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METABOLIC N- AND C-OXIDATION: IN VITRO AND IN VIVO STUDIES OF AMPHETAMINE ANALOGS

by

© GARY WESLEY DAWSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

IN

BIOPHARMACY

(DRUG METABOLISM)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA

SPRING 1977

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Metabolic N- and C-oxidation ~~-~~ In vitro and in vivo studies of amphetamine analogs" submitted by Gary Wesley Dawson in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biopharmacy.

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DEDICATION

To Barbara,

for unending patience and understanding.

ABSTRACT

The *in vitro* and *in vivo* metabolism of N-(n-propyl)amphetamine (NPA) and some related amphetamines was investigated.

In vitro metabolism of (±)-NPA at pH 7.4 using the 12,000 x g supernatant of homogenized rat liver fortified with the normal cofactors but *not* nicotinamide, resulted in the formation of various products including two N-oxygenated compounds identified as N-hydroxy-1-phenyl-2-(n-propylamino)propane, *i.e.* N-hydroxy-NPA, and N-[(1-methyl-2-phenyl)ethyl]-1-propanimine N-oxide, *i.e.* NPA-nitrone. These two metabolites were identified by comparison of their t.l.c., g.l.c. and combined g.l.c./m.s. properties with those of authentic samples of each. Other metabolites identified in the same manner were amphetamine, phenylacetone, phenylacetone oxime and a previously unreported metabolite, 2-(2-hydroxypropylamino)-1-phenylpropane. Methods employed in the quantitation of *in vitro* metabolic N-oxidation are discussed in detail.

Increasing the pH of the incubation mixture from 7.4 to 8.4, or *in vivo* pretreatment of rats with 3-methylcholanthrene resulted in an increase in the *in vitro* metabolic N-oxidation of NPA, whereas pretreatment with phenobarbital or NPA did not. The presence of SKF 525-A in incubation mixtures reduced the amount of C-oxidation and to a lesser degree, N-oxidation. Including nicotinamide in *in vitro* incubation mixtures significantly reduced N-oxidation of NPA.

When (+)-NPA or (-)-NPA was used as the substrate in vitro, the rate of C-oxidation was greater with (+)-NPA than (-)-NPA whereas the contrary was true for N-oxidation. The presence of EDTA or ascorbic acid in the incubation mixtures had little effect on the N-oxidation of NPA.

In vivo metabolism in the rat of NPA or its analogs, i.e. amphetamine, N-methylamphetamine (NMA), N-ethylamphetamine (NEA), or N-n-butylamphetamine (NBA), resulted in the formation of phenolic metabolites identified as the para-hydroxylated derivatives of the respective substrates. In the case of NPA, NEA, and NBA, a previously unreported ring-hydroxylated and methoxylated metabolite was also identified in urine extracts by g.l.c. and combined g.l.c./m.s. and by comparison to authentic samples of each.

The metabolism of 1-phenyl-2-propanone oxime, a known metabolite of amphetamine, was also investigated. In vitro metabolism of this oxime with the 12,000 x g supernatant from homogenized rat liver gave one major and two minor metabolites which were identified as 2-nitro-1-phenylpropane, benzyl alcohol and 1-phenyl-2-propanone, respectively, by means of g.l.c., combined g.l.c./m.s., and by comparison with authentic samples of each.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to Dr. R. T. Coutts, who, as a friend and supervisor, provided direction to, and a personal interest in me, as a person and graduate student.

To the other members of my committee, the late Dr. C. W. Nash, Dr. L. I. Wiebe, Dr. R. A. Locock, Dr. M. W. Wolowyk and Dr. D. A. Cook, I wish to express my appreciation for their comments and continued interest during the course of this study.

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I. INTRODUCTION

Drug metabolism or drug biotransformation may be defined as a study of the chemical modification of drugs or other foreign compounds (xenobiotics) by enzyme systems. It can be studied in either in vivo, i.e., whole body experiments, or in vitro where specific enzyme systems are isolated from tissues or organs. The in vitro system is of particular value when the substrate in question is toxic or carcinogenic in the whole animal or if individual metabolites or metabolic steps are of interest.

Azo-reduction and oxidative N-demethylation of aminoazo dyes (fig. 1) were among the first examples of in vitro metabolism of xenobiotics by homogenized liver tissue (1,2). These experiments and others (3,4) demonstrated that there was a specific fraction of a liver homogenate that could be obtained by differential centrifugation which was responsible for the metabolism observed. The portion of the homogenized liver used in these in vitro metabolism studies is now commonly referred to as the microsomal fraction or the 10,000 x g supernatant. It required nicotinamide adenine dinucleotide phosphate ($NADP^+$), nicotinamide adenine dinucleotide (NAD), Mg^{++} and molecular oxygen for maximal metabolic activity.

Microsomal fractions can be obtained from tissues other than liver although in vitro metabolism studies generally employ liver preparations.

