine release. Although it is at present not possible to state with certainty that these effects are mediated through a specific neurohormone, a brief summary of the available evidence will be given.

The mechanism of the hyperthermic response to amphetamine is currently the subject of much controversy and will be discussed in greater detail in the following chapter. Hill and Horita (1971), in the rabbit, and Matsumoto and Griffin

Table 8:1. Pharmacological Effects of Amphetamine and the Respective Neurotransmitters through which these Effects are thought to be Mediated

Mediator	Peripheral	Central
Noradrenaline	Hyperthermia*	
	Cardiovascular effects	Anorexia*
	Plasma FFA	(Locomotor activity)
	Urinary noradrenaline	
Dopamine		(Hyperthermia)
		Stereotypy*
		Locomotor activity*
5-Hydroxytryptamine		(Anorexia)

* Effects monitored in current study.

(1971), in the rat, have provided evidence for a role of central dopamine release in producing the rise in temperature following amphetamine dosage. In these experiments hyperthermia was inhibited by pimozide, a 'selective' dopamine receptor blocking agent (Anden et al., 1970). However, Sulser (1974) demonstrated that pimozide was not a selective dopamine receptor antagonist because it also antagonised the cyclic-AMP response to noradrenaline in the limbic forebrain of rats, thus the case for dopamine involvement in the hyperthermic response to amphetamine is more equivocal. Nevertheless, Costa and Groppetti (1970) have also implicated dopamine in the hyperthermia by demonstrating that in rats, low doses of amphetamine, which alter the turnover of dopamine but not noradrenaline in brain, produce a rise in temperature. Much of the work, however, in rats suggests that the hyperthermia in this species is related to the peripheral release of noradrenaline. Gessa, Clay and Brodie (1969) concluded that the temperature elevation was a calorogenic effect resulting from the peripheral mobilisation of fat and that noradrenaline was involved in this mechanism. On the other hand, Matsumoto and Shaw (1971) were able to differentiate between hyperthermia and free fatty acid (FFA) release by showing that desmethylinipramine, which blocks the elevation in plasma FFA, potentiates the rise in body temperature after amphetamine.

Experiments with 6-hydroxydopamine (6-OHDA) administered by intravenous injection in doses known to deplete peripheral but not central noradrenaline (Smookler & Clarke, 1972) showed that this treatment almost completely abolished the hyperthermic response to amphetamine, which suggests that the essential mechanism is mediated through the release of noradrenaline from peripheral sympathetic neurons (see Chapter 9).

Tolerance to this hyperthermia develops on repeated administration of amphetamine in rats (Harrison, Ambrus & Ambrus, 1952; Brodie et al., 1969;

Lewander, 1971d) and the last two authors implicated *p*-hydroxynorephedrine in the development of this phenomenon.

That the administration of amphetamine causes loss in body weight was first reported by Nathanson (1937), and Harris, Ivy and Searle (1947) produced evidence that the weight loss was largely due to an anorectic effect. A predominant role of catecholamines, particularly noradrenaline, as mediators of this anorexia has been suggested by Goldman, Lehr and Friedman (1971); Leibowitz (1970); Berger, Wise & Stein, (1971) and Lewander (1971d). Frey and Schultz (1973) who showed that both α -methyl-*p*-tyrosine and disulfiram antagonise the anorexia, again point to an essential role for noradrenaline in this effect. These authors also implicate 5-hydroxytryptamine since the anorexigenic response was antagonised by *p*-chloro-phenylalanine and also by cyproheptadine in a low dose at which only tryptaminergic receptors are blocked.

Tolerance to the anorexic effect on repeated dosage with amphetamine has been shown to develop within three to five days in rats (Tormey & Lasagna, 1960; Lewander, 1971d) but although this effect may be mediated through noradrenaline release, the latter author concluded that *p*-hydroxynorephedrine was not involved in the subsequent attenuation of the response with chronic administration.

Ehrich and Krumbhaar (1937) reported that Benzedrine (dextroamphetamine) (5-20 mg/kg) caused behavioural changes in rats characterised by excessive locomotor activity and 'gnawing at the iron wires of their cages'. This latter effect is probably the first record of a form of stereotyped behaviour subsequently described and characterised by many authors including Randrup and Munkvad (1970). Stereotyped activity consists of a continuous repetition of one or a few items of behaviour which in the rat takes the form of repetitive sniffing at the bottom of the cage, accompanied by movements of the head and forelegs. Sniffing may also be associated with licking or biting of the wires of the cage. There ^{are} ~~is~~ little data on

activity recordings in the guinea pig, but at doses in excess of 15 mg/kg of amphetamine locomotor activity is increased, and repetitive purposeless small movements occur.

The involvement of central dopamine and noradrenaline release in the mechanism of the hyperactivity produced by amphetamine has been more fully discussed in the preceding chapter 1. At this point it is sufficient to summarise the evidence as follows. In the rat, reserpine (7.5 mg/kg s.c.) administered 18 hours prior to amphetamine challenge increases the behavioural stimulant effect of this drug, but α -methyl-p-tyrosine, which inhibits the ongoing biosynthesis of both dopamine and noradrenaline abolishes this. In the reserpinised animal it has been shown that amphetamine still interacts with dopamine release and metabolism as shown by an increase in the levels of methoxytyramine, the O-methylated metabolite, while the influence of noradrenaline metabolism was strongly reduced (Scheel-Krüger, 1972).

In a previous paper, (Randrup & Scheel-Krüger, 1966), it was demonstrated that diethyldithiocarbamate, which blocks the conversion of dopamine to noradrenaline, markedly reduced the gross locomotor activity produced by amphetamine, but stereotyped behaviour was unaffected. In conjunction with the evidence that DOPA reverses the effect of α -methyl-p-tyrosine (vide supra) these authors conclude that dopamine rather than noradrenaline is associated with stereotypy, while the gross motility may be more related to noradrenaline release. Further evidence supporting this is provided by Janssen et al. (1968) who showed that pimozide inhibits stereotyped behaviour, thereby implicating dopamine in this phenomenon. Croase and Iversen (1973) found that amphetamine induced stereotypy and locomotor activity were reduced in adult rats that had received 6-hydroxydopamine (6-OHDA) by cerebral intraventricular injection in the neonatal period. These rats responded normally to apomorphine, a dopamine receptor stimulant, indicating that this effect of 6-OHDA was

presynaptic in origin, which correlated well with the observed depletion of striatal tyrosine hydroxylase, which showed that at least 98% of the dopaminergic terminals were non-functional. Intraventricular injections of 6-OHDA in adult rats, however, did not block the locomotor response to amphetamine and when 6-OHDA was injected into the substantia nigra the locomotor response was normal but stereotypy inhibited. Whether, therefore, an alternative dopaminergic pathway or a noradrenergic mechanism or indeed both are responsible for mediating the gross locomotor stimulation remains to be clarified.

Information regarding the development of tolerance to locomotor stimulation and stereotyped behaviour shows certain discrepancies. After daily s.c. injections (20-30 mg/kg) of amphetamine for 1-2 weeks to rats, locomotor stimulation appeared to diminish (Ehrich & Krumbhaar, 1937), although no evidence for the development of such tolerance was reported by later workers (Tormey & Lasagna, 1959; Kosman & Unna, 1968; Lu, Ho & McIsaac, 1972). There are isolated reports that hyperactivity increases with repeated administration of amphetamine (Schuster & Zimmerman, 1961; Zieve, 1937), but it must be stressed that in none of the above studies were attempts made to discriminate, by activity monitoring, gross locomotor activity from stereotyped movements (vide infra), in addition to which, no information is available on long term administration to the guinea pig.

Experimental Procedure

A total of fifty rats and sixty guinea pigs were used in these experiments. Animals were housed in plastic cages (see Chapter 7) in groups of three-five at constant ambient temperature, and exposed to light from 0700 to 1900 hours each day. Food and water were provided ad libitum, and in some experiments the amounts were monitored.

Drugs and administration

(+)-Amphetamine sulphate was administered to rats and guinea pigs twice daily (morning and evening) by the intraperitoneal route for periods varying between 5 and 15 days. In the rat a 5 mg/kg dose produced significant changes in body temperature, activity and food intake. However, the guinea pig required a large dose (20 mg/kg) in order to produce comparable hyperthermia and locomotor stimulation and in this species, therefore, monitoring was performed at two dose levels (5 mg/kg and 20 mg/kg). Control animals of each species received an equivalent volume of saline twice daily i.p.

Chronic dosage in the rat

Rats were weighed daily at 9 a.m. and the total amount of food consumed over the previous 24 hour period was recorded for groups of 3 animals. Colonic temperatures were recorded immediately prior to and at $\frac{1}{2}$, 1, 2 and 4 hours after the first of the two daily injections of amphetamine. Animal activity was monitored at two levels (discriminating between gross locomotor stimulation and stereotyped fine movements) on animals housed singly, for the period $\frac{1}{2}$ -1 $\frac{1}{2}$ hours following amphetamine administrations. This was performed at approximately the same time each morning, in an attempt to minimise any diurnal variation in behaviour.

Chronic dosage in the guinea pig

Animals were weighed at 9 a.m., but it proved difficult to obtain measurements of food consumption in view of the pellet form in which guinea pig food is prepared, in addition to which the eating habits of this species made such measurements virtually impossible. Colonic temperatures were recorded as for rats, and animal activity was monitored at two levels for the period $\frac{1}{2}$ -2 hours after amphetamine administration on animals housed singly. Animals were tested at about the same time each morning.

Statistical analyses

Comparison of results was performed with the Student t test for unpaired data. Data were processed with a Varian Data 620/1 computer which was programmed for the above test and for the determination of regression lines by the least squares method by Mr. J.R. Idle of this department.

Results - Rat

Body temperature

Representative temperature curves following the administration of amphetamine on days 1, 4, 7 and 9 are shown in Fig. 8:1. It can be seen that throughout this period there is a progressive attenuation in the hyperthermic response. By day 9, it is apparent that the shapes of the curves are similar to those produced after administration of saline, and show no significant difference when compared statistically.

The maximum rise in temperature for each animal was determined over the 4 hour test period and the mean value obtained for each day of the experiment is shown in Fig. 8:2. The maximum rise in temperature decreased progressively over the treatment period from 2.4°C on day 1 to 0.2°C on day 9, which also suggests that tolerance develops to this effect of amphetamine. Saline treated control animals showed small variations in temperature ($\pm 0.5^\circ\text{C}$) throughout the test period.

Body weight

Data are presented in Fig. 8:3. Daily increments in body weight were recorded for each animal. When the raw data were graphically represented, plotting the mean values for weight change each day against time, it appeared that the amphetamine treated animals showed little variation in weight for the first five days, but thereafter their increase in weight paralleled that of the saline treated group.

Fig. 8:1.

Representative temperature curves following the administration of (+)-amphetamine twice daily to rats and guinea pigs.

Tolerance to Amphetamine Hyperthermia

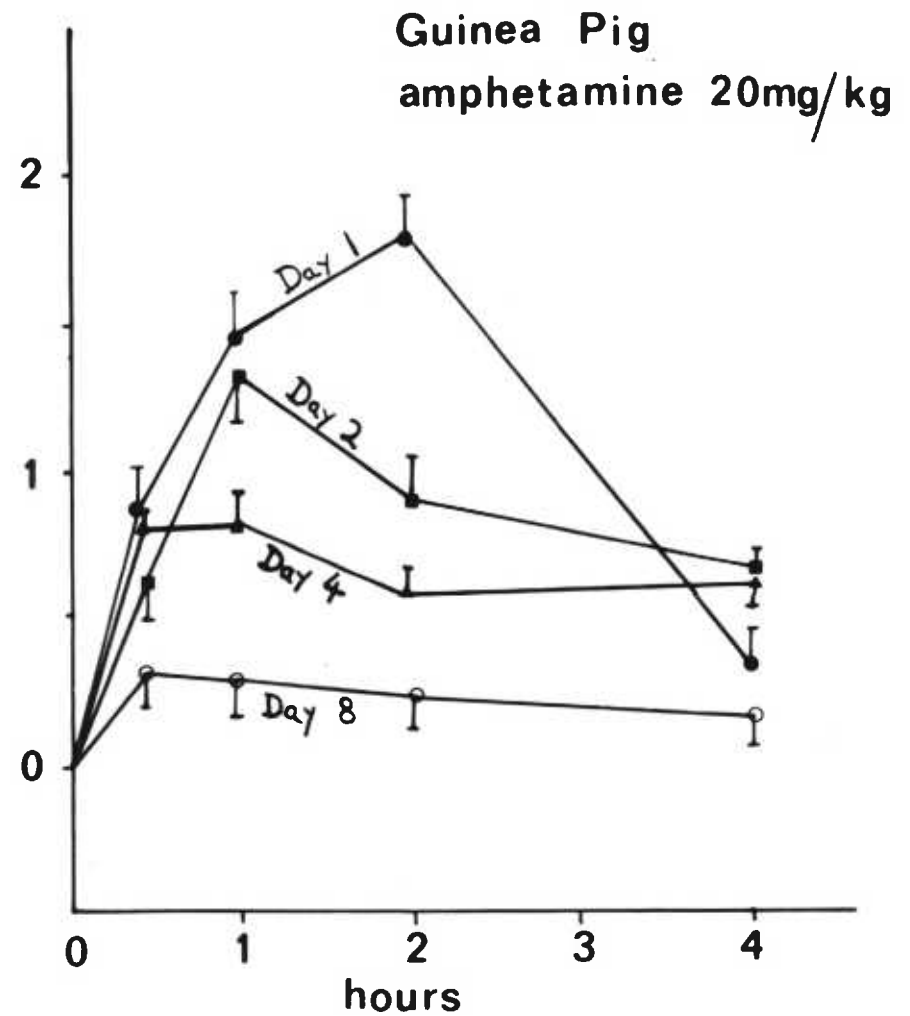
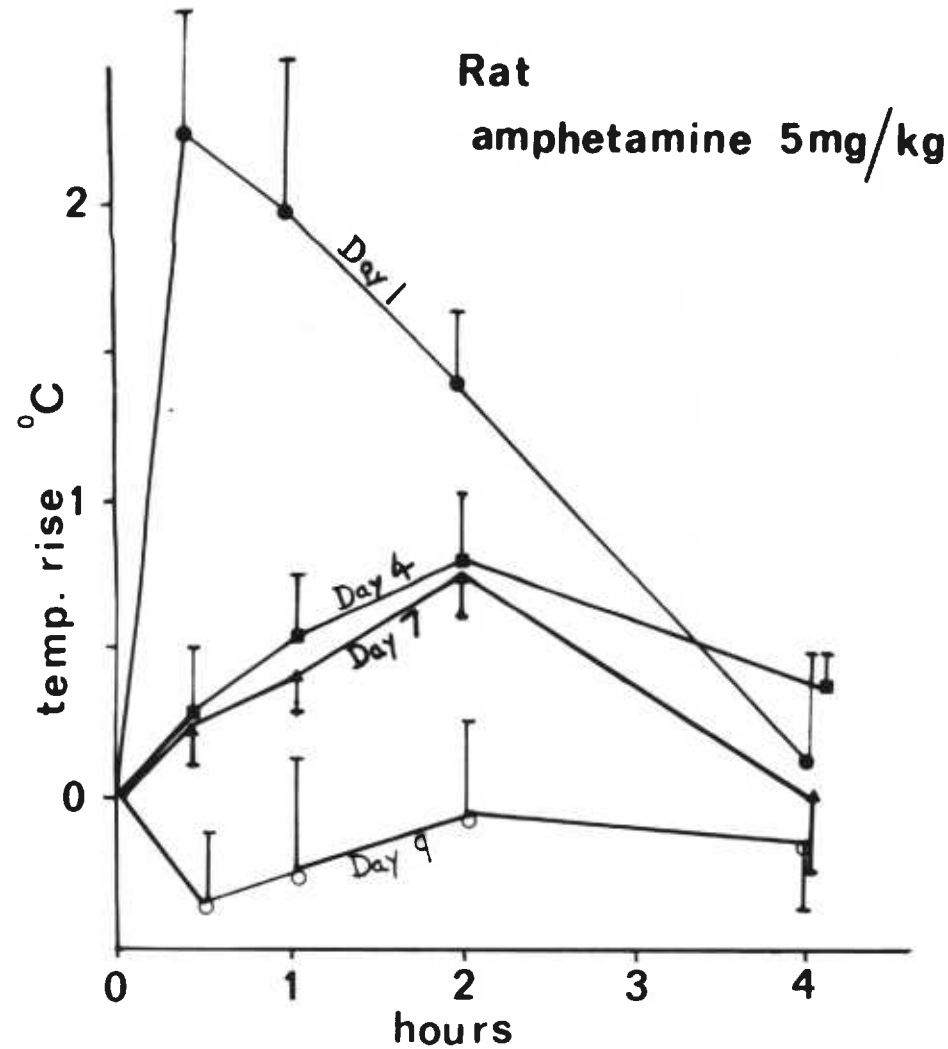


Fig. 8:1

Representative temperature curves (+ S. E.) following the administration of
(+)-amphetamine twice daily to rats and guinea pigs.

For Rats:

n = 8 (amphetamine treated)

n = 6 (saline treated)

Comparing Days 1, 4 and 7, amphetamine treated with respective saline
controls, $P < 0.01$

Comparing Day 9 amphetamine treated with saline control, P is not significant.

For Guinea Pigs:

n = 5 (amphetamine treated)

n = 5 (saline treated)

Comparing Days 1, 2 and 4, amphetamine treated with saline controls,
 $P < 0.01$

Comparing Day 8 amphetamine treated with saline control, P is not significant.

Fig. 8:2. Mean maximum temperature rise following administration of (+)-amphetamine twice daily to rats and guinea pigs.

Tolerance to Amphetamine Hyperthermia

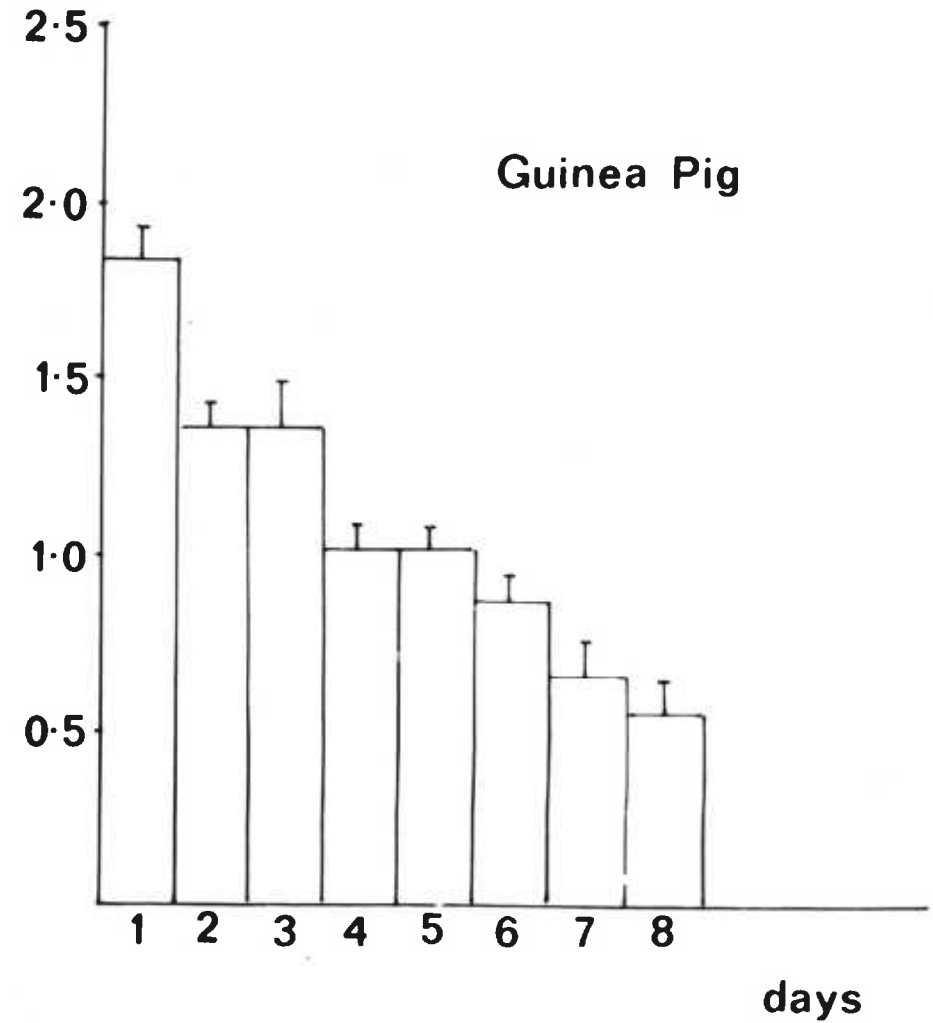
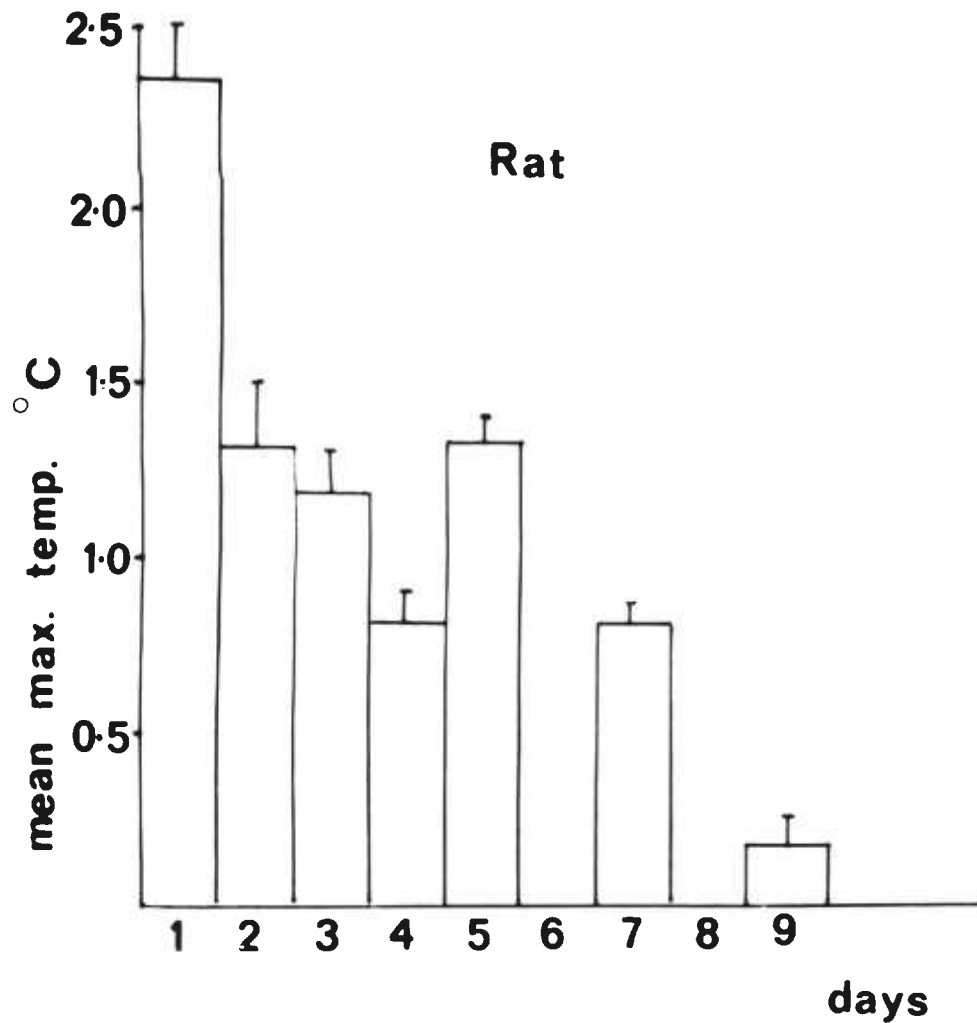


Fig. 8:2

Mean maximum temperature rise ($\pm$ S. E.) following administration of

(+)-amphetamine twice daily to rats and guinea pigs. Dose - see Fig. 8:1.

For Rats:

n = 4-8 (amphetamine treated)

n = 3-6 (saline treated)

Comparing values for days 1-7 amphetamine treated with respective saline controls, $P < 0.05$

Comparing values for day 9 amphetamine treated with saline controls,
P is not significant.

For Guinea Pigs:

n = 5 (amphetamine treated)

n = 5 (saline treated)

Comparing values for days 1-6 amphetamine treated with saline controls
 $P < 0.01$

Comparing values for day 8 amphetamine treated with saline control,
P is not significant.

Fig. 8:3

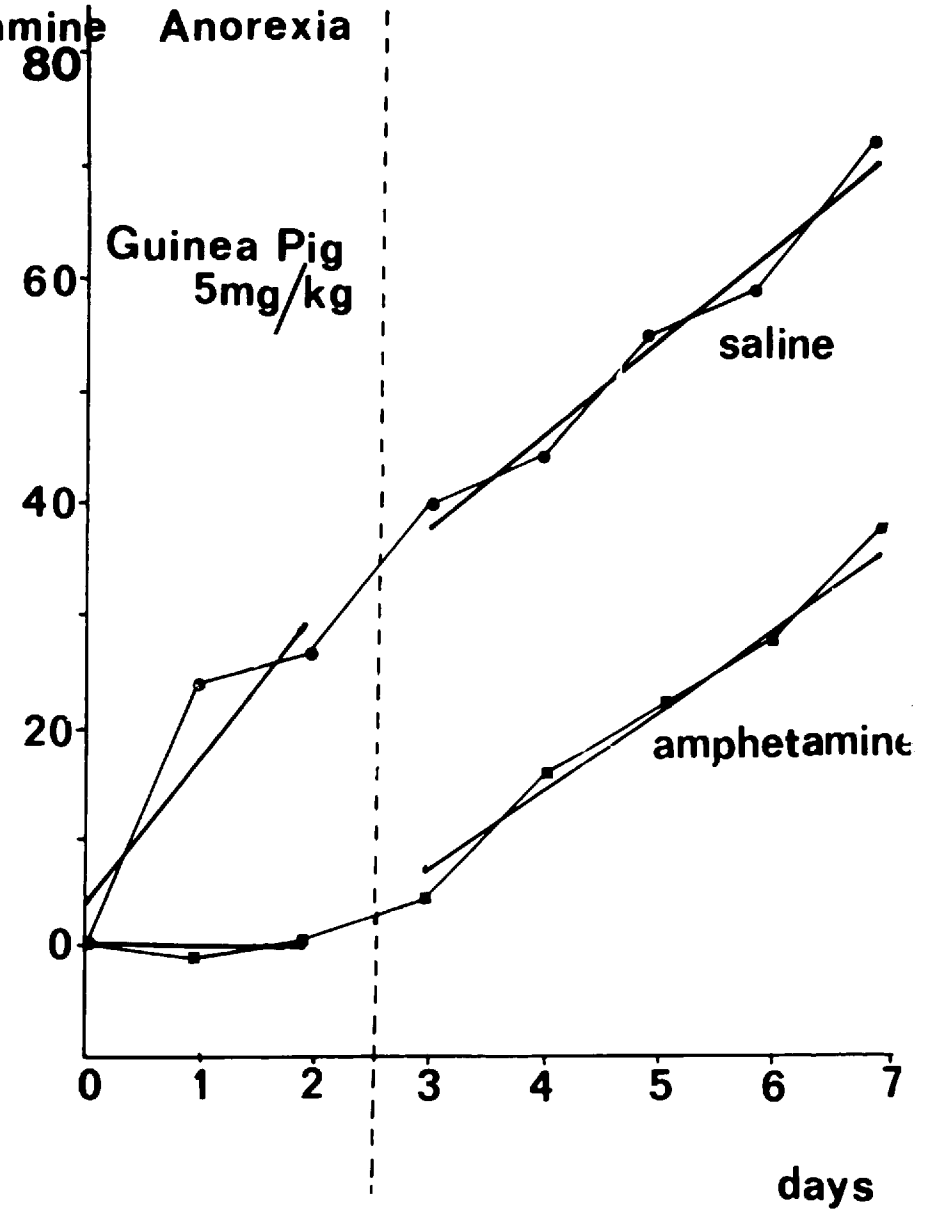
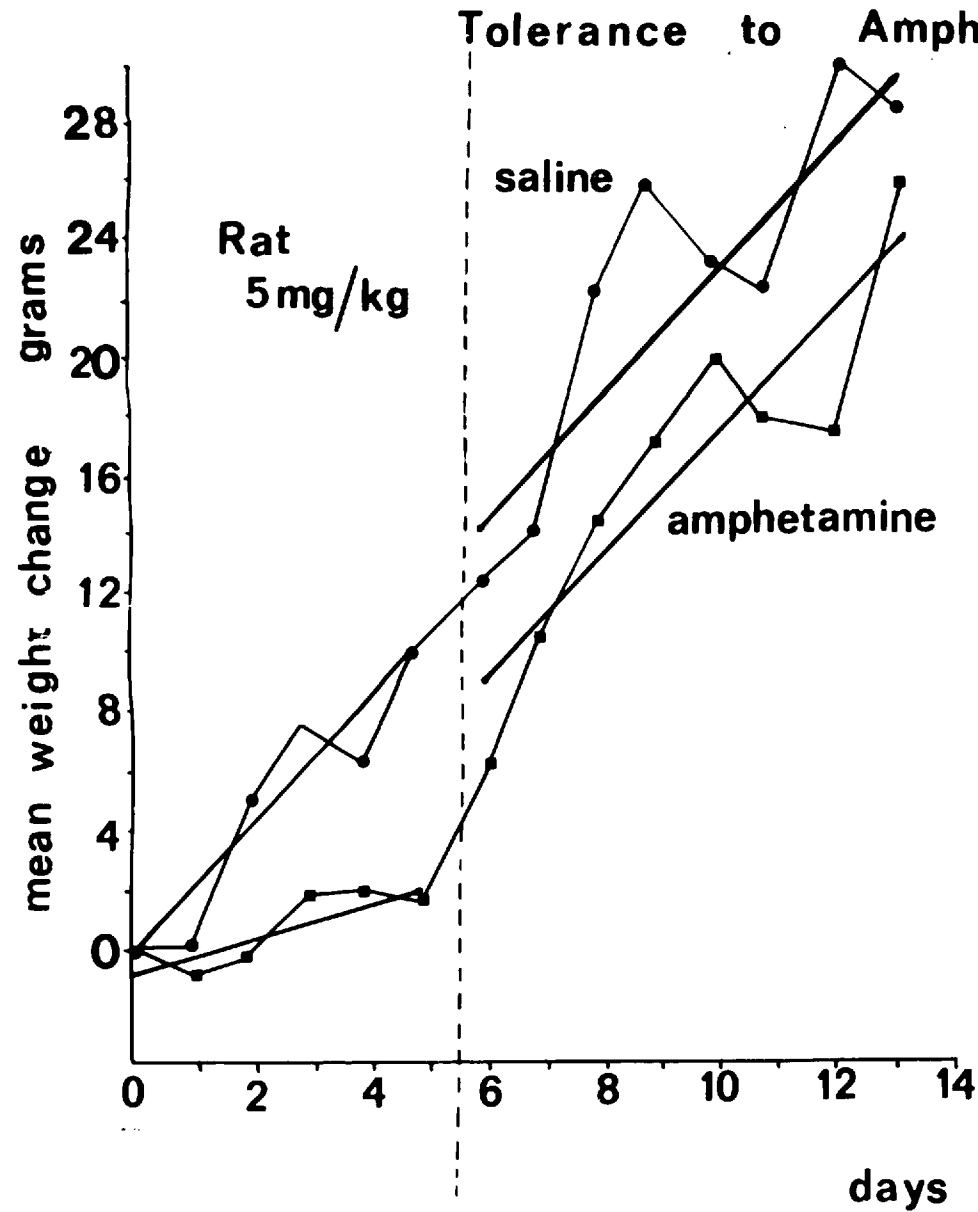


Fig. 8:3

Mean daily weight change in rats and guinea pigs receiving (+)-amphetamine,
5 mg/kg twice daily compared with saline treated controls.

For Rats:

n = 12 (amphetamine treated)
n = 12 (saline treated)

Comparing the slopes of regression lines constructed from data derived
from amphetamine treated rats with saline controls

Day 0-5 $P < 0.0005$

Day 6-14 P is not significant.

For Guinea Pigs:

n = 15 (amphetamine treated)
n = 15 (saline treated)

Comparing the slopes of the regression lines

Day 0-2 (amphetamine versus saline) $P < 0.0005$

Day 3-7 (amphetamine versus saline) P is not significant.

Regression lines were therefore calculated for the two groups over the time periods 0-5 days and 6-13 days. During the first period there was a significant difference between the two groups but during the latter period 6-13 days, the weight increase was very similar for both amphetamine and saline treated animals.

Food intake

Data are presented in Table 8:2. The mean daily food intake by rats dosed with saline shows little variation during the course of the experiment (55 g per 3 rats, range 49-61 g), and there is no significant difference between the food intake from days 1-5 and days 6-13.

Amphetamine treated rats consume considerably less food during the first 5 days of treatment (mean 39 g per 3 rats) although there is a gradual increase in daily food intake throughout this period. During the second period (days 6-14), despite daily variations, food intake remains fairly steady (48 g, range 41-54). When these two treatment periods are compared there is a highly significant difference between food consumption during days 1-5 and days 6-14.

The experimental data presented suggest, therefore, that tolerance develops to the anorectic effect of amphetamine if the parameters of weight gain and food intake can be taken as true measurements of anorexia.

Animal activity

In a preliminary study a small group of three rats were monitored on alternate days for 15 days (Fig. 8:4). Despite considerable inter-individual variations in the behavioural response to amphetamine there was a trend for gross locomotor activity to decline during the early stages of the treatment period, which did not, however, achieve statistical significance.

Repeated investigations were therefore undertaken with a group of twelve rats which were treated as previously described and monitored daily for 4 days after an

Table 8:2. Mean Daily Food Intake by Four Groups of Three Rats Chronically Treated with (+)-Amphetamine 5 mg/kg or Saline Twice Daily

The values quoted were obtained after pooling data from days 1 to 5 and from days 6 to 14 inclusive, $\pm$ S.E.

<u>Treatment</u>	<u>Days 1-5</u>	<u>Days 6-14</u>
	<u>Mean Food Intake per</u> <u>3 rats per day (g)</u>	<u>Mean Food Intake per</u> <u>3 rats per day (g)</u>
Amphetamine	39.3 $\pm$ 1.2	47.8 $\pm$ 1.3
Saline	54.7 $\pm$ 1.5	55.2 $\pm$ 1.1

Comparing days 1-5 with days 6-14 for amphetamine treated animals, $P < 0.001$

Comparing days 1-5 with days 6-14 for saline treated animals, P is not significant.