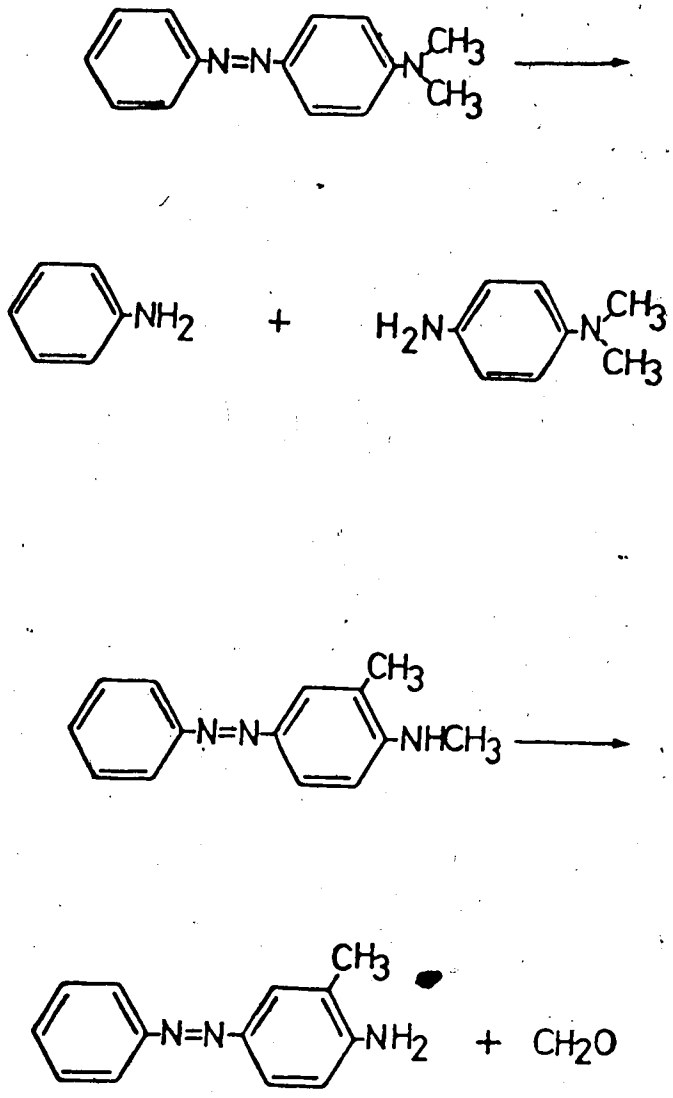


Fig. 1. The reductive cleavage of 4-dimethylaminoazobenzene (top) and N-demethylation of 3-methyl-4-monomethylaminoazobenzene (bottom) by rat liver homogenates.

Among the other sources of microsomes are lung, adrenals, brain, testes, and kidney (5-7).

The microsomal fraction is not the only portion of the liver homogenate that is of interest in drug metabolism. Purified microsomal protein is obtained by ultracentrifugation (100,000 x g for 60 min) of the 10,000 x g supernatant (fig. 2). The so-called microsomal pellet obtained by this method is used to study the properties of cytochrome P-450 (8-10), to determine microsomal protein (11), and as a source of purified enzymes for kinetic studies (9,12). Although some metabolic reduction is catalyzed by microsomal enzymes, reductases tend to be localized in the mitochondria (13), which can be isolated as shown in fig. 2. The supernatant obtained by centrifugation of a liver homogenate at 600 x g has been used as an adjunct in subcellular localization of enzymatic activity but is of little use in other studies of drug metabolism due to its relative impurity (11).

Brodie et al. (3) demonstrated that it was the reduced form of $NADP^+$ (i.e. NADPH) that was required for the activity of the microsomal system and that NADPH could be generated from $NADP^+$ in the presence of glucose-6-phosphate (G-6-P) and the enzyme glucose-6-phosphate dehydrogenase (fig. 3). This requirement for NADPH complicates experiments which utilize the 100,000 x g microsomal pellet rather than the 10,000 x g supernatant. Glucose-6-phosphate dehydrogenase is present in the 10,000 x g supernatant but is lost