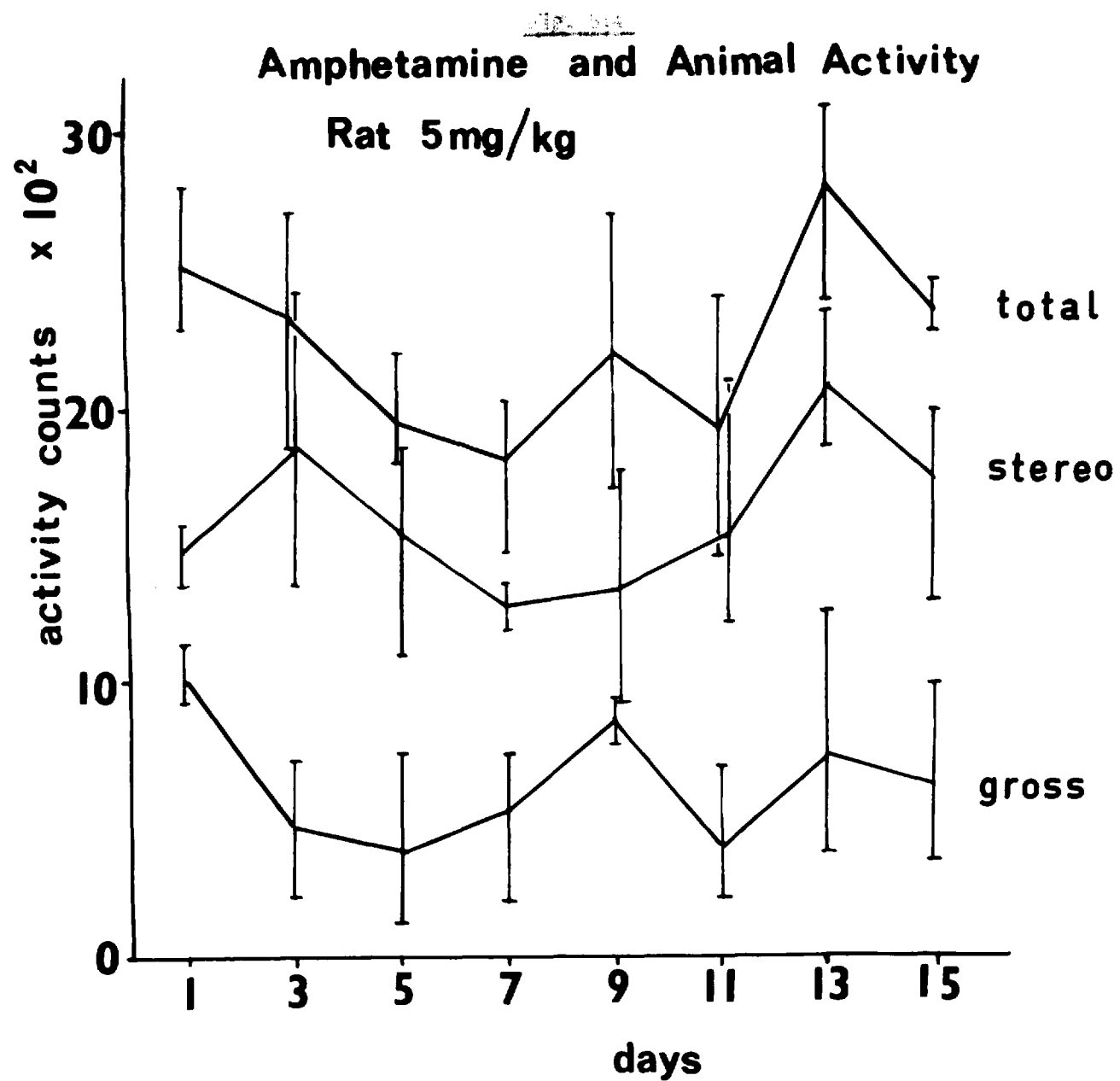


Fig. 8:4

Electronic animal activity monitoring in a preliminary study of 3 rats receiving 5 mg/kg (+)-amphetamine twice daily. Activity counts ($\pm$ S.E.) are given for total, gross and stereotyped movements. For details see text.

Statistically significant changes were not achieved.

initial (day 0) period of acclimatisation to the test environment following a saline challenge (0.5 ml i.p.). For further confirmation of behavioural effects animals were scored with a scale modified from that of Quinton and Halliwell (1963). In these studies (Fig. 8:5) it was apparent that when activity was separated into the two components, over the treatment period, measurements of gross locomotor activity showed a progressive fall with repeated dosage. Significant diminution in activity was achieved when days 2, 3 and 4 were compared with day 1. Surprisingly, stereotyped monitored activity showed a gradual but significant increase with dosage such that total activity counts were unchanged over the 4 day period. There was excellent correlation between visual and monitored activity for gross movements, however, the visual scores for stereotypy did not show the same upwards trend as did the monitored data.

Results - Guinea Pig

Body temperature

Representative temperature curves following the administration of amphetamine on days 1, 2, 4 and 8 are shown in Fig. 8:1. As in the comparable experiment with rats, there is a progressive attenuation in the hyperthermia, and again by day 8 there is no significant difference in the temperature curve following the administration of amphetamine when compared with that from saline treated animals. The mean maximum rise in temperature over the 4 hour test period on each day is shown in Fig. 8:2. On day 1 the maximum rise produced by amphetamine was 1.8°C and this progressively diminished to 1.0°C on day 4 and 0.5°C on day 8. These results show, therefore, that tolerance develops to the hyperthermic effect of amphetamine in this species and indeed resembles closely the tolerance seen in the rat.

Body weight

At a dose of 20 mg/kg (+)-amphetamine animals lost weight rapidly over the

Fig. 8:5

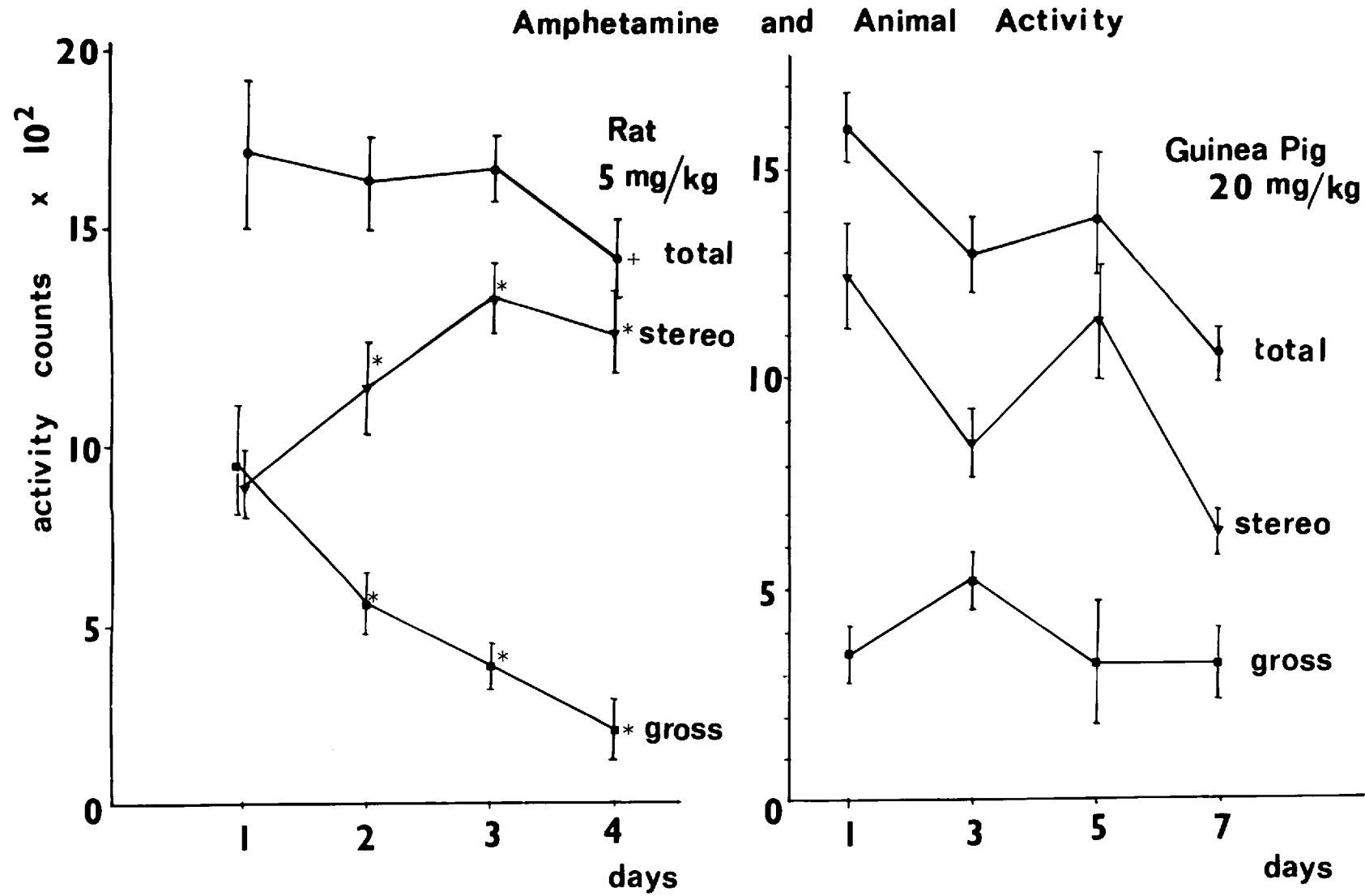


Fig. 8:5Electronic activity monitoring in rats and guinea pigs receiving (+)-amphetamine
twice daily

Activity counts $\pm$ S.E. are given for total, gross and stereotyped movements.
For details see text.

For Rats:

n = 12

The total counts recorded for saline treated controls (n = 12) were 160 ± 60

* $P < 0.01$ when compared with day 1

+ $P < 0.05 > 0.01$ when compared with day 1

For Guinea pigs:

n = 5

The total counts recorded for saline controls (n = 5) were 230 ± 40

When day 5 values were compared with day 1, P was not significant.

(Statistical analysis was not applied to values obtained on day 7, see text).

first 2 days, and thereafter continued to lose weight, albeit more slowly. When animals were dosed with 5 mg/kg (+)-amphetamine twice daily, body weight remained stable for the first 2 days but thereafter rapidly increased and resembled the pattern observed in saline treated animals. Data are shown in Fig. 8:3.

In view of the similarity of these results to those obtained in the rat experiment, the data were processed in the same way, regression lines being derived from body weight values for days 0-2, and days 3-7. During the first 2 days the amphetamine treated animals gained significantly less weight than the saline treated controls, however, during the 3-7 day period, the weight gain per day for the two groups did not differ significantly, suggesting that tolerance had developed to this effect of amphetamine.

Animal activity

Data are shown in Fig. 8:5. It was apparent that during the first 5 days of treatment, no change occurred in either gross locomotor activity or stereotyped behaviour. On day 7, the final day of treatment, total counts for the measurement were diminished at both levels, but a closer examination of the 10 minute counts showed that there was a progressive fall in counts during the 1-2 hour period, which correlated with an observed deterioration in the general condition of these animals. Following the test period on this day three of the five guinea pigs died and the experiment was therefore terminated.

Discussion

The results presented here show that in both species tolerance develops to some effects of amphetamine but not to others. For the rat, the rapid acquisition of tolerance to the hyperthermia and anorexia confirms similar reports in the literature (Harrison, Ambrus & Ambrus, 1952; Tormey & Lasagna, 1960; Lewander, 1971g). The data on gross locomotor activity suggest that tolerance develops to this

effect in rats with repeated dosage of amphetamine, contrasting with stereotyped behaviour, which increases on chronic treatment. The results of these activity studies are at variance with other workers who report that tolerance does not develop to the locomotor stimulant effect of amphetamine (see introduction). One possible explanation for this finding is that, in the present study, careful attempts have been made to discriminate between gross locomotor (exploratory) movements and stereotyped activity, and the increase in the latter fully compensated for the decrease in gross activity.

It will be appreciated that some experiments undertaken in guinea pigs were performed at higher dose levels in order to obtain satisfactory pharmacological and behavioural responses. In this species the disposition of amphetamine following administration by the intraperitoneal (and presumably oral) route differs from that found in the rat. According to Lewander (1971c) amphetamine is successively absorbed from the peritoneal cavity during the first two hours reaching a peak plasma level at 2 hours after injection which contrasts with a peak plasma level at 15 mins. in rats. In addition, the disappearance curves for plasma in guinea pig show a $t_{1/2}$ of 2.5-3.1 hours compared with 40 mins. in the rat.

Thus, in guinea pigs it was found necessary after certain preliminary experiments to measure animal activity and body temperature following a dose of 20 mg/kg (+)-amphetamine, whereas for anorexia a dose of 5 mg/kg twice daily was sufficient to demonstrate the acute effect. In this species tolerance developed rapidly to both hyperthermia and anorexia, however, data on both gross locomotor activity and stereotypy showed little change with repeated dosage.

How, then, can these results be reconciled with the false transmitter hypothesis? The results presented are in accord with the proposal that tolerance might be expected to develop to those effects of amphetamine mediated via

noradrenergic neurons in both periphery and brain. The only exception in the current study was the failure of guinea pigs to become tolerant to gross locomotor activity, and since dopamine has been suggested as the neurotransmitter responsible for stereotypy, it was not surprising that tolerance did not develop to this effect.

The implication that the false neurotransmitter, *p*-hydroxynorephedrine is involved in the persistent noradrenaline depletion and the development of tolerance to noradrenergic effects of amphetamine on chronic dosage is difficult to believe in the light of the results in the guinea pig, since the formation of *p*-hydroxynorephedrine is not a significant metabolic route in this species. Most authors (see Chapter 4) have failed to identify *p*-hydroxynorephedrine as a metabolite of amphetamine in guinea pigs, and although small amounts were found in the urine in experiments presented in this thesis, Lewander (1971c) was unable to identify this metabolite in either brain or heart after administration of amphetamine. Thus, although chronic dosage of amphetamine in both species causes a marked depletion of tissue catecholamines, noradrenaline and dopamine (which appears to be a prerequisite for the development of tolerance) in the guinea pig the false transmitter, *p*-hydroxynorephedrine, cannot be implicated and alternative mechanisms must be involved. Since the temporal development of tolerance is very similar in both species, it may be that even in the rat false transmitters are not essential for this phenomenon.

A less likely suggestion is that in the guinea pig, the *p*-hydroxylated metabolite, norephedrine, could play the role of the false transmitter. Such a function for this compound was suggested by Iversen (1967), however, although Thoenen et al. (1966) found this metabolite of amphetamine in cat spleen, it was not identified in the perfusate on nerve stimulation. These authors, however, pointed out that the quantity of norephedrine present in the spleen was very small, and the amount possibly liberated may have escaped detection. Nevertheless, the criteria laid down by Kopin for false neurotransmitter function cannot at present be claimed for this metabolite.

The extrapolation of information derived from animal experiments to the human situation is of course hazardous. A major difficulty encountered previously, has been the apparent lack of correlation between the tolerance acquired in man to the euphoriant effects of amphetamine and the failure to demonstrate tolerance to the central effects of this drug in animals. However, the results on motor activity in the rat may shed a little light on this problem. If the euphoriant effect of amphetamine in man is mediated through noradrenaline release (a not unreasonable suggestion when the contrasting pattern of behaviour, depression, may be associated with impaired noradrenergic mechanisms; Bunney & Davis, 1965) and the psychotic behavioural patterns associated with stereotypy mediated through dopamine release, the animal correlates of these two effects would appear to be gross exploratory locomotor activity (noradrenergic) and stereotypy (dopaminergic), with tolerance developing only to the former. A fuller discussion on the possible importance of the finding of an increase in dopamine mediated effect with repeated dosage is given in the final chapters.

CHAPTER NINE

PRETREATMENT STUDIES

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Pretreatment Studies

a) The Mechanism of Amphetamine Hyperthermia

Introduction

In the latter part of this chapter a series of experiments will be described in which precursors of the false noradrenergic neurotransmitter, *p*-hydroxynorephedrine, have been given to rats, which were then challenged with amphetamine. If *p*-hydroxynorephedrine is incorporated into the noradrenergic granules with replacement of noradrenaline, and is released by a subsequent dose of amphetamine, the resultant pharmacological effect will be less marked than that produced by amphetamine alone, provided the pharmacological response monitored is indeed mediated through the release of noradrenaline.

In the rat, a dose dependent hyperthermia of some 3-4 hours duration follows the administration of amphetamine, and although much evidence supports the hypothesis that this is due to the release of noradrenaline, controversy exists as to whether other neurotransmitters such as dopamine and 5-HT may be involved, or indeed whether this effect of amphetamine, if noradrenergic, is central or peripheral in origin. Previous work on the mechanism of the hyperthermia is discussed in Chapter 8.

In order to elucidate this mechanism further, rats were subjected to a range of pretreatment schedules designed to modify individual neurotransmitters in both central and peripheral nervous systems and subsequently challenged with amphetamine.

Experimental Procedure

A total of 160 male and female Wistar albino rats were used. Housing, food, control of environmental temperature and recording of colonic temperature have already been described (Chapter 8). Table 9:1 gives details of the various

pretreatment schedules, and the animals were subsequently challenged with (+)-amphetamine sulphate (5 mg/kg i.p.) or an equivalent volume of saline. Body temperature was recorded immediately prior to, and at 1 hour after the challenging injection. The results are expressed as the mean temperature rise after amphetamine, and statistical analysis was by the Student *t*-test for unpaired data.

Results

The results are given in Table 9:1. α -Methyl-*p*-tyrosine, which inhibits the ongoing biosynthesis of both dopamine and noradrenaline in the dose administered (Buethin *et al.*, 1972), totally abolished the hyperthermia produced by amphetamine. Peripheral sympathectomy, which was achieved by a single i.v. injection of 6-OHDA, similarly attenuated the rise in temperature on subsequent amphetamine challenge, whereas when desmethylinipramine was administered prior to 6-OHDA, a drug combination which selectively depletes peripheral dopamine (Breese, Cooper & Smith, 1974), the combination had no effect on the amphetamine induced hyperthermia. Ganglionic blockade with tetraethylammonium bromide was ineffective in preventing the rise in temperature suggesting that this effect of amphetamine is postganglionic, and finally, the depletion of central serotonin with *p*-chloroamphetamine (Miller *et al.*, 1970) failed to modify the hyperthermia, indicating that serotonin is not involved in this effect of amphetamine.

None of the above pretreatment schedules altered baseline temperatures, and when the challenging agent was saline, the maximum rise in temperature recorded after any of the pretreatment regimes was 0.4°C.

Discussion

On the basis of the results presented, it would appear that intact peripheral noradrenergic neurons are a prerequisite for the hyperthermic action of amphetamine in the rat. The mechanism by which this hyperthermia is produced may differ in

Table 9:1. Influence of Various Pretreatments Designed to Impair Nerve Function on Amphetamine-Induced Hyperthermia

Values are mean maximum temperature rise after challenge in each group, $\pm$ S. E. M. n is given in parentheses

<u>Pretreatment</u>	<u>Effect of pretreatment</u>	Mean maximum temperature rise in:			
		<u>Female challenged with</u>		<u>Male challenged with</u>	
		<u>Amphetamine</u>	<u>Saline</u>	<u>Amphetamine</u>	<u>Saline</u>
α -Methyl-p-tyrosine methyl ester (150 mg/kg i. p. 2 x daily for 3 days; last dose 12 h prior to challenge)	inhibits catecholamine biosynthesis	0.1 $\pm$ 0.1 (8)*	0.1 $\pm$ 0.1 (5)	0.1 $\pm$ 0.1 (5)*	0.1 $\pm$ 0.2 (5)
Saline as above		2.0 $\pm$ 0.3 (6)	-	2.1 $\pm$ 0.1 (10)	-
6-Hydroxydopamine (100 mg/kg i. v. 48 h prior to challenge)	depletes peripheral catecholamines	0.8 $\pm$ 0.3 (7)* ⁺	0.2 $\pm$ 0.1 (6)	0.5 $\pm$ 0.2 (5)* ⁺	0.0 $\pm$ 0.0 (5)
Saline as above		2.2 $\pm$ 0.2 (10)	-	2.0 $\pm$ 0.3 (7)	-
Desmethylinipramine (15mg/kg i. p.) followed 30 min. later by 6-hydroxydopamine (100 mg/kg i. v.); challenge given 48 h later.	depletes peripheral dopamine	1.9 $\pm$ 0.2 (5) ⁺	0.4 $\pm$ 0.2 (5)	2.4 $\pm$ 0.7 (4) ⁺	0.4 $\pm$ 0.1 (5)
Saline as above		2.4 $\pm$ 0.3 (10)	-	2.1 $\pm$ 0.2 (8)	-
p-Chloroamphetamine (3 mg/kg i. p.) 72 h prior to challenge	depletes central serotonin	2.4 $\pm$ 0.3 (5) ⁺	0.3 $\pm$ 0.2 (5)	2.1 $\pm$ 0.2 (5) ⁺	0.3 $\pm$ 0.1 (6)
Saline as above		2.3 $\pm$ 0.1 (6)	-	2.0 $\pm$ 0.1 (8)	-
Tetraethylammonium bromide (30 mg/kg i. p.) 30 min prior to challenge	ganglion blocker	2.7 $\pm$ 0.1 (6) ⁺	0.6 $\pm$ 0.1 (3)	2.4 $\pm$ 0.5 (6) ⁺	0.4 $\pm$ 0.1 (5)
Saline as above		2.4 $\pm$ 0.2 (6)	-	2.1 $\pm$ 0.1 (9)	-

* P < 0.01 compared with saline pretreated amphetamine challenge control. ⁺ P < 0.01 compared with similarly pretreated saline challenge control

other species for in the mouse peripheral sympathectomy with 6-OHDA does not influence the amphetamine hyperthermia (Wolf & Bunce, 1973). Evidence that central dopamine release is required for amphetamine to evoke this effect in the rabbit is provided by Hill and Horita (1971) (see Chapter 8), and although a similar conclusion has been reached by Matsumoto and Griffin (1971) in the rat, the latter authors give neither an indication of the numbers of animals tested nor statistical analysis of their results. Whether the rise in body temperature after amphetamine results from both central dopamine and peripheral noradrenaline release is therefore uncertain.

b) The Effects of the False Transmitter, p-Hydroxynorephedrine and its Precursors p-Hydroxyamphetamine and Norephedrine, on Amphetamine Hyperthermia

Introduction

It has been reported previously that p-hydroxyamphetamine is able to confer a degree of protection on rats against certain effects of amphetamine (Lewander, 1971d) and this has been attributed to the accumulation of the metabolite of p-hydroxyamphetamine, namely p-hydroxynorephedrine which can act as a false transmitter (see Chapter 3). In the course of a further investigation of this protective effect, the two precursors of p-hydroxynorephedrine, p-hydroxyamphetamine and norephedrine (see Table 9:2), and p-hydroxynorephedrine itself were administered to rats prior to amphetamine challenge. The observed sex difference in the ability of these drugs to protect rats against the hyperthermic effect of amphetamine is discussed in relation to possible sex differences in drug metabolism. In addition, female guinea pigs were also pretreated with either p-hydroxyamphetamine or p-hydroxynorephedrine and the hyperthermic response to subsequent amphetamine administration determined.

Table 9:2. The Metabolic Formation of p-Hydroxynorephedrine from Amphetamine and its Derivatives in Male and Female Rats, *in vivo*

<u>% ¹⁴C dose:</u>				
<u>Compound</u>	<u>Sex</u>	<u>in 0-24 h urine</u>	<u>as p-hydroxy-norephedrine</u>	<u>Source</u>
Amphetamine	M	-	-	(Dring, Smith & Williams, 1970)
	F	79	0.3	
p-Hydroxyamphetamine	M	92	2	(Chapter 3, this thesis)
	F	84	3	
Norephedrine	M	84	27	(J. Caldwell, unpublished data) (Sinsheimer, Dring & Williams, 1973).
	F	92	28	

The significance of the findings in these experiments in relation to the false transmitter hypothesis is outlined.

Experimental Procedure

A total of 105 female and male rats and 40 female guinea pigs were used. The experimental procedure was similar to that already described for pretreatment studies. Drugs were given by the i. p. route 20 hours prior to challenge with amphetamine. This time interval was chosen specifically with respect to p-hydroxyamphetamine, since Lewander (1971b) demonstrated in the rat that 20 hours following the injection of p-hydroxyamphetamine the only metabolite identifiable in the brain and peripheral tissues was p-hydroxynorephedrine. This author concluded, therefore, that only p-hydroxynorephedrine could be responsible for the observed attenuation in the hyperthermic response to subsequently administered amphetamine.

Results

The results are summarised in Table 9:3, Figs. 9:1 and 9:2, which show that while the temperature rise after amphetamine is considerably reduced in male rats, pretreated with p-hydroxyamphetamine, this is not the case in the females. In both the Wistar albino and Sprague-Dawley strains there is a statistically significant difference between the temperature rise in male rats pretreated with saline and those pretreated with p-hydroxyamphetamine. However, there is no significant difference between the two values obtained in similarly pretreated female rats. In addition, the temperature rise after amphetamine in p-hydroxyamphetamine pretreated male rats is significantly less than in similarly pretreated females.

Although a strain difference may be apparent in that male Wistar albino rats show a small temperature rise when amphetamine is given after p-hydroxyamphetamine compared with male Sprague-Dawley rats in which the hyperthermia is totally abolished by pretreatment, the sex difference is extremely clear cut.

Fig. 9:1. The effect of p-hydroxyamphetamine pretreatment on amphetamine hyperthermia in the rat.

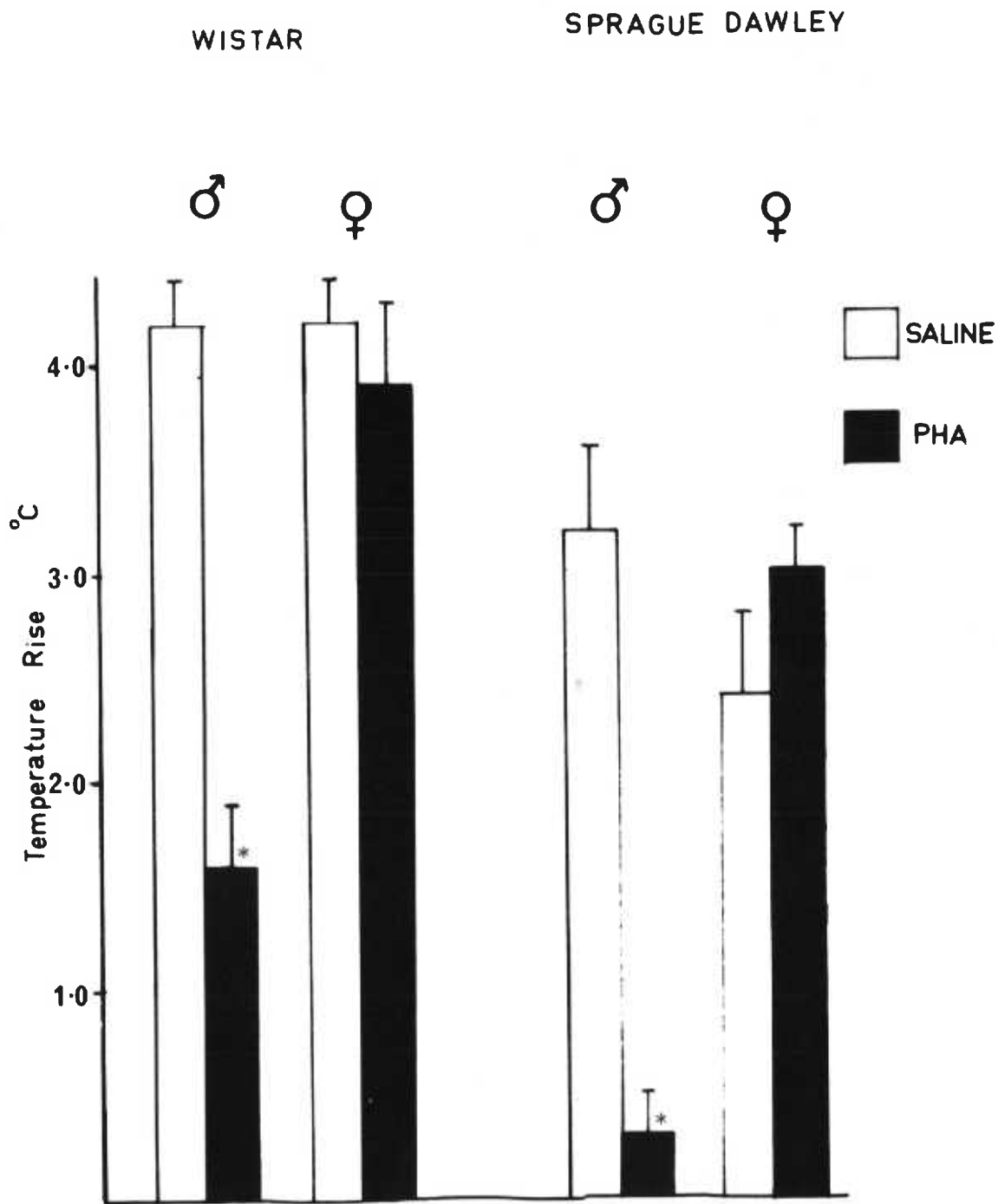


Fig: 9:1

The effect of p-hydroxyamphetamine pretreatment on amphetamine hyperthermia
in the rat

Rats were pretreated with p-hydroxyamphetamine (PHA) (40 mg/kg, i. p.) or saline (0.5 ml i. p.) 20 h prior to challenge with (+)-amphetamine (20 mg/kg i. p.)

Columns represent the mean rise in colonic temperature ($\pm$ S. E.) in 1 h after amphetamine. n = 5 or 6 for each group.

* Compared with saline pretreated male $P < 0.001$

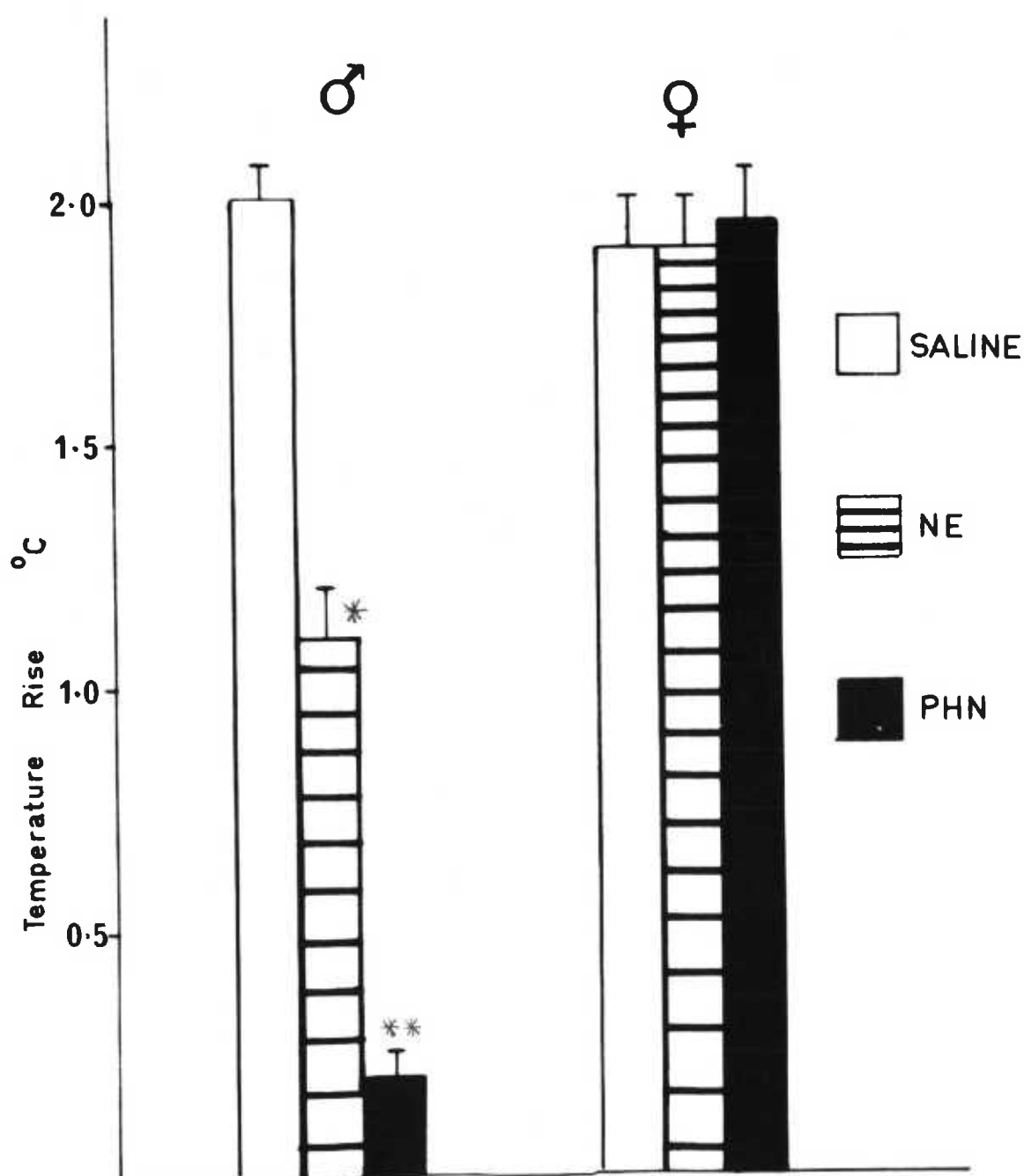
Compared with PHA pretreated female $P < 0.001$

Control animals of both sexes and strains were injected with saline after either saline or PHA pretreatment as described in the text showed a temperature change in 1 h after the challenge of $\pm 0.3^{\circ}\text{C}$.

Fig. 9.2 The effect of norephedrine and p-hydroxynorephedrine pretreatment on amphetamine hyperthermia in the rat

Rats were pretreated with norephedrine (NE) (15 mg/kg i.p.), p-hydroxynorephedrine (PHN) (5 mg/kg i.p.) or saline (0.5 ml i.p.) 20 h prior to challenge with (+)-amphetamine (5 mg/kg i.p.) (n = 10-13 for each group)

Columns represent the mean rise in colonic temperature ($\pm$ S.E.) in 1 h after amphetamine.



* Compared with saline pretreated male $P < 0.01$
 Compared with NE pretreated female $P < 0.05$

** Compared with saline pretreated male $P < 0.001$
 Compared with PHN pretreated female $P < 0.001$

Control animals injected with saline after saline, PHN or NE pretreatment showed a temperature change in 1h after the challenge of $\pm 0.3^{\circ}\text{C}$.

When control animals of both sexes and strains were injected with saline after either saline or *p*-hydroxyamphetamine pretreatment, as described, the temperature rise after the challenge was 0.3°, which was not a significant change in any case examined.

Although no rats died during the 1 hour measurement period, in many of the experiments where the animals were challenged with amphetamine, deaths occurred 1-4 hours after dosing (see Table 9:3). It is apparent that while *p*-hydroxyamphetamine pretreatment protected male rats against the lethal effects of this dose of amphetamine, similarly pretreated female rats died, as did control animals. No deaths occurred in rats of either sex or strain challenged with saline after either of the pretreatments.

The results for experiments in which either norephedrine or *p*-hydroxynorephedrine were administered prior to amphetamine are similar to those already described for *p*-hydroxyamphetamine (Fig. 9:2). Again, male animals are partially protected by pretreatment, whereas females are not. For male rats the hyperthermia is significantly attenuated compared with saline pretreated controls (for both *p*-hydroxynorephedrine and norephedrine), but in females there is no statistically significant difference in the temperature rise produced by amphetamine after either pretreatment drug. In female guinea pigs neither *p*-hydroxyamphetamine nor *p*-hydroxynorephedrine afforded any protection against the hyperthermia produced by amphetamine (Fig. 9:3). The temperature rise of ca. 2°C in this species following a dose of 20 mg/kg (+)-amphetamine was not modified by either pretreatment.