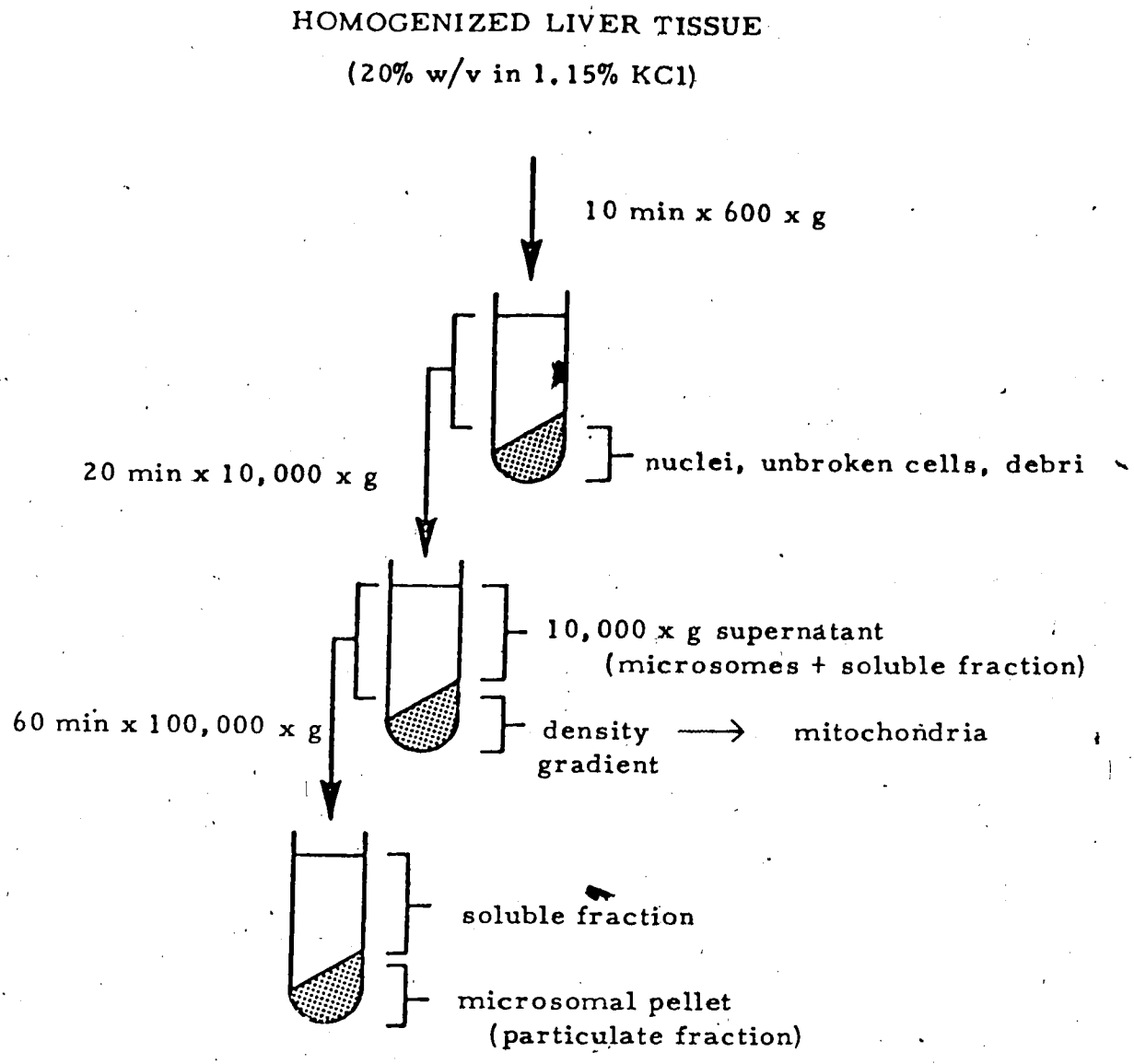


Fig. 2. Schematic representation of the methods used to prepare various fractions of liver homogenate for use in in vitro drug metabolism studies.

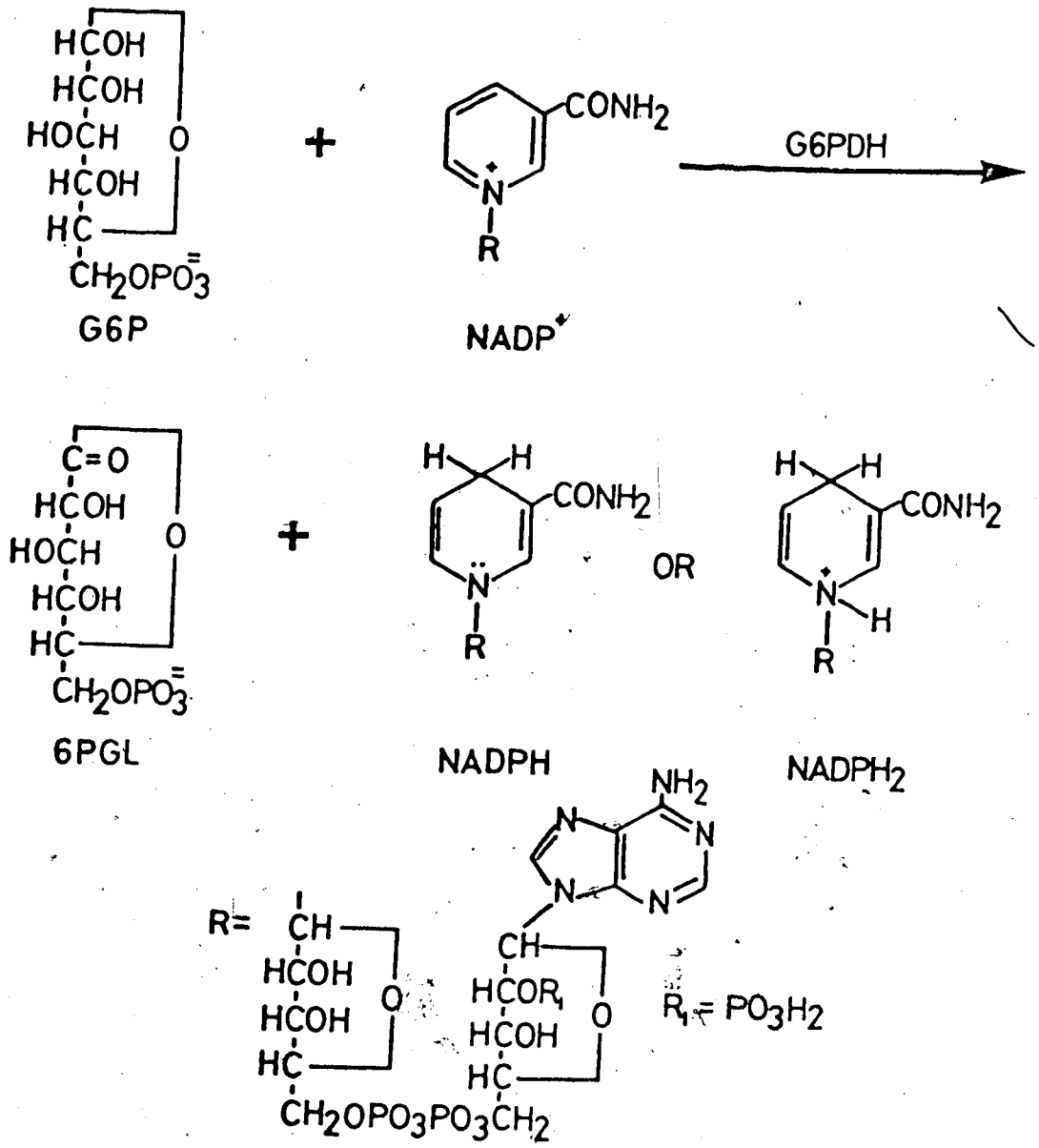


Fig. 3. Schematic representation of the relationship between glucose-6-phosphate (G-6-P) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) and the production of reduced nicotinamide adenine dinucleotide phosphate (NADPH). G6PDH: glucose-6-phosphate dehydrogenase. 6PGL: 6-phosphogluconolactone.

when the 100,000 x g pellet is prepared (11). As a result, glucose-6-phosphate dehydrogenase must be added, along with G-6-P and $NADP^+$, to the solutions when the resuspended 100,000 x g pellet is used. NADPH may be added directly to the incubation mixture but due to its relative instability (i.e. conversion to $NADP^+$) it must usually be added in aliquots over the duration of the incubation.

Since these early reports, numerous studies of the microsomal enzyme systems and drug metabolism have been published. Detailed treatment of the more current advances can be found in the published proceedings of two symposia devoted to microsomes and drug oxidations (14,15).