Discussion

The hypothesis that the accumulation of *p*-hydroxynorephedrine, a metabolite of amphetamine, might be involved in the tolerance to amphetamine as a false transmitter in peripheral and central noradrenergic neurons, was tested by

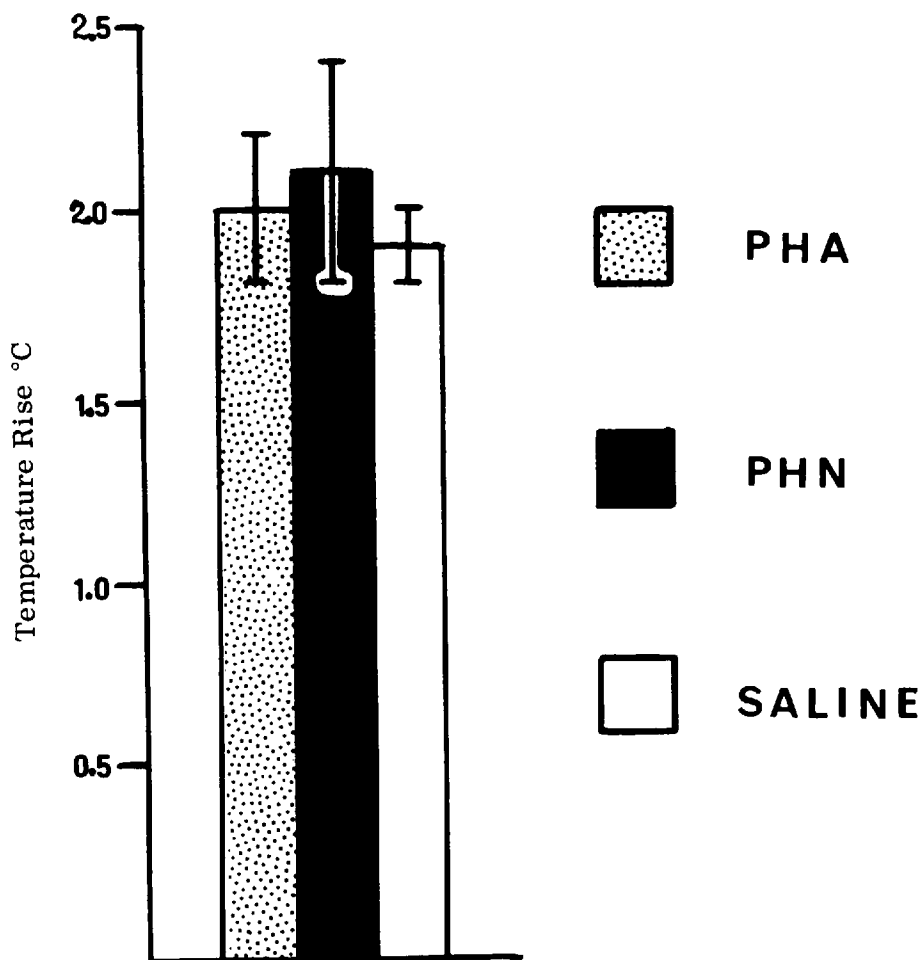
Table 9:3. Mortality in p-Hydroxyamphetamine Pretreated Rats Challenged with Amphetamine

Doses as for Figure 9:1.

		<u>Pretreatment</u>	
		<u>Saline</u>	<u>p-Hydroxyamphetamine</u>
Wistar albino	Male	3/5	0/5
	Female	6/6	3/6
Sprague-Dawley	Male	5/6	0/6
	Female	2/5	4/5

Fig. 9:3

PROTECTION AGAINST AMPHETAMINE HYPERTHERMIA BY
p-HYDROXYNOREPHEDRINE PRECURSORS - FEMALE GUINEA PIG



(Continued overleaf)

Fig. 9:3. Protection against amphetamine hyperthermia by p-hydroxynorephedrine precursors - female guinea pig

Guinea pigs were pretreated with p-hydroxyamphetamine (PHA) (40 mg/kg i.p.), p-hydroxynorephedrine (PHN) (5 mg/kg i.p.) or saline (0.5 ml i.p.) 20 h prior to challenge with (+)-amphetamine (20 mg/kg i.p.)

n = 5 for each group

Columns represent mean maximal temperature rise occurring up to 4h after amphetamine ($\pm$ S. E.)

No significant differences were recorded for either pretreatment group when compared with saline pretreated animals.

Control guinea pigs received a saline challenge after pretreatment with saline, PHN or PHA and the temperature from 0.4 h after injection was $\pm 0.5^{\circ}\text{C}$.

pretreating rats and guinea pigs with not only precursors of the false transmitter, but also *p*-hydroxynorephedrine itself, and subsequently challenging these animals with amphetamine.

The investigations presented here revealed a sex difference in the protective effect of *p*-hydroxyamphetamine, *p*-hydroxynorephedrine and norephedrine in rats. That this difference could result from a sex difference in amphetamine response can be excluded by reference to Figs. 9:1 and 9:2, which show that the extent of hyperthermia in rats treated with amphetamine alone is similar in both sexes. Alternatively, as an explanation for the observed sex difference, the metabolic fate of either amphetamine or the pretreatment drugs in the two sexes could differ. The former possibility is negated by the study of Lowander and Jonsson (1972) who showed no significant differences in the pattern of distribution of urinary metabolites of amphetamine between the sexes. A sex difference in the pharmacokinetics of amphetamine has, however, been noted (Groppetti & Costa, 1966), the $T_{1/2}$ for amphetamine in males being approximately half that found in females.

With respect to *p*-hydroxyamphetamine, Dingell *et al.* (1974) have reported the sex difference in the ability of liver microsomal preparations from Wistar rats to conjugate *p*-hydroxyamphetamine with glucuronic acid and this sex difference has been subsequently confirmed in Sprague-Dawley strains. An examination of the *p*-hydroxyamphetamine conjugations *in vivo* (see Chapter 3) provides further evidence that sex differences in *p*-hydroxyamphetamine conjugation may be causally related to the observed sex difference in the protective effects of *p*-hydroxyamphetamine against amphetamine hyperthermia. It may be that female rats conjugate injected *p*-hydroxyamphetamine so rapidly that no free drug is available to protect against amphetamine, whereas in the males, conjugation proceeds more slowly, allowing some free *p*-hydroxyamphetamine to exert its protective action. However,

p-hydroxynorephedrine is thought to be the molecular species responsible for this protection, and from Table 8:2, it will be noted that despite variations in p-hydroxyamphetamine conjugation, both sexes produce small amounts of p-hydroxynorephedrine, the metabolite claimed to be responsible for the protective effect. The data presented argue, then, that this effect may not be related to false transmitter production, but to some other mechanism involving p-hydroxyamphetamine itself and not its metabolite, p-hydroxynorephedrine.

The ability of p-hydroxyamphetamine to protect male rats against amphetamine hyperthermia may not be related to the mechanism of tolerance to amphetamine in rats. As in the male rats examined by Lewander (1971d), female rats have been found to become tolerant to the hyperthermic effect of amphetamine (Chapter 8). Thus the failure of p-hydroxyamphetamine to protect females against amphetamine hyperthermia is apparently not related to a deficiency in the mechanism giving rise to tolerance. This argument applies also to the guinea pig, since in females of this species, neither p-hydroxyamphetamine nor p-hydroxynorephedrine protected the animals against a subsequent amphetamine challenge.

At present the exact nature of the observed sex difference in the rat is equivocal. It is possible that variations in drug handling between the sexes is involved or that there is no involvement of noradrenergic false transmitters in tolerance to amphetamine induced hyperthermia in the rat.

From Table 8:3, it is noted that p-hydroxyamphetamine protected male but not female rats against the lethal effects of amphetamine and this reflects the protection seen against the hyperthermic action. It could be suggested on that evidence that hyperthermia and lethality are causally related.

Although no data is available on the metabolism of p-hydroxynorephedrine in male and female rats, when the fate of norephedrine is considered in the two sexes,

little difference is seen in the amounts of *p*-hydroxynorephedrine excreted in urine (27% male, 28% female) and the extent of conjugation of this metabolite with glucuronic acid is similar (12% male, 17% female). The sources of this data are shown in Table 9:2.

In view of the similar sex differences in the protective effect against amphetamine hyperthermia afforded by these two drugs and *p*-hydroxyamphetamine, it is suggested that metabolic differences between the two sexes are not of major significance, and that the data presented here militate against the false transmitter hypothesis for amphetamine tolerance.

c) The Effect of *p*-Hydroxyamphetamine Pretreatment on Locomotor Activity

Induced by Amphetamine

Introduction

As discussed previously (Chapter 8), evidence has been presented for the role of both dopamine and noradrenaline in the mediation of the increased locomotor activity that occurs after the administration of amphetamine. Following attempts to modify the hyperthermic effect of amphetamine by pretreatment with *p*-hydroxyamphetamine, experiments were designed to determine whether such pretreatment affected animal activity on subsequent amphetamine challenge, despite the evidence to the contrary presented by Lewander (1971d) who pretreated male rats with a single dose of *p*-hydroxyamphetamine (40 mg/kg) 20 hours prior to (+)-amphetamine (20 mg/kg).

Experimental Procedure

A total of 35 Wistar rats (male and female) were housed as previously described. *p*-Hydroxyamphetamine was administered either as a single dose (40 mg/kg i.p.) 20 hours prior to (+)-amphetamine (5 mg/kg i.p.) or in a total of 4 doses (40 mg/kg i.p. 2 times daily for 2 days), the last dose 20 hours prior to

amphetamine (5 mg/kg i.p.). Control animals were pretreated with equivalent volumes of saline.

The motor activity of individual rats (gross locomotor activity and stereotypy) was recorded on the animal activity monitor for 10 minute intervals for the period of $\frac{1}{2}$ -1 $\frac{1}{2}$ hours after amphetamine injection, the 0- $\frac{1}{2}$ hour period being used for acclimatisation (Chapter 7).

The results are expressed as the mean activity (gross locomotor or stereotyped) per 10 minute period per animal.

Results

The data are given in Table 9:4. For both male and female rats there appears to be a successive diminution in the gross locomotor activity, with increasing doses of p-hydroxyamphetamine pretreatment, whereas the figures for stereotyped activity are equivocal. Statistical interpretation of data derived from behavioural experiments is difficult in view of the wide interindividual variations in drug response and the difficulty in obtaining rigidly controlled environmental factors, thus standard errors are large. However, when comparing saline pretreated rats with those animals that had received 4 doses of p-hydroxyamphetamine, the fall in gross locomotor counts achieved statistical significance but no statistically significant change occurred with stereotyped behaviour.

Discussion

The results presented are in contrast to the study of Lewander (1971d) who failed to show any change in activity when rats were challenged with (+)-amphetamine (20 mg/kg), after a single dose (40 mg/kg) of p-hydroxyamphetamine. In the current experiment with 4 doses of p-hydroxyamphetamine and a smaller dose of amphetamine there appeared to be a depression in gross locomotor stimulation in both sexes. In the absence of the previously reported data on the effects of

Table 9:4. The Effects of p-Hydroxyamphetamine Pretreatment on Amphetamine**Activity**Data are presented as mean activity counts $\pm$ S. E.

	<u>Gross Locomotor Activity</u>	<u>Stereotyped Behaviour</u>	
<u>Male Wistar</u>			
Saline/Amphetamine	1309 $\pm$ 182	966 $\pm$ 180	n = 5
p-Hydroxyamphetamine (40 mg/kg)/amphetamine	1025 $\pm$ 225	1236 $\pm$ 178	n = 5
p-Hydroxyamphetamine (4 x 40 mg/kg)/amphetamine	672 $\pm$ 137*	1069 $\pm$ 60	n = 5
<u>Female Wistar</u>			
Saline/Amphetamine	1159 $\pm$ 85	1413 $\pm$ 103	n = 5
p-Hydroxyamphetamine (40 mg/kg)/amphetamine	962 $\pm$ 243	1187 $\pm$ 113	n = 7
p-Hydroxyamphetamine (4 x 40 mg/kg)/amphetamine	320 $\pm$ 95*	960 $\pm$ 116*	n = 5

* $P < 0.05$ compared with saline pretreated controls; all other values are not significant.

p-hydroxyamphetamine on amphetamine hyperthermia, one might interpret these findings as evidence for the accumulation of p-hydroxynorephedrine in central noradrenergic neurons resulting in an attenuation of the locomotor activity following subsequently administered amphetamine. The apparent lack of effect of such pretreatment on stereotyped behaviour is in accordance with the view that this action of amphetamine is mediated through dopamine release.

It is of interest that this data correlates well with the observations presented in the previous chapter, which showed that tolerance developed to gross locomotor activity, in the rat, with repeated administration of amphetamine, whereas no such change in stereotypy occurred.

These results, while in accordance with the false transmitter hypothesis, are difficult to reconcile with the apparent inability of p-hydroxyamphetamine to protect female animals against amphetamine hyperthermia, which militate against a role of p-hydroxynorephedrine in amphetamine tolerance (Chapter 8).

CHAPTER TEN

DISCUSSION AND CONCLUSIONS

Chapter Ten**Discussion and Conclusions**

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Discussion

The Significance of the formation of hydroxylated metabolites of amphetamine

Most studies reported in the literature on the possible mechanisms by which amphetamine produces its pharmacological and biochemical effects have concentrated upon the interrelationship between the parent drug amphetamine and the disposition of the catecholamines. Many of these studies have been performed in the rat, a species which metabolises amphetamine largely by aromatic hydroxylation rather than oxidative deamination of the side chain, a pathway occurring to a greater extent in most other species including the guinea pig and man. However, since the hydroxylated metabolites of amphetamine possess intrinsic pharmacological activity, it is important to consider their role in the overall response of animals or man to administered amphetamine.

Although amphetamine itself is not stored in sympathetic neurons, the introduction of the aromatic hydroxyl function permits selective concentration of *p*-hydroxy-amphetamine in peripheral tissues and brain (Iversen, 1967) and is reflected by the increased half-life of this metabolite in brain after intraventricular injection ($t_{1/2}$ 78 min.) as compared with the parent amine ($t_{1/2}$ 15 min.) (Taylor & Sulser, 1973). With substitution of a hydroxyl group in the β -position of the side chain, the resultant metabolite, *p*-hydroxynorephedrine, has a considerably prolonged tissue half-life, in excess of 2 hours (Taylor & Sulser, 1973; Lewander, 1971b; Costa & Groppetti, 1969) and probably results from intraneuronal binding in granules similar to those which store noradrenaline (see Chapter 1).

Certain peripheral pharmacological effects of *p*-hydroxyamphetamine have already been considered (see Chapter 3), and it is interesting to speculate upon the role this metabolite and/or its β -hydroxylated derivative could play in the CNS stimulant effects of amphetamine. Such a role has been alluded to by Costa and

Groppetti (1970) and Gunne, Jonsson and Anggard, (1972) and is further suggested by Taylor and Sulser (1973) who administered *p*-hydroxyamphetamine and *p*-hydroxynorephedrine into the cerebral ventricles of rats. The former compound produced intense gross locomotor stimulation and the latter mainly stereotyped behaviour. Similar CNS stimulation was observed by Fog (1970) when *p*-hydroxyamphetamine was injected into the corpus striatum of reserpinised rats, and by Clymer and Seifter (1947) who injected *p*-hydroxyamphetamine systemically to young chickens in which the blood brain barrier is not fully developed. In both these studies, stereotypy was the major response. Whether *p*-hydroxyamphetamine in this situation acts directly or whether it has to be converted to *p*-hydroxynorephedrine is uncertain, but the former is more likely since *l*-*p*-hydroxyamphetamine was found to be almost as potent as the *d*-isomer (Taylor & Sulser, 1973) and the *l*-antipode is not a substrate for dopamine- β -hydroxylase (Goldstein, McKereghan & Lauber, 1964).

Freeman and Sulser (1972) reported that pretreatment of rats with iprindole, an inhibitor of aromatic hydroxylation of amphetamine, actually enhanced the central effects of amphetamine. A closer examination of these observations, however, shows that the effect of such pretreatment was only to prolong, rather than to enhance, the central stimulation. Nevertheless, it appears that *p*-hydroxyamphetamine is not essential for the acute CNS stimulant effects of amphetamine.

There is evidence that with repeated dosage of amphetamine, the degree of central stimulation is unrelated to brain levels of amphetamine (Magour, Coper & Fährdrich, 1974) and the inference must be drawn that metabolites may be involved. It is interesting to speculate therefore upon the possible significance of these studies in relation to the human amphetamine psychosis. This phenomenon is discussed by Connell (1958) and recently studied by Griffith *et al.* (1970). In this study, cumulative doses of *d*-amphetamine (120-200 mg) were administered to amphetamine

addicts over a period of 1-5 days. Initial euphoria and hyperactivity followed a 10 mg i. v. dose. With continued oral administration after 50 mg total, all subjects became depressed and hypochondriacal, refusing to answer questions, confining themselves to their rooms, anorectic, unstable and fault finding. This was followed with increasing dosage by a prepsychotic phase lasting several hours when patients became paranoid and taciturn. The onset of psychosis was abrupt with loquacious discussion of their paranoid ideas. Agitation was present in only one of six patients and all others exhibited blunted affect. Organised delusions were apparent and ideas of reference were common. Orientation remained and states of consciousness were unaltered. Visual and auditory hallucinations did not occur in this study but were noted in 10 of 12 patients examined by Jonsson and Gunne, (1970). In addition to these features, stereotyped, repetitive activities have been described during the psychotic episode such as continuous chewing or teeth grinding movements, repetitive polishing of nails and dismantling and reassembling clocks or motors (Randrup & Munkvad, 1970).