Microsomes are known to be involved in a wide variety of metabolic oxidative reactions including deamination, O-, N-, and S-dealkylation, aliphatic C-oxidation, ring hydroxylation and N-oxidations. Examples of these and other metabolic reactions can be found in recent reviews (13,16,17). Azo- and nitro-reductases are also present to a certain extent in tissue microsomal fractions.

The terms "mixed-function oxidase" or "mono-oxygenase" have been frequently used to describe the microsomal drug metabolizing system. Both of these terms simply mean that for every molecule of oxygen consumed during the course of a metabolic oxidation, one atom is incorporated into one molecule of substrate and one atom undergoes reduction to water (18). Although the term mixed-function oxidase (MFO) is non-descriptive, much is known of the

chemistry of the reactions involved in drug metabolism. It is thought that MFO is a complex arrangement of enzymes and enzyme cofactors of which some component (identified as 'A' in scheme 1) is reduced by NADPH. This reduced component (AH_2) then reacts with molecular oxygen to form what is referred to as an "active oxygen" intermediate. It is generally thought that this active intermediate can then react with a substrate and subsequently transfer the oxygen to that substrate. Based on this theory, Gillette (19) formulated the reaction sequence illustrated in scheme 1. A major limitation of this sequence is that it does not explain the mechanism of oxygen incorporation into the substrate.

$$\text{NADPH} + \text{A} + \text{H}^+ \longrightarrow \text{AH}_2 + \text{NADP}^+$$

$$\text{AH}_2 + \text{O}_2 \longrightarrow \text{ACTIVE OXYGEN}$$

$$\text{ACTIVE OXYGEN} + \text{DRUG} \longrightarrow \text{DRUG-OH} + \text{A} + \text{H}_2\text{O}$$

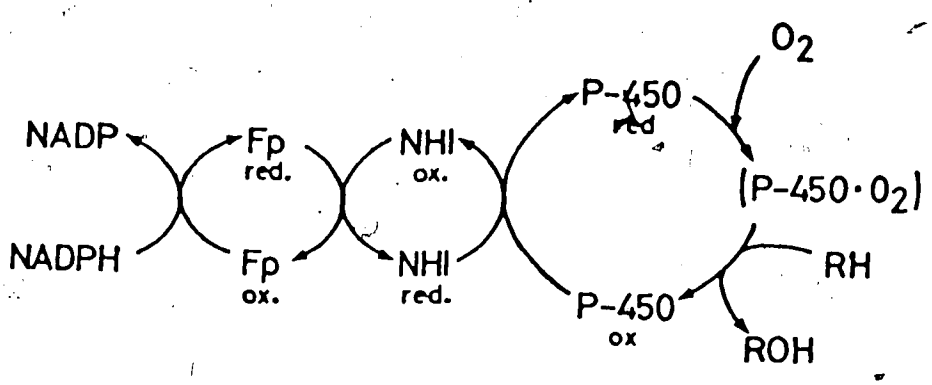
$$\text{NADPH} + \text{O}_2 + \text{DRUG} + \text{H}_2\text{O} \longrightarrow \text{NADP}^+ + \text{H}_2\text{O} + \text{DRUG-OH}$$

Scheme 1

It has long been accepted that the hemoprotein cytochrome P-450 (usually simply termed P-450) plays a major role in microsomal drug metabolism and it is now believed that P-450 is the identity of "A" in scheme 1. C-oxidation and to some extent N-oxidation require the involvement of P-450 but most N-oxidative steps, i.e.

N-hydroxylation of phenylalkylamines, appear to involve an enzyme system other than P-450. This alternative N-oxidation enzyme will be discussed later. P-450 is abundant not only in liver microsomes but also in microsomes and mitochondria of the adrenal cortex (20,21), kidney (5,6), intestinal mucosa (5) and mitochondria of the corpus leuteum (22).

The precise relationship between NADPH oxidation and P-450 reduction was not clearly understood until Omura et al. (23,24), working with beef adrenal cortex, were able to separate P-450 from other hemoproteins. They prepared a particulate fraction containing only P-450 and a soluble fraction which retained the activity of the NADPH-cytochrome P-450 reductase system. The soluble fraction contained non-heme iron (NHI) and a flavin-adenine dinucleotide (FAD)-linked flavoprotein (now referred to as cytochrome c reductase) both of which were required for reconstitution of the NADPH-cytochrome P-450 reductase activity. On the basis of this information, Omura postulated that the components of the soluble fraction (i.e., NHI and cytochrome c reductase) were involved in P-450 reduction and [illegible] the pathway shown in scheme 2 to illustrate the transfer of electrons from NADPH to P-450 and subsequent oxidation of a substrate. Although this theory is based on information from adrenal tissue, other evidence suggests that the system is also present in hepatic tissue (25).



Scheme 2

This scheme, however, does not explain how the oxygen is incorporated into the substrate. A more complete representation of the function of P-450 and the mechanism of oxygen incorporation into the substrate (R-H) can be visualized from figure 4. This scheme suggests that a P-450-substrate complex is formed <u>before</u> oxygen is incorporated. This, however, is in direct contradiction to the scheme of Omura (scheme 2).

Figure 4 was devised by Coutts (26) from literature data (27) and illustrates how P-450 superoxide is believed to be involved in metabolic C-oxidation. In agreement with the earlier mechanisms proposed for metabolic oxidation (<u>vide supra</u>) the essential features of the sequence depicted in figure 4 are the involvement of two one-electron transfer reactions, and the cleavage of the oxygen molecule such that one atom appears in the substrate and the other as water.

(Explanation Fig. 4). Cytochrome P-450 (a) in the oxidized state, combines with a substrate, RH, to form a complex which exhibits uv spectra characteristic of the mode of binding with RH, i.e. type I or type II binding spectra. The mechanism of complexation is not known but thought to involve hydrogen bonding. The subsequent reaction (b) involves the transfer of one electron, presumably from NADPH and cytochrome c reductase, and reduction of P-450. This electron transfer is believed to be the rate-limiting step in the overall cycle. Reduced P-450 can be quantitated by exposure to carbon monoxide (c) followed by uv spectral measurement.