These authors considered that noradrenaline release might cause a hyperstimulation of internal arousal mechanisms but noted that many of their subjects did not appear hyperstimulated with larger doses. At this later stage, perhaps depletion of catecholamines by amphetamine itself or a metabolite occurs, resulting in depression.

The following proposal outlines possible pharmacological events leading to a state of neurohormone imbalance which it is suggested may be associated with the development of the psychosis. Following repeated administration of large doses of amphetamine, with short time intervals between doses, it may be construed that the initial pharmacological effects of amphetamine, resulting from the liberation of the catecholamines noradrenaline and dopamine would be modified by the presence

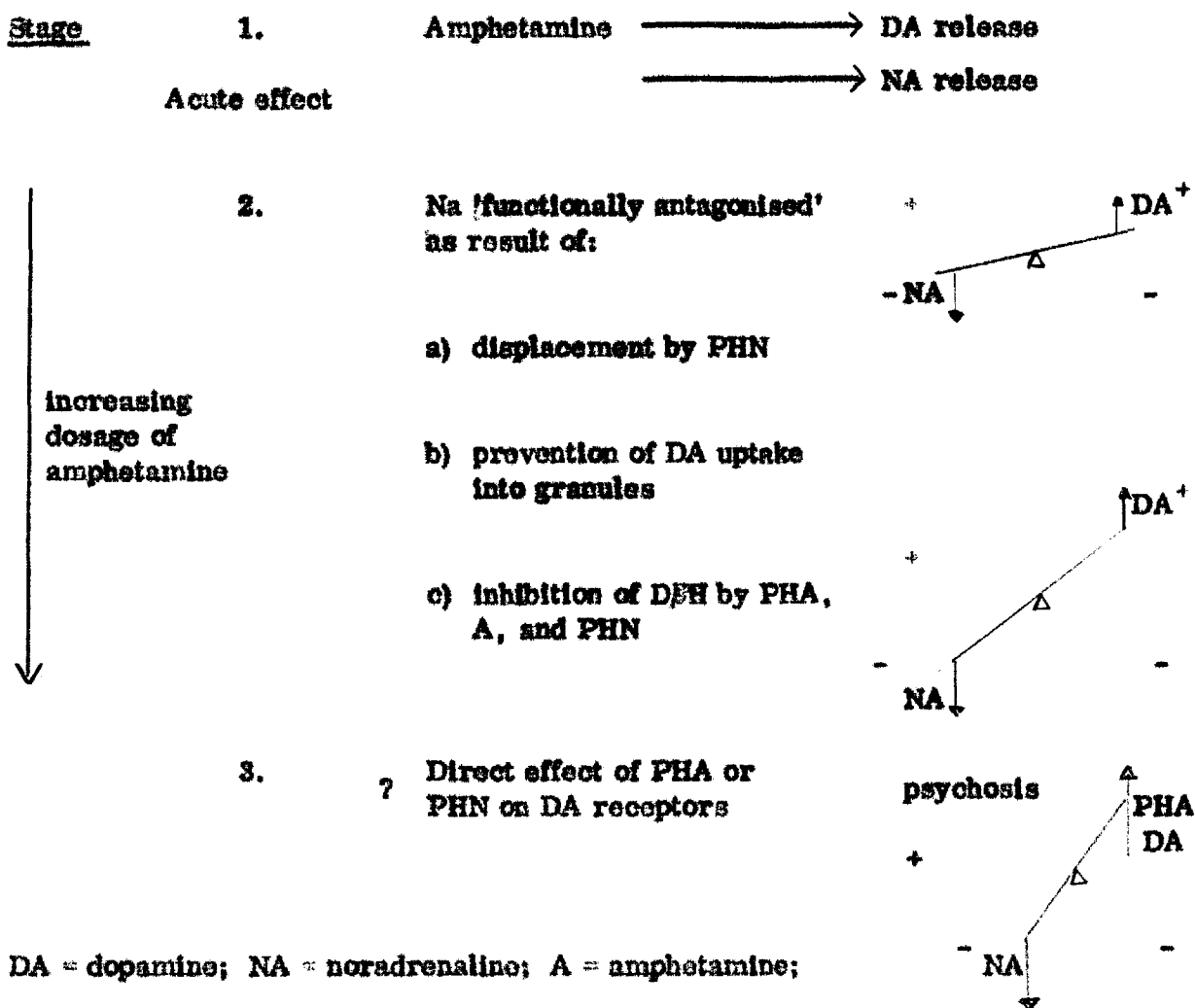
within the central neurons of the metabolites *p*-hydroxyamphetamine and *p*-hydroxynorephedrine. Although *p*-hydroxynorephedrine has an agonist activity on peripheral noradrenergic receptors only 1/500th that of noradrenaline (Brodie, Cho & Gessa, 1970), the previously mentioned study of Taylor and Sulser (1973) suggests that its direct effect centrally may be more significant.

Alternatively, it is possible that the CNS normally functions in a state of homeostasis dependent upon a balance of neurohormones, and that the relationship between dopamine and noradrenaline is critical. With repeated doses of amphetamine the persistent depletion of noradrenaline (*vide infra*) would upset the balance in favour of dopamine. Furthermore, this imbalance would be potentiated by inhibition of dopamine- β -hydroxylase. Creveling *et al.* (1962) showed that both amphetamine and *p*-hydroxyamphetamine were competitive inhibitors of this enzyme, in addition to which, the formation of *p*-hydroxynorephedrine within the adrenergic granules could be expected to suppress the activity of dopamine- β -hydroxylase by end product inhibition. Thus the ongoing biosynthesis of noradrenaline is prevented and dopamine accumulates.

A final and highly speculative factor is to attribute a role for *p*-hydroxyamphetamine as a dopamine receptor agonist perhaps ultimately responsible for the state of hyperdopaminism characterised by stereotypy and psychotic behaviour. Evidence for this is provided by the observations on stereotyped behaviour which increase when rats are chronically dosed with amphetamine (Chapter 6).

Hypothesis

Stages leading to amphetamine psychosis.



DA = dopamine; NA = noradrenaline; A = amphetamine;

PHA = p-hydroxyamphetamine; PHN = p-hydroxynorephedrine;

DβH = dopamine-β-hydroxylase.

Since the neuroleptic drugs which have antipsychotic effects both in patients with schizophrenia (Goodman & Gilman, 1976) and with the amphetamine psychosis, (Geerlings, 1972), probably exert their effects by dopamine receptor antagonism (Matthysse, 1973) the case for a state of dopamine preponderance in these conditions is therefore strengthened.

Undoubtedly further studies will explore the validity of the proposed animal model for the human psychosis, but it must be emphasised that the rat is not the ideal

species for such studies, in view of the major differences in metabolism of amphetamine in this species when compared with man (see Chapter 1).

Hydroxylated metabolites of amphetamine and catecholamine depletion

Several studies have shown that (+)-amphetamine lowers the brain and heart concentration of noradrenaline (Costa & Groppetti, 1970). In view of the prolonged depletion of noradrenaline found in rat heart and brain, which contrasts with the rapid disappearance of amphetamine from these tissues, it has been postulated that a metabolite of amphetamine is responsible for this depletion (Costa & Groppetti, 1970; Brodie, Cho & Gessa, 1970). Both *p*-hydroxynorephedrine and *p*-hydroxyamphetamine when administered to rats cause a more pronounced fall in heart noradrenaline than equimolar amounts of amphetamine (Costa & Groppetti, 1970), and after (+)-amphetamine, *p*-hydroxynorephedrine has also been identified in both brain and peripheral tissues in amounts related to the noradrenaline deficit (Costa & Groppetti, 1970; Brodie, Cho & Gessa, 1970). Studies with (-)-amphetamine which is not metabolised to *p*-hydroxynorephedrine (Goldstein & Anagnoste, 1965) reveal a more short lived noradrenaline depletion (Brodie, Cho & Gessa, 1970).

Following inhibition of aromatic hydroxylation of amphetamine with iprindole (Freeman & Sulser, 1972), peripheral and central noradrenaline levels still decline but return to normal values within 24 hours, in contrast to a persistent decline of up to 48 hours whilst amphetamine is given alone. The studies of these authors taken in conjunction with those of Costa and Groppetti (1970), who found that in guinea pigs, which do not metabolise amphetamine significantly by ring hydroxylation, the noradrenaline depletion has a similar time course relating only to the presence of amphetamine in the tissues, suggest that prolonged noradrenaline depletion requires the presence of the metabolite, *p*-hydroxynorephedrine. Costa and Groppetti (1970) conclude that the delayed effects of amphetamine, including psychoses and tolerance,

are in part dependent upon the storage of *p*-hydroxynorephedrine in brain where it would be released not only by successive doses of amphetamine but also by nerve activity.

It is interesting to point out here that like the other false transmitters investigated by Kopin, Weis and Sedvall (1968), *p*-hydroxynorephedrine diminishes the turnover rate of cerebral noradrenaline in contrast to the parent amine amphetamine which increases the turnover (*vide infra*). Thus, with accumulation of false transmitter, both displacement of noradrenaline and diminished synthesis could occur and, perhaps, relate causally to the known CNS effect of amphetamine on repeated dosage.

Studies on tyrosine hydroxylase activity suggest that the activity of this enzyme is increased by a negative feedback mechanism, resulting from synthesis of new enzyme, which can be blocked by protein synthesis inhibition (Mandell & Morgan, 1970). Although tyrosine hydroxylase has been considered to be the rate limiting enzyme for noradrenaline biosynthesis (Levitt *et al.*, 1965), attention has recently been diverted to dopamine- β -hydroxylase, which may have more immediate control over noradrenaline formation (Mollinoff & Orcutt, 1974). Studies on this enzyme in states of amphetamine tolerance have not however been performed, but from previously mentioned studies, inhibition of this enzyme by amphetamine, *p*-hydroxyamphetamine or *p*-hydroxynorephedrine, would prevent any increase in the biosynthesis of noradrenaline to compensate for the depletion and account for the low turnover rates observed in the tolerant state. These findings suggest that the disposition of dopamine would differ from that of noradrenaline, but perusal of the literature provides conflicting evidence. Lewander (1971c), in rats made tolerant to amphetamine, demonstrated that dopamine levels were diminished but turnover unchanged, however, Costa and Groppetti (1970) showed that turnover of dopamine was increased by repeated small doses of amphetamine. The converse was however reported by Glowinski *et al.* (1974) who

observed a decrease in turnover of dopamine with larger doses. The case, therefore, is unresolved.

Irrespective of dopamine, it may be construed that the changes occurring within the nerve terminals would favour a diminished biosynthesis of noradrenaline such that repeated dosage of amphetamines would release less noradrenaline from the nerve ending. It is not essential at this point to involve *p*-hydroxynorephedrine as a false neurotransmitter.

Table 10:1 summarises the available data on the effects of acute and chronic amphetamine treatment upon the disposition of noradrenaline. Although certain vital data are lacking, it can be seen that the only consistent finding in tolerant animals is the enhanced depletion of noradrenaline with chronic dosage (Lewander, 1968; 1971g), neither the prolonged depletion of noradrenaline, nor the formation of *p*-hydroxynorephedrine being a prerequisite for tolerance.

Regulation of the noradrenergic neuron

There is little doubt that within the sympathetic nervous system control mechanisms exert their influence at various levels in order to maintain a state of homeostasis. Although overall control of sympathetic activity is from the CNS, the neurons themselves may regulate the release of noradrenaline from the nerve terminals (see Smith, 1972). With chronic administration of amphetamine and because of the diverse effects of this drug on the neuron, it is probable that control mechanisms for noradrenaline synthesis, uptake, storage and release are deranged.

In the preceeding paragraphs, the effects of amphetamine and its metabolites upon certain presynaptic mechanisms, which govern the biosynthesis and storage of noradrenaline, have been considered. The following discussion will outline the possible role of false neurotransmitters in relation to the noradrenergic neuron and will be concluded by a summary of the evidence for and against the false transmitter hypothesis for amphetamine tolerance.

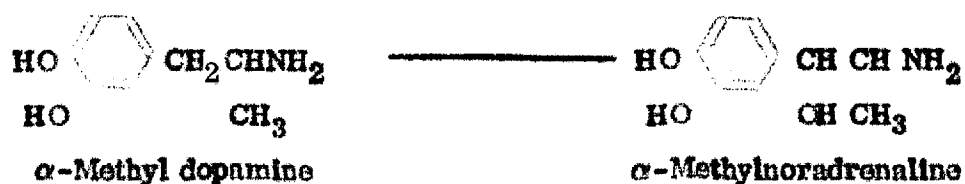
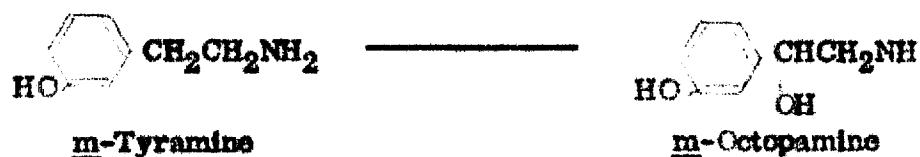
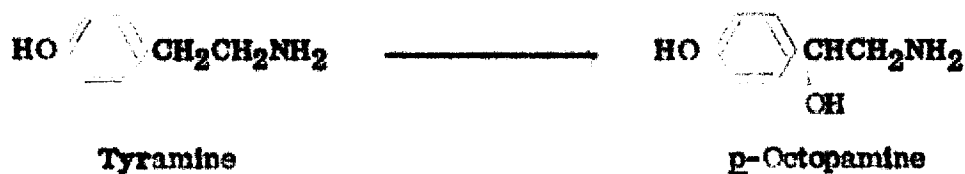
Table 10:1 The Effects of Acute and Chronic Amphetamine Treatment upon the Disposition of Noradrenaline

	d-Amphetamine (rat)	l-Amphetamine (rat)	d-Amphetamine + iprindole (rat)	d-Amphetamine (guinea pig)
Initial depletion of noradrenaline	+	+	+	+
Prolonged depletion of noradrenaline	+	-	-	-
Enhanced depletion of noradrenaline on chronic dosage	+	?	?	+
p-Hydroxynorephedrine formation in tissues	+	-	-	-
Tolerance	+	+	?	+

An assessment of the structure and function of 'false transmitters'

The structure activity relationships for false transmitters were reviewed by Kopin in 1968.

As referred to previously in this thesis, many phenylethyl- and phenyliso-propyl- amines may be converted to their β -hydroxylated derivatives by dopamine- β -hydroxylase (*in vivo*), e.g.



If β -hydroxylation of these amines is prevented by the administration of disulfiram, which inhibits this reaction, but does not interfere with noradrenaline binding, these amines are not retained by the noradrenergic vesicles (Musacchio, Snyder & Kopin, 1964; Musacchio, Fischer & Kopin, 1966). These authors conclude therefore that storage of these compounds within the sympathetic neuronal granules is at least dependent upon the presence of the β -hydroxyl group.

Comparison of the retention of various β -hydroxylated amines by particulate fractions of sympathetic neurons reveals that, apart from the β -hydroxyl group, the disposition of the aromatic hydroxyl groups is important, catechol groups being retained to a greater extent than monophenolic groups. However, α -methyl substitution did not alter the binding since retention by α -methyltyramine and tyramine was similar (Kopin, 1968).

With respect to false transmitter release from sympathetic neurons there are several reports suggesting that the false transmitter is selectively released in favour of endogenous noradrenaline (Crout & Shore, 1965) (by nerve stimulation and by indirect acting sympathomimetics) and, according to Pösch & Kopin (1966), might explain tachyphylaxis to indirectly acting sympathomimetics. The effects of these false transmitters on catecholamine synthesis may depend upon whether the compounds formed during the false transmitter synthesis compete with the enzymes in the noradrenaline biosynthetic pathway, e.g. α -methyldopa. With non catechols, which are metabolised to false transmitters, the resultant displacement of noradrenaline from storage sites and rise in free noradrenaline would tend to inhibit noradrenaline synthesis by feedback inhibition, while with catechols, the reduction in noradrenaline synthesis may occur as a result of competition for the enzymes involved in the noradrenaline biosynthetic pathway.

The total effects of the false transmitter and its precursors on noradrenaline

disposition are further complicated by the fact that some of these compounds may compete for noradrenaline reuptake into the nerve terminals (Uptake₁; see Chapter 1).

Finally, the resultant pharmacological effects will depend upon the agonist activity of the particular false transmitter, and although many of these compounds are several orders of magnitude less potent than noradrenaline, particularly with respect to peripheral effects, Day *et al.* (1973) suggested that, on central noradrenergic neurons, α -methylnoradrenaline may be almost as potent as noradrenaline.

Lewander's observation, that *p*-hydroxyamphetamine protects against only peripheral effects of amphetamine (presumably following its conversion to *p*-hydroxynorephedrine), could be explained by the fact that the effect of *p*-hydroxynorephedrine on central noradrenergic receptors is almost as potent as that of the endogenous transmitter noradrenaline.

Could norephedrine function as a false transmitter?

The evidence for norephedrine functioning as a false transmitter has been considered previously (Chapter 6). This compound is undoubtedly formed metabolically from amphetamine in several species including rat, guinea pig and cat (Caldwell, Dring & Williams, 1972; Lewander, 1971a; Thoenen *et al.*, 1966; Chapter 4 of this thesis). Thoenen *et al.* (1966) have identified this metabolite in homogenates of cat spleen following administration of amphetamine and since norephedrine is produced by β -hydroxylation of amphetamine, this reaction presumably takes place within the catecholamine storage granules. However, retention within the granules appears to be dependent also upon the presence of phenolic or catechol groups and the failure to detect norephedrine on splenic nerve stimulation following amphetamine administration (Thoenen *et al.*, 1966) suggests that norephedrine is unlikely to function as a false transmitter.