The reduced form of the P-450-substrate complex (d) now reacts with molecular oxygen to produce e which is not believed to be sufficiently reactive to mediate C-oxidation. A second electron transfer, believed to come from NADH reduction of cytochrome b_5 and subsequent transfer to e, alters the oxidation state of e and allows formation of a reactive free radical species (f) which may be in equilibrium with g. Structures f and g are sometimes referred to as P-450 superoxide. Subsequent protonation to h results in oxidation of substrate at which point, ROH, water and reduced P-450 (a) are produced.

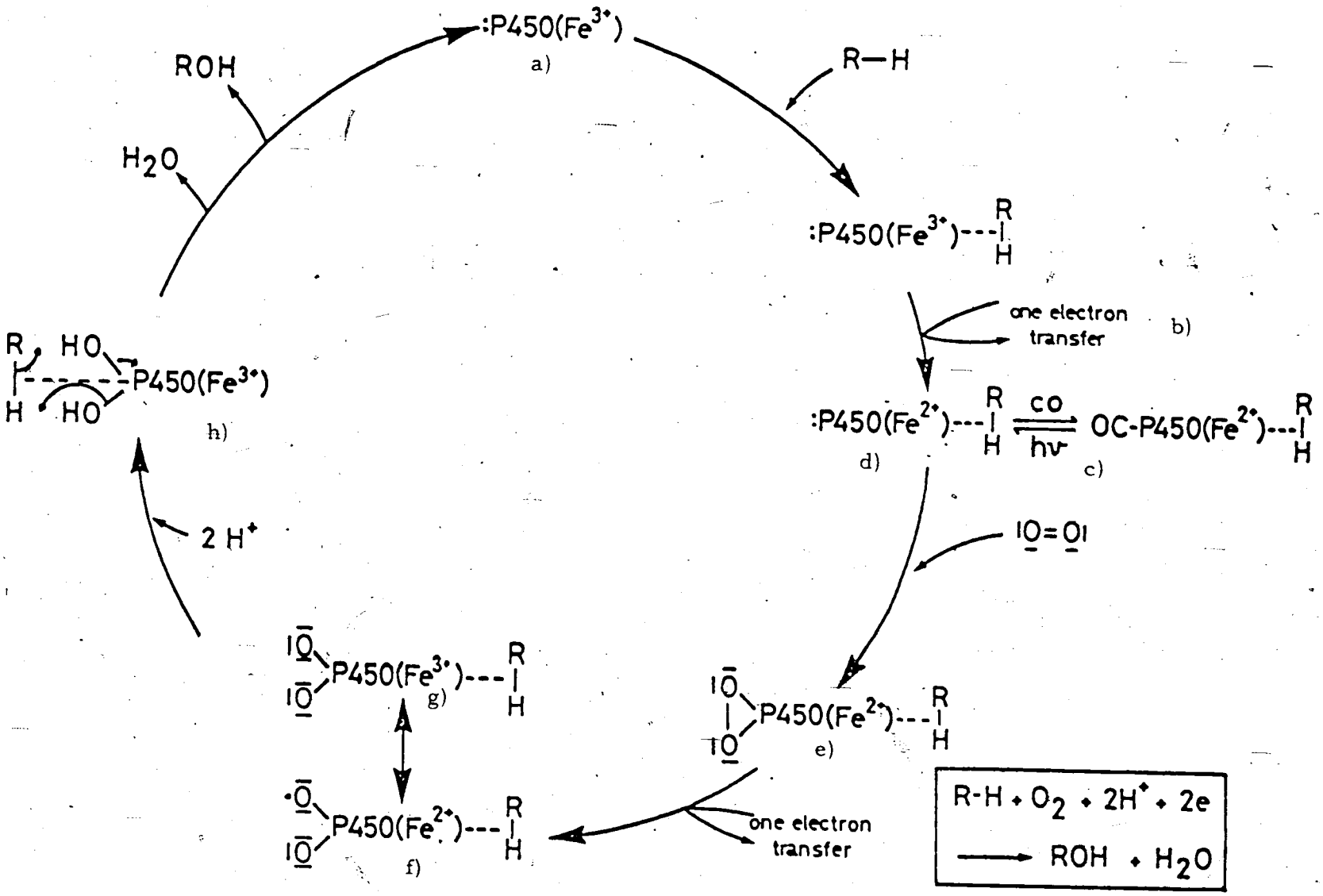
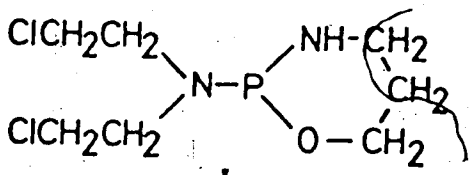


Fig. 4. Schematic representation of the involvement of cytochrome P-450 in metabolic C-oxidation. See opposite page for explanation.

The pathways represented in schemes 1 and 2 suggest that equivalent amounts of NADPH, oxygen and substrate are utilized in the reaction. Although a stoichiometric relationship has been observed for the metabolism of some substrates (28-30), others deviate from this rule (31, 32). This discrepancy may at least in part be explained by the involvement of another heme molecule, cytochrome b_5 (31). As pointed out by earlier investigators (1, 2) NADH is required for certain microsomal oxidative reactions. It is now thought that cytochrome b_5 may be reduced by NADH analogous to the NADPH reduction of cytochrome c (Fp in scheme 2). Cytochrome b_5 may then in turn supply the second electron required for P-450 mediated oxidation as shown in figure 4. Sasame et al. (33) have published evidence in further support of this view. The difference in stoichiometric requirements for the metabolism of various substrates may in part be explained by a recent observation of the existance of at least four distinct forms of P-450 each of which exhibit preferential binding to different substrates (34). That more than one "cytochrome P-450" may be involved in drug metabolism has been suspected for some time from information derived from spectral studies but this is the first report of the isolation of several forms of P-450 where the spectral properties are all identical. It is conceivable that the dependence of individual forms of P-450 on cytochrome b_5 for the second electron reaction may vary and thus explain the stoichiometric differences

seen with different substrates. Further, the occurence of several forms of liver microsomal P-450 with different subunit molecular weights may account for the variety of metabolic activities attributed to this cytochrome.

Induction and inhibition of microsomal drug metabolizing enzymes and their resultant effects on the duration and intensity of action of many drugs have been the subjects of numerous reviews (35-40). For the most part, the significance of the metabolic pathway of a given drug depends largely upon whether or not the metabolite is more pharmacologically active or toxic than the parent compound. For example, cyclophosphamide (I) is inactive as an alkylating agent until it is metabolized by the liver by cleavage of the cyclic P-N bond or P-O bond followed by cleavage of the phosphamide bridge (41). Loss of liver metabolic function therefore decreases the effectiveness, and enzyme induction increases the toxicity of cyclophosphamide.



I

Many drugs or chemicals may induce or enhance their own metabolism or the metabolism of other compounds. Two such compounds of particular

interest are phenobarbital (PB) and 3-methylcholanthrene (3-MC). These two compounds differ in their mechanism of induction and in the types of enzymatic reactions they enhance (38, 42). Treatment of animals *in vivo* with either of these compounds leads to increased metabolic activity, with PB having a somewhat more non-selective, broader range of inducing capability than 3-MC. Administration of both simultaneously produces an additive inducing effect. There are several theories of enzyme induction but they are generally based upon either increased *de novo* synthesis of protein or a decreased turnover of existing protein (36, 43).

Inhibitors of drug metabolism, like the inducers, vary markedly in their structures (fig. 5). Inhibition mediated by these compounds may occur in various ways (35), including:

1) combination with the enzyme at the active site (competitive inhibition) or at a site other than the active site (non-competitive inhibition, *e.g.* SKF 525-A);

or 2) alteration of coenzyme concentration by depletion or interference with synthesis;

or 3) interference with protein synthesis in general (antibiotics, *e.g.* Actinomycin D).

SKF 525-A is probably the most frequently used metabolic C-oxidation inhibitor. It is interesting to note that this compound is metabolically N-dealkylated to another potent inhibitor, SKF 8742-A (44). Chronic treatment of animals *in vivo* with SKF 525-A actually leads to enzyme induction but to a lesser degree than with PB (45). The significance and biochemistry of enzyme inhibition have been the subject of a number of reviews (35, 42).

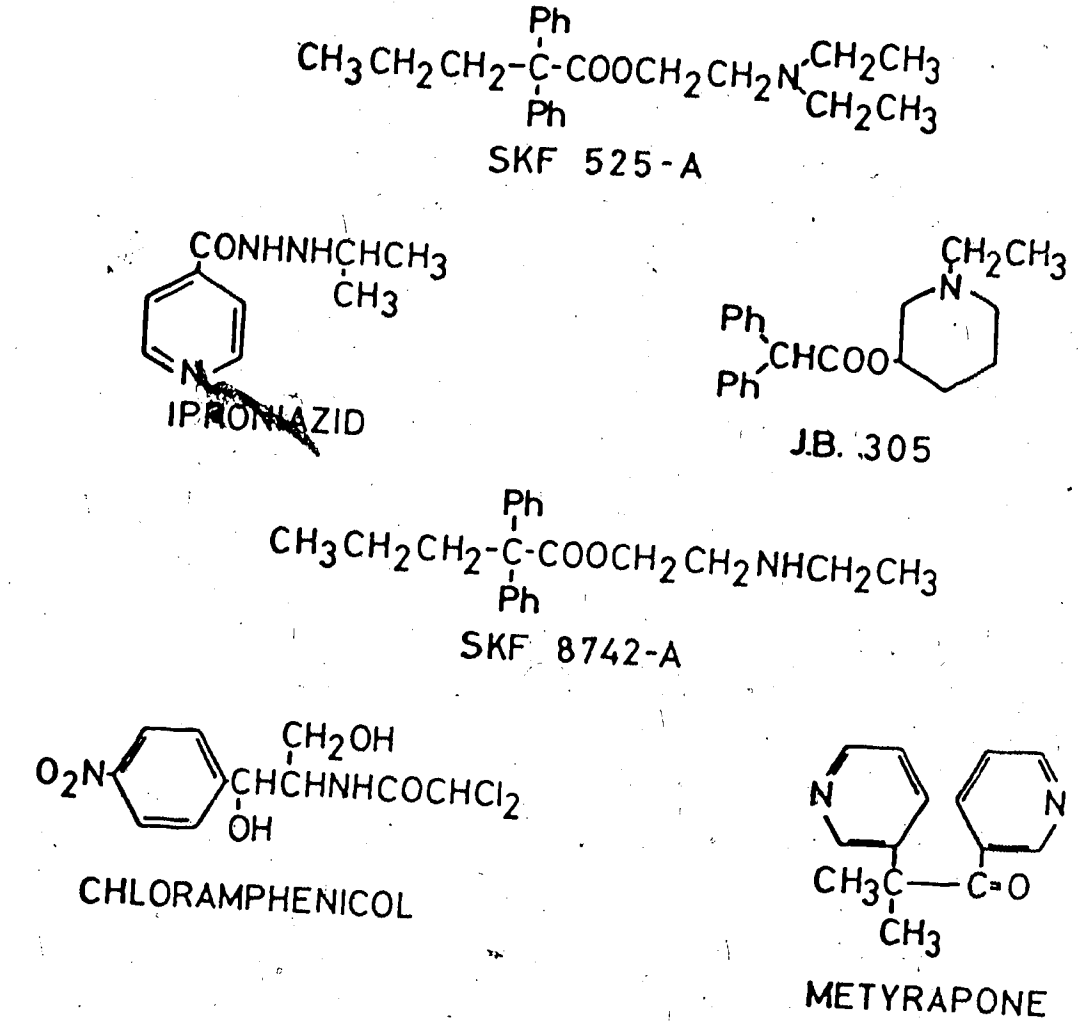


Fig. 5. Examples of some compounds which inhibit microsomal drug metabolism (see ref. 35,42).

The metabolism of foreign compounds is generally considered to be a detoxification mechanism in the excretory process. It is accepted for the most part that incorporation of an oxygen atom into a molecule tends to increase the water solubility and consequently promote more rapid excretion. Furthermore, most pharmacologically active compounds are inactivated as a result of their metabolism. Occasionally, however, the pharmacological activity and/or the toxicity of some foreign compounds increase as a result of metabolism (fig. 6; 46). The idea of metabolism altering the pharmacology of a compound has also been applied to producing drugs (termed pro-drugs) whose metabolites possess greater potency than the parent compounds. The use of metabolism in this manner to produce a pharmacologically active product is a means of circumventing physico-chemical problems which may be associated with administration, absorption, formulation, etc., of the active compound.

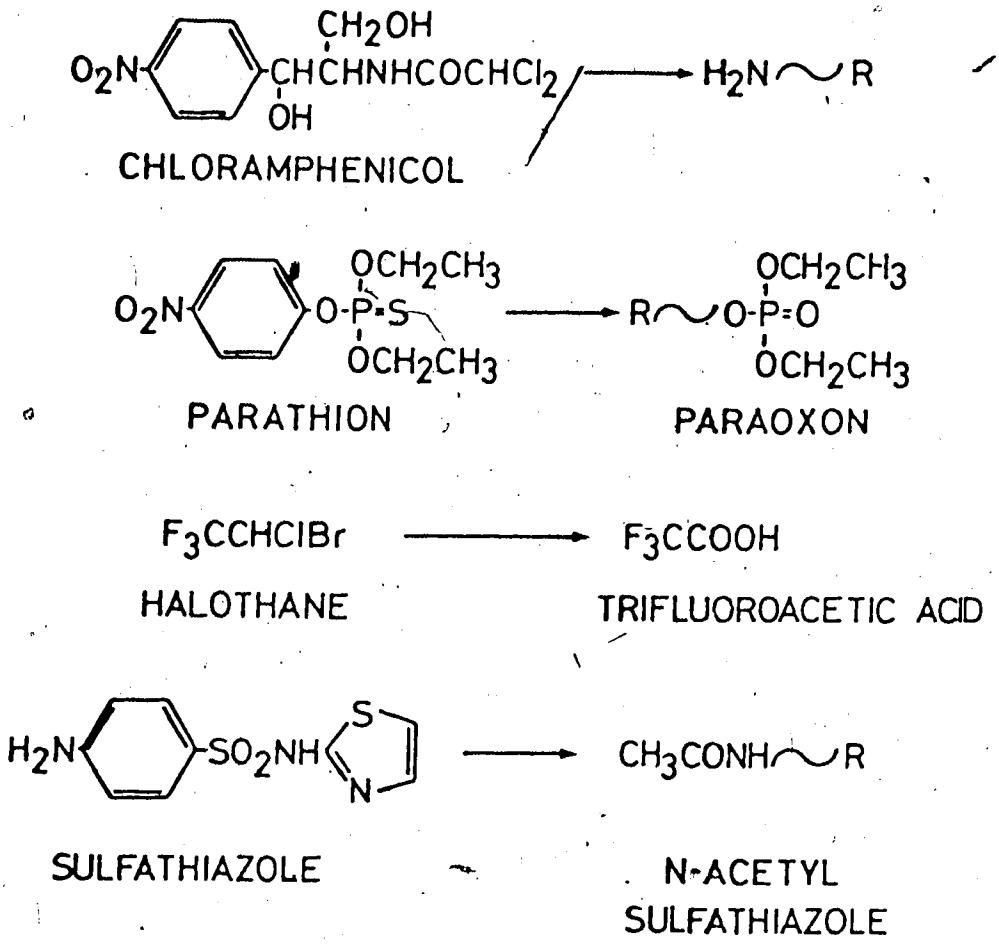
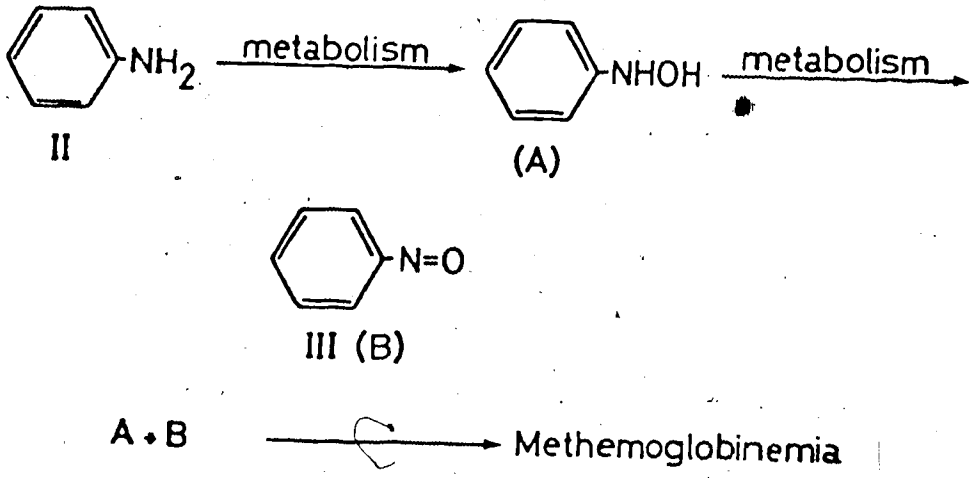


Fig. 6. Examples of the metabolism of drugs in which the metabolites are either more toxic or pharmacologically more active than the parent compound (see ref. 46).