There have been few considerations of the possibility of false dopaminergic neurotransmitters. However, Shore and Dorris (1974) commented that α -methyl--m-tyramine may occupy such a function at dopamine receptors. In relation to noradrenaline, it has been discussed that β -hydroxylation is a requirement for storage, but in dopaminergic neurons, the storage characteristics must be unrelated to the β -hydroxyl group, and if one accepts that p-hydroxynorephedrine is a false transmitter in noradrenaline containing neurons, why not p-hydroxy-amphetamine in dopamine containing neurons?

The resultant effect would again be dependent upon the agonist activity of p-hydroxyamphetamine.

Summary of the evidence for p-hydroxynorephedrine as a false transmitter

There is no dispute that in many species p-hydroxynorephedrine is formed metabolically from its precursor p-hydroxyamphetamine by the action of dopamine- β -hydroxylase, an enzyme occurring mainly within the noradrenaline storage granules (Goldstein & Anagnoste, 1965; Cavanaugh, Griffith & Oates, 1970; Brodie, Cho & Gessa, 1970). Unequivocal evidence has also been provided by Thoenen et al. (1966) and Fischer, Horst and Kopin (1965) for the liberation of p-hydroxynorephedrine from perfused cat spleen preparations on sympathetic nerve stimulation. Finally, Brodie, Cho and Gessa (1970) confirm the observation of Trendelenburg et al. (1962) that p-hydroxynorephedrine possesses only weak intrinsic sympathomimetic activity (1/500 that of noradrenaline). Thus the evidence for p-hydroxynorephedrine as a false transmitter would appear to be unassailable, nevertheless to assign a role for p-hydroxynorephedrine in the development of amphetamine tolerance is more difficult.

Evidence in favour of the false transmitter hypothesis in amphetamine tolerance

This may be summarised as follows:

1. Tolerance develops in rats to some effects of amphetamine but not to others, namely hyperthermia, anorexia, cardiovascular effects and the increase in urinary noradrenaline excretion (Lewander, 1971d) and most of the available evidence suggests that these effects are mediated through noradrenaline release.
2. Enhanced noradrenaline depletion in peripheral tissues and brain is associated with the development of tolerance (Lewander, 1968).
3. *p*-Hydroxynorephedrine is present in these tissues in amounts that relate to the noradrenaline deficit (Brodie, Cho & Gessa, 1970).
4. *p*-Hydroxynorephedrine is released preferentially by amphetamine in respect to noradrenaline (Brodie, Cho & Gessa, 1970).
5. *p*-Hydroxyamphetamine and *p*-hydroxynorephedrine pretreatment attenuates the peripheral effects of noradrenaline, possibly by incorporation of *p*-hydroxynorephedrine into the noradrenergic granules with displacement and depletion of noradrenaline (but this is true for male rats only).

Much of this evidence is circumstantial and leaves the following questions unanswered:

1. Why are certain central effects of amphetamine allegedly mediated via noradrenaline and to which tolerance develops, not attenuated by the presence of *p*-hydroxynorephedrine in central neurons?
2. Does *p*-hydroxynorephedrine accumulate in sufficient amounts to block the actions of amphetamine, since Brodie, Cho and Gessa (1970) failed to show any accumulation of *p*-hydroxynorephedrine in heart after seven repeated doses of amphetamine? These authors suggested that each new dose of amphetamine released most of the *p*-hydroxynorephedrine accumulated in storage granules.

Evidence against the involvement of p-hydroxynorephedrine in amphetamine tolerance

From the work presented in this thesis, it is possible to argue against the false transmitter hypothesis for amphetamine tolerance, on the following grounds:

1. A species which does not metabolise amphetamine to any significant extent by aromatic hydroxylation (the guinea pig) and in which p-hydroxynorephedrine cannot be identified at tissue sites, rapidly develops tolerance with repeated administration of amphetamine and shows little difference from the rat, a species which metabolises amphetamine to p-hydroxynorephedrine.
2. Although both male and female rats become tolerant to amphetamine and indeed convert both norephedrine and p-hydroxyamphetamine to the false transmitter, p-hydroxynorephedrine, pretreatment of animals with either precursor or p-hydroxynorephedrine itself only protects male animals against a subsequent amphetamine challenge; females being unaffected by such pretreatment. Thus the metabolic production of p-hydroxynorephedrine from amphetamine appears unrelated to tolerance, since the sex difference in protection is not paralleled by a deficiency in the tolerance mechanism.
3. Ephedrine, a drug to which tolerance rapidly develops with repeated administration, is not metabolised to the false transmitter in man (see Chapter 5).
4. Other amphetamine analogues such as phenmetrazine, which do not form p-hydroxynorephedrine metabolically (Franklin, 1974) give rise to tolerance on repeated exposure (Kalant, Le Blanc & Gibbins, 1971).
5. Tolerance develops to the hyperthermic response in rats chronically dosed with l-amphetamine, which is not metabolised to p-hydroxynorephedrine (Lowander, 1974).

In summary, therefore, it appears that despite much circumstantial evidence claiming a causal relationship between the formation of the false transmitter,

p-hydroxynorephedrine, and the development of tolerance to amphetamines, many questions have been unresolved, and from the evidence presented in this thesis, the false transmitter hypothesis is no longer tenable, and alternative mechanisms must be sought to explain amphetamine tolerance.

Little attention has been focussed on post-synaptic or receptor initiated regulatory mechanisms for noradrenaline release, which may be involved in the tolerance mechanism, and in the light of recent studies, these will be discussed briefly.

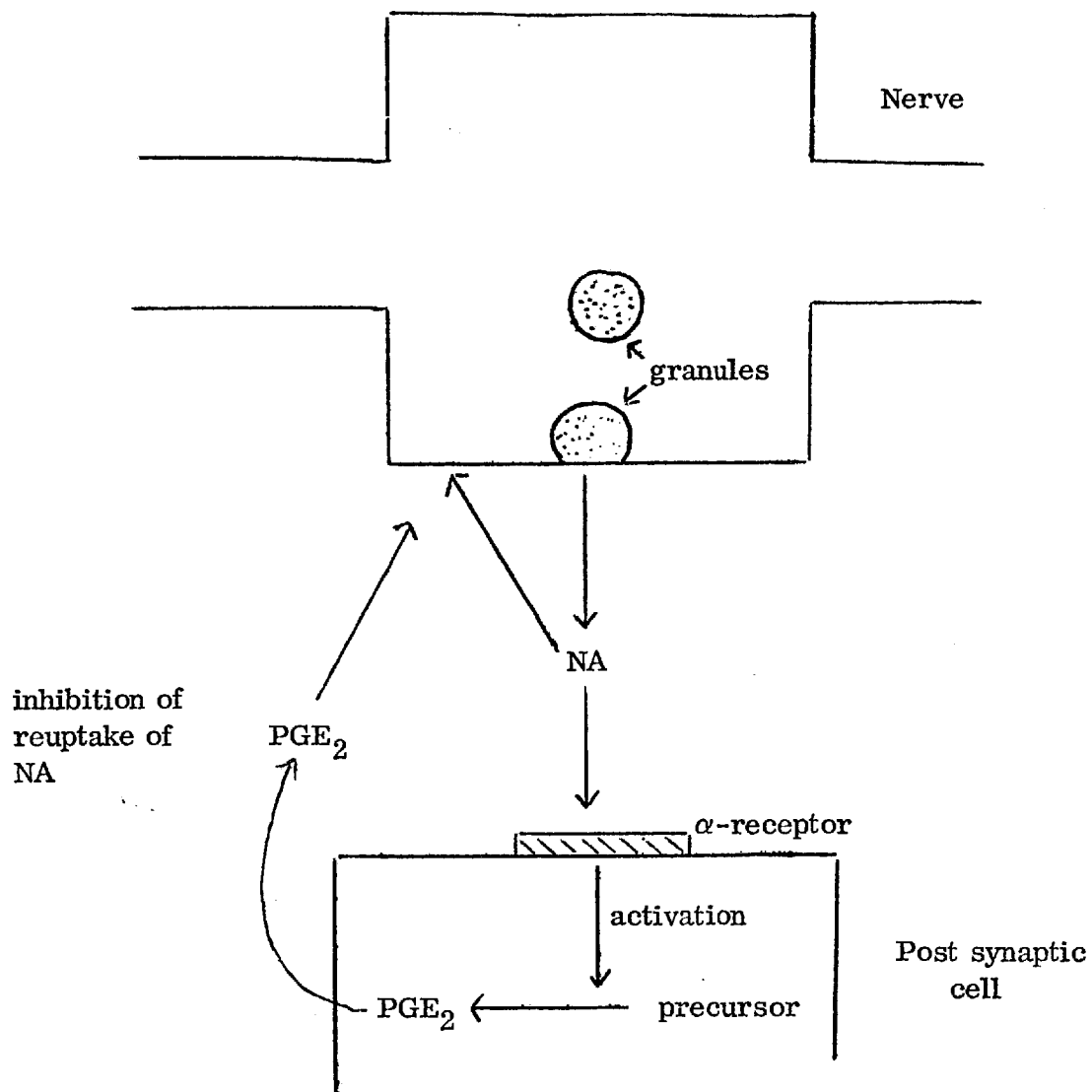
Postsynaptic events

Hedqvist (1970a) has proposed that prostaglandins (PG) of the E group notably PGE₂, may play a specific role in the regulation of the 'sympathetic neuro-effector system' by 'inhibition of the release of noradrenaline from sympathetic neurons'. Many subsequent investigators have produced evidence supporting this hypothesis (see Smith, 1972) and studies with prostaglandin synthesis inhibitors show that noradrenaline release from nerve terminals is enhanced by pretreatment with the specific inhibitor of PG synthetase (elcosa 5,8,11,14-tetraynoic acid). The postulated feedback loop involving prostaglandins is shown in Fig. 10.1.

Early studies with exogenous PGE₂ failed to influence the amount of noradrenaline released by the indirectly-acting sympathomimetic tyramine as opposed to nerve stimulation (Hedqvist, 1970). However, tyramine may not be truly representative of the indirectly-acting sympathomimetics, and thus it would be of interest to determine whether the effects of amphetamine were modified by interruption of PG mediated feedback loop.

The results of such a study carried out subsequent to the preparation of this thesis are published elsewhere (Sever & Trelinski, 1974). In this investigation,

Fig. 10:1. Possible Mechanisms in the feedback control of Release of Noradrenaline
by Prostaglandins



After Smith (1972)

NA = noradrenaline

PGE₂ = Prostaglandin E₂

the possibility that noradrenergic effects of amphetamine could be potentiated by pretreatment of rats with the prostaglandin inhibitor, indomethacin, was explored. Female Wistar rats were dosed with either (+)-amphetamine sulphate (5 mg/kg i.p.) alone, or after pretreatment with indomethacin (5 mg/kg) administered by gastric tube 2 h prior to amphetamine challenge. Colonic temperature was recorded as described in Chapter 7, $\frac{1}{2}$, 1 and 2 hours after amphetamine injection. The results are shown in Fig. 10:2. The mean maximum temperature ~~also~~ evoked by amphetamine after indomethacin pretreatment is significantly greater ($P < 0.05 > 0.01$) than that produced by amphetamine alone. Control experiments confirmed that indomethacin had no effect on baseline temperatures.

Enhancement of the negative feedback mechanism controlling noradrenaline release could be involved in the development of tolerance that is seen with chronic dosage of amphetamines. This was investigated by dosing rats with indomethacin (5 mg/kg twice daily) and amphetamine (5 mg/kg twice daily) for 5 days. Colonic temperature was recorded after the first dose of amphetamine on each day. Results are shown in Fig. 10:3.

From the data recorded it is apparent that the tolerance produced to the hyperthermic effect of amphetamine with repeated exposure is partially prevented by indomethacin, a potent inhibitor of prostaglandin synthesis (Ferreira, Moncada & Vane, 1971). The studies reported in Chapter 8 with repeated administration of amphetamine alone, show that a marked attenuation of the hyperthermic response occurs within 24-36 hours, and, although indomethacin prevents this early acquisition of tolerance, on the fourth day of treatment with the combination of drugs, tolerance develops.

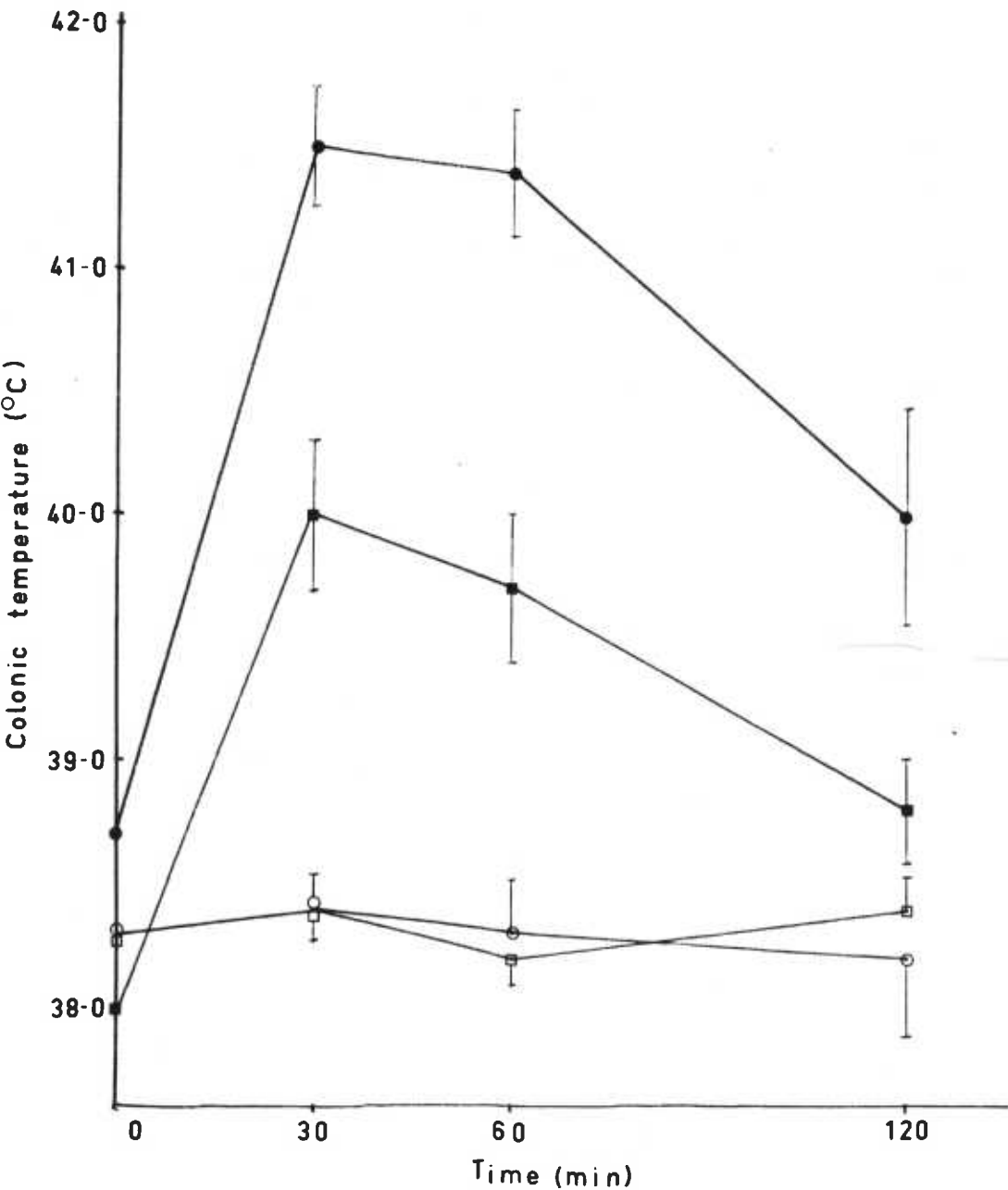
It could be suggested that indomethacin was producing its effect by inhibiting amphetamine metabolism. To eliminate this possibility, [^{14}C]-(+)-amphetamine

Fig. 10:2 The effect of indomethacin on the time course of amphetamine
induced hyperthermia

Rats were pretreated with indomethacin (5 mg/kg orally) or normal saline (0.5 ml orally) 2 h prior to injection of (+)-amphetamine (5 mg/kg i.p.) or saline.

Each curve represents the mean colonic temperature ($\pm$ S. E.) of rats receiving:

- Indomethacin before Amphetamine (n=10)
- Saline before Amphetamine (n=13)
- Indomethacin before Saline (n=9)
- Saline before Saline (n=6)



Comparing temperature rise evoked by amphetamine after indomethacin pretreatment with that evoked by amphetamine after saline pretreatment
 $P < 0.05 > 0.01$.