II. LITERATURE SURVEY

A. Metabolic N-oxidation - toxicological implications

Early investigations of toxicities of organic compounds were attempts to explain why aromatic amines caused methemoglobinemia. In 1913, Heubner (47) proposed that aniline (II) was metabolically oxidized to a hydroxylamine which possessed a significant capability to oxidize hemoglobin. Little comment followed Heubner's hypothesis until 1959, when Kiese reported that there was indeed a relationship between methemoglobinemia and N-oxidation of aniline to nitrosobenzene (III, scheme 3; 48).



Scheme 3

Further interest in the toxicological implications of metabolic N-oxidation arose as a result of the discovery of N-hydroxy-2-acetylaminofluorene (IV) in the urine of rats which had been fed the

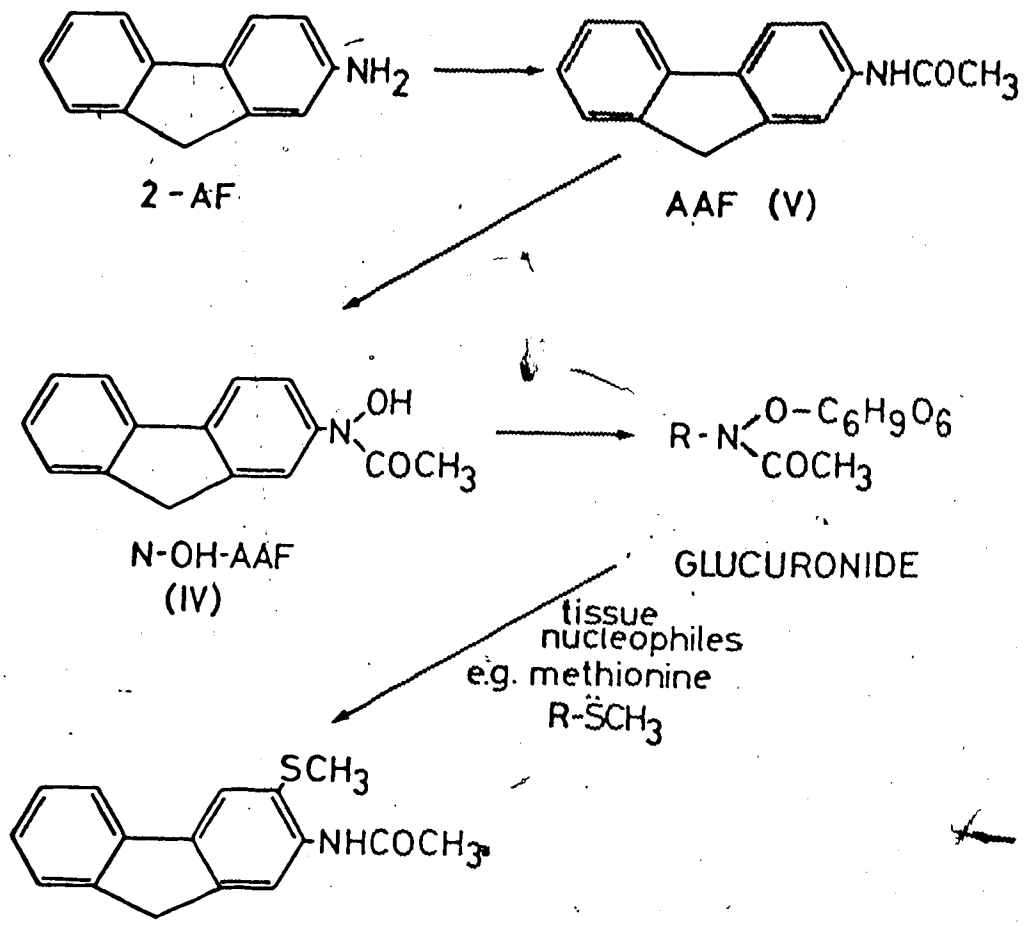


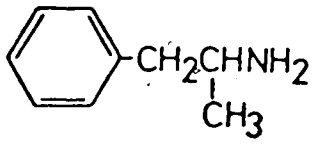
Fig. 7. Metabolism of 2-aminofluorene (2-AF) via N-acetylation (2-AAF) and subsequent N-oxidation to the proximate carcinogen (N-OH-AAF) which produces its effect by reacting with tissue nucleophiles.

known carcinogen, 2-acetylaminofluorene (AAF, V; 49). It could be shown that the N-hydroxy-AAF reacted strongly with tissue nucleophiles and was the proximate carcinogen (fig. 7; 50-52).

After this discovery, reports of other carcinogenic amines and amides began to appear (53-58) and it is now known that many amines undergo metabolic N-oxidation but the toxicity of most of these compounds is still open to question. Two excellent reviews summarize the recent knowledge of N-oxidation and its biological implications (59, 60).

B. Metabolic N-oxidation of aliphatic amines

Aromatic and tertiary aliphatic amines have undergone the most intensive investigations in metabolic N-oxidation studies (59, 60). For the most part these classes of compounds require the involvement of P-450 for N-oxidation to procede; the reaction is subject to the same induction and inhibition as other P-450 mediated reactions (61). It was not until 1971 (62) that metabolic N-oxidation of a primary aliphatic amine, amphetamine (VI) was reported. Since that time



VI

numerous reports have appeared indicating that N-oxidation of primary and secondary aliphatic amines is a general in vitro metabolic route (63-70).

Zeigler et al. (71) reported the isolation of an enzyme from pig liver microsomes which catalyzed N-oxidation but was free of any detectable P-450. Now termed amine oxidase, this enzyme contains FAD and can oxidize a wide variety of tertiary amines (i.e. amines of group Ic and IIc, fig. 8) to