Fig. 10:3. The effects of indomethacin on the development of tolerance to amphetamine induced hyperthermia

(Continued overleaf)

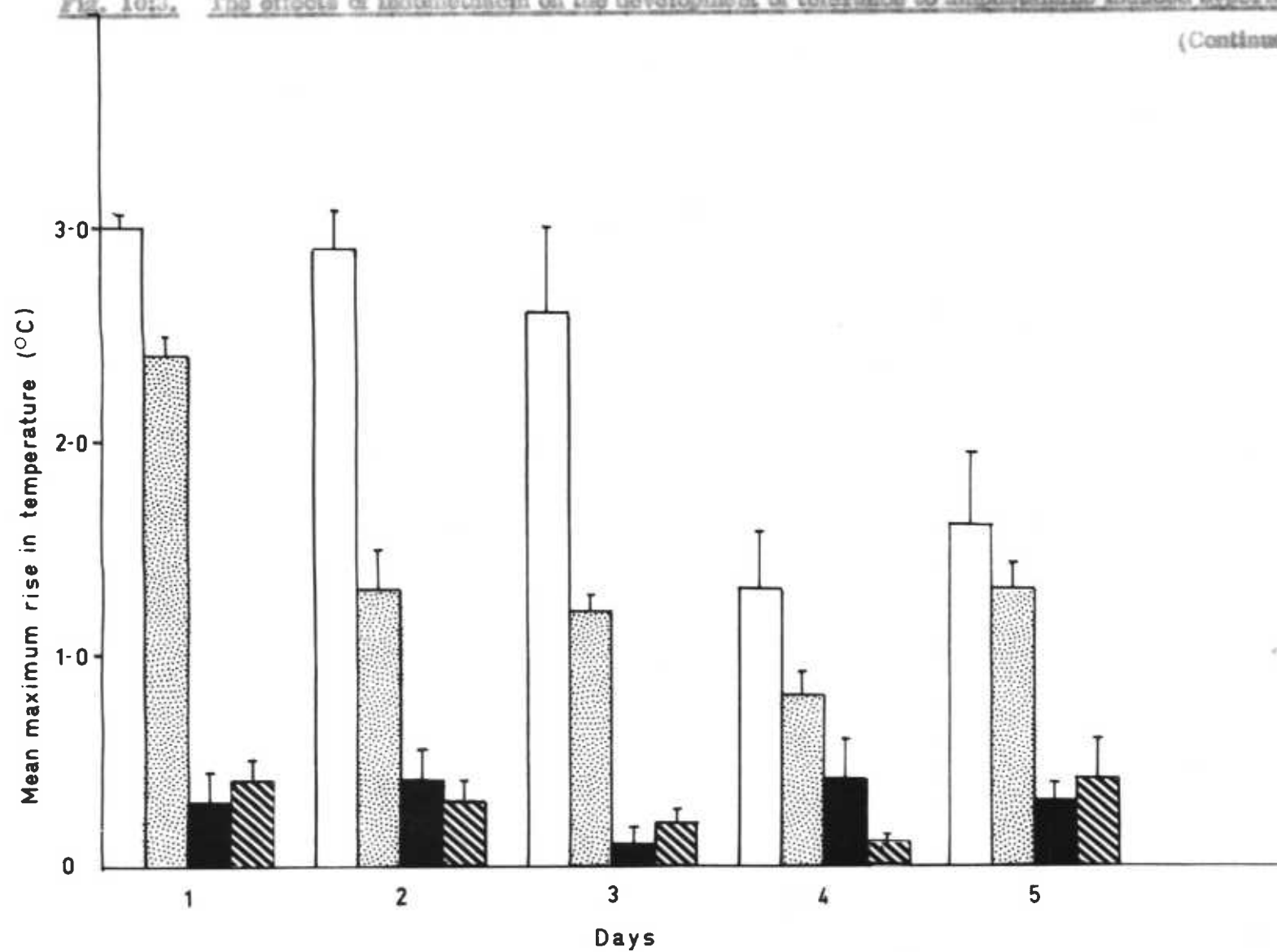


Fig 10.3. The effects of indomethacin on the development of tolerance to amphetamine induced hyperthermia

Rats were treated twice daily with indomethacin (5 mg/kg orally) or normal saline (0.5 ml) 2 h before injection of (+)-amphetamine (5 mg/kg i.p.) or normal saline (0.5 ml)

Each bar represents the mean maximal temperature elevation produced from 0-2h after the first dose of amphetamine or saline on days 1-5.

□ Indomethacin and amphetamine (n = 8-25)

▣ Indomethacin and saline (n = 4-9)

▤ Saline and amphetamine (n = 5-8)

▥ Saline and Saline (n = 6)

Vertical bars represent S. E.

sulphate (5 mg/kg i.p.; 1 μ Cl per animal) was administered to rats 2 hours after pretreatment with either indomethacin (5 mg/kg orally) or an equivalent volume of saline orally. The total unchanged amphetamine and metabolites excreted in the first 24 hour urine after dosing was determined by solvent extraction and paper chromatography essentially as described in Chapter 2. The percentage of dose excreted as amphetamine in 2 indomethacin pretreated rats was 13 and 18% compared with 15 and 14% in the 2 saline pretreated animals. No quantitative difference in the urinary excretion of amphetamine metabolites was found when the two pairs of rats were compared. The recovery of ^{14}C in the 0-24 hour urine from the 4 rats varied from 70-80%. Thus it would appear that indomethacin does not have an inhibitory effect upon the metabolism of amphetamine.

These results imply that prostaglandin synthesis may be required for the initial development of tolerance to the hyperthermic effect of amphetamine, however, the finding that tolerance subsequently develops (by day 4 in this experiment) suggests that alternative mechanisms have to be invoked for the later development.

Conclusion

The metabolic studies presented in the first part of this thesis confirm that *p*-hydroxynorephedrine (the putative false transmitter metabolite of amphetamine) is excreted as a urinary metabolite of *p*-hydroxyamphetamine in all species investigated, and of amphetamine in tolerant human subjects. It is not found following the administration of ephedrine, a drug to which, like paradrine and amphetamine, tolerance develops rapidly in man.

It is realised that the identification and quantitation of drug metabolites in the urine may be unrepresentative of their tissue disposition, and the argument proposed for the possible significance of the excretion of *p*-hydroxynorephedrine in human subjects tolerant to amphetamine exemplifies this point. Conversely, the identification of *p*-hydroxynorephedrine in guinea pig urine was perhaps misleading, since this metabolite of amphetamine has not been identified in the tissues of this species when chronically dosed with amphetamine, and therefore cannot be implicated in the development of tolerance.

Nevertheless, tolerant human subjects in whom experimental studies are naturally limited, provided no convincing evidence that alterations in drug disposition contributed to the development of tolerance and more specifically no evidence for an enhanced production of the false neurotransmitter, *p*-hydroxynorephedrine was found. Those changes occurring with prolonged exposure to amphetamine were in opposition to that which might be anticipated, i.e. less metabolism with repeated dosage rather than enhanced biotransformation.

The object of the latter part of this thesis was to explore the proposed relationship between the metabolic formation of false transmitter metabolites of amphetamine and the development of tolerance to these drugs. Many studies on drug tolerance have concentrated upon the influence that repeated dosage with amphetamine has upon the disposition of the catecholamines. Whilst the importance

of such studies cannot be denied, it remains conjectural as to whether changes in neurotransmitter disposition can provide the solution to the elusive problem of amphetamine tolerance. However, in view of the intimate relationship between the amphetamines and the catecholamines, such investigations have been reviewed where appropriate.

The multitude of references to false transmitters in relation to amphetamine tolerance warranted an investigation into the validity of this hypothesis.

Consideration of the effects of chronic amphetamine challenge upon the noradrenergic neuron suggests that depletion of noradrenaline may occur with or without the formation of p-hydroxynorephedrine.

That tolerance is related to the metabolic production of the false transmitter can be discounted from the observations presented in this thesis that:

1. Animals failing to produce the false transmitter from amphetamine develop tolerance.
2. Precursors of the false transmitter (other than amphetamine) fail to protect against a subsequent amphetamine challenge in female animals known to possess the ability to become tolerant to amphetamines.

Furthermore, a recent study by Lewander (1974) reveals that tolerance develops in the rat to (-)-amphetamine which (see Chapter 1) is not metabolised to p-hydroxynorephedrine.

Little attention has been focussed upon receptor adaptation and post synaptic regulatory mechanisms and preliminary studies with prostaglandin synthesis inhibitors suggest that the post synaptic regulatory negative feedback mechanism controlling noradrenaline release may be, in part, important in the initial development of tolerance. However, in view of the subsequent development of this phenomenon, even when prostaglandin synthesis is prevented, other mechanisms must be invoked.

That diminished receptor sensitivity could play a part in amphetamine dependence has been largely ignored. The studies presented in this thesis were not designed to explore this possibility, however, the biological phenomenon of tolerance is unlikely to be unique for any one class of foreign compounds, and since receptor adaptation has been postulated in the development of tolerance to other psychoactive drugs, it would seem reasonable to implicate such a mechanism in the state of amphetamine tolerance.

Future studies should be designed to evaluate the effects of chronic amphetamine administration on the short and long term regulatory mechanisms for neurohormone synthesis and release. In addition, with the recent developments of refined histochemical techniques and the mapping of noradrenergic and dopaminergic pathways in the animal brain, it should be possible with the aid of sophisticated stereotactic techniques to stimulate or lesion selected pathways, such that a more complete understanding of the mechanism of action of the psychotropic drugs of the amphetamine group is acquired. This naturally has widespread implications in that a more refined evaluation of the biochemical and pharmacological abnormalities occurring after high doses of amphetamines may provide insight into the underlying mechanisms in the paranoid psychoses, particularly since the drug amphetamine is capable of producing a mental disorder indistinguishable from schizophrenia, the aetiology of which has defied investigators for decades.

APPENDIX

SYNTHESES OF (+)-[¹⁴C]-p-HYDROXYAMPHETAMINE AND
(-)-[¹⁴C]-EPHEDRINE

Appendix Syntheses of (+)-[¹⁴C]-p-Hydroxyamphetamine and (-)-[¹⁴C]-Ephedrine

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Synthesis of (+)-[¹⁴ C]-p-Hydroxyamphetamine hydrochloride	215
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Appendix

Syntheses of [^{14}C]-labelled compounds used in the metabolic studies reported in Chapters 3 and 5

The syntheses of (+)-[^{14}C]-p-hydroxyamphetamine hydrochloride and (-)-[^{14}C]-ephedrine hydrochloride were carried out by Dr. L. G. Dring of this Department. At the present time neither of these syntheses has been published in the literature, and therefore they will be included in this appendix.

Synthesis of p-Hydroxyamphetamine

[Carboxyl- ^{14}C]-Anisic acid (1.35 g, m.p. 178°C) was prepared from $\text{Ba}^{14}\text{CO}_3$ (2.41 g, 6 mCi; Radiochemical Centre, Amersham, Bucks., U.K.) according to the method of Reid and Jones (1948). This was converted to [carbonyl- ^{14}C]-anisaldehyde (0.92 g) using the method described by the same authors. To the radioactively labelled aldehyde was added inactive anisaldehyde (1.80 g) together with nitroethane (1.80 ml), *n*-butylamine (0.10 ml), piperidine (0.15 ml) and benzene (12.5 ml). This reaction mixture was refluxed for 13 hours and the water formed in the condensation was removed with a Dean and Stark trap. On cooling, the mixture was washed with 2×5 ml 2M HCl and 3×10 ml water and the benzene solution dried over Na_2SO_4 . Evaporation of the solvents under reduced pressure yielded impure 1-(4'-methoxyphenyl)-2-nitro-[1- ^{14}C]-prop-1-ene (1.4 g) as a light brown crystalline solid. This nitro compound (1.1 g) was dissolved in dry ether (20 ml) and added over a period of 30 mins. to a stirred solution of lithium aluminium hydride (0.92 g) in dry ether (50 ml) and the mixture was refluxed for 2.5 hours to complete the reaction. Excess reagent was destroyed by the addition of water (10 ml) and, after addition of 2M NaOH (10 ml) the ether layer was separated.

Further base was extracted from the aqueous solution with ether (2 x 10 ml).

The bulked ethereal solutions of the amine were extracted with 2M HCl (2 x 20 ml) and the acidic solution washed with ether and made alkaline with 10M NaOH (15 ml). The liberated base was extracted with ether (3 x 10 ml) and dried over Na₂SO₄. On addition of an excess of an ethereal solution of dry HCl gas the base hydrochloride precipitated as an oil which on standing gave white crystals of (+)-1-(4'-methoxyphenyl)-[1-¹⁴C]-2-aminopropane hydrochloride (0.58 g, m.p. 204°C and a specific activity of 0.76 μCi/mg; Hoover & Hass (1947) give 207°C). The radiochemical purity of the compound was determined by paper chromatography and by reverse isotope dilution analysis with authentic (+)-1-(4'-methoxyphenyl)-2-aminopropane hydrochloride (Smith, Kline and French Laboratories, Welwyn Garden City, Herts, U.K.) and in both cases the purity was greater than 98%.

[¹⁴C]-p-Hydroxyamphetamine was prepared from the above methoxy compound (0.25 g) by heating it in a sealed tube together with 10M HCl (10 ml) for 2.5 hours at 140°C. The resulting straw coloured liquid was taken to dryness under reduced pressure, the residue dissolved in water (10 ml) and again evaporated to dryness, this procedure being repeated three times to remove HCl. The solid obtained was triturated with acetone and on filtration gave white crystals of (+)-1-(4'-hydroxyphenyl)-2-aminopropane hydrochloride (0.117 g, m.p. 167-169°C, specific activity 0.80 μCi/mg; Alles (1932) gives m.p. 171-172°C). The radiochemical purity of the compound was determined by paper chromatography and reverse isotope dilution analysis with the authentic compound (Paredrine; Smith, Kline and French Laboratories) as the N,O-dibenzoyl derivative (Dring, Smith & Williams, 1970) and in both cases the purity was greater than 98%.

Synthesis of (-)-2-Methylamino-1-phenyl-[1-¹⁴C]-propan-1-ol ([1-¹⁴C]-ephedrine)

Racemic ephedrine was synthesised using a modification of the route outlined by Nagase *et al.* (1961).

1-Oxo-1-phenyl-[1-¹⁴C]-propane ([¹⁴C]-propiophenone; 57 mg, 3 μ Cl/mg, Sinsheimer, Dring & Williams, 1973) and inactive propiophenone (840 mg) were dissolved in 10 ml of benzene which was then heated to 70°C. Bromine (1.12 g), dissolved in 5 ml of benzene, was added dropwise, allowing the yellow colour to dispel between each addition. Finally the mixture was refluxed for one hour. On cooling, the benzene was removed in vacuo, the resulting oil rinsed with benzene and taken to dryness once more to remove any bromine and hydrogen bromide and finally taken into 10 ml of benzene. This solution of 2-bromo-1-oxo-1-phenyl-[1-¹⁴C]-propane was cooled in ice and a solution of methylamine in ethanol (1.032 g, 8 ml) was added dropwise over 15 min. The mixture was left to stand for a day at room temperature, evaporated to dryness in vacuo, redissolved in 10 ml of benzene and extracted with 2M HCl (3 x 15 ml). After cooling in ice the bulked acid extract was treated carefully with 10M NaOH (10 ml). The liberated base was extracted with ether (3 x 20 ml) dried over Na₂SO₄, evaporated in vacuo to 5 ml and treated carefully with a solution of HCl in ethanol (7.5M) until a slight excess of acid was present. Crystallisation of the resulting oil was induced by evaporating the mixture almost to dryness in vacuo and the addition of 3 ml of acetone. The oil dissolved and crystals of (+)-2-methylamino-1-oxo-1-phenyl-[1-¹⁴C]-propane hydrochloride (348 mg, m.p. 176-178°C with a red melt characteristic of amino-ketones, lit. m.p. 176-177°, Hyde Browning & Adams, (1928)) soon appeared.

The above aminoketone hydrochloride was dissolved in 20 ml of absolute ethanol in which was suspended 350 mg of 5% palladium on charcoal and reduced with hydrogen at a pressure of 210 KN/m² (30 lbf/in²) for three hours. The

catalyst was filtered off (Whatman No. 44 paper), the solvent evaporated to a small volume and acetone added. White crystals of $(\pm)$ -2-methylamino-1-phenyl- ^{14}C -propan-1-ol hydrochloride ($(\pm)$ - ^{14}C -ephedrine hydrochloride) soon crystallised out from the mixture (264 mg, m.p. 185-187°C, lit. 189-190°C, Hyde, Browning & Adams (1929); specific radioactivity 0.22 $\mu\text{Ci}/\text{mg}$). The amino alcohol was further characterised by its i.r. spectrum.

Resolution of the $(\pm)$ - ^{14}C -ephedrine hydrochloride was performed in a manner outlined by Nagase *et al.* (1967) after the method of Manske and Johnson (1929). $(\pm)$ -Ephedrine hydrochloride (0.264 g of radioactive compound + 0.348 g of non-radioactive compound) was dissolved in 2 ml of water and treated with 10M NaOH (1.0 ml). The liberated ephedrine base was extracted with diethyl ether (3 x 10 ml) and dried over anhydrous Na_2SO_4 . The solvent was removed under reduced pressure and the base dissolved in 2 ml of ethanol. $(-)$ -Mandelic acid was added to the mixture with stirring and on standing at 5°C for several hours the $(-)$ -ephedrine- $(-)$ -mandelate crystallised. This was filtered and recrystallised from ethanol to give 0.307 g of this salt (m.p. 166°C; Manske & Johnson give 167-169°C). Treatment of the salt, after suspension in 2 ml of water, with excess 10M NaOH liberated ephedrine base which was extracted with ether (3 x 10 ml) and the extract washed with water (3 x 2 ml) and dried over anhydrous Na_2SO_4 . A slight excess of an ethereal solution of HCl gas was added. Evaporation of the ether to 5 ml caused the crystallisation of $(-)$ - ^{14}C -ephedrine hydrochloride (0.180 g), m.p. 210°C (Nagai & Kanao (1929) give 216-217°C for the non-radioactive compound). The radiochemical purity as determined by paper chromatography was 98%, and the optical purity by reverse isotope dilution (Evans *et al.*, 1963) with authentic $(-)$ -ephedrine was 99%. The optical rotation in water of a sample of $(-)$ -ephedrine hydrochloride prepared from non-radioactive $(\pm)$ -ephedrine in the same way as the

radioactive material was $[\alpha]_D^{20} = -33.7^\circ$, $C = 3.85$, Nagai and Kanao (1929)

give $[\alpha]_D^{20} = -34.5^\circ$, $C = 9.0$ for the natural product.

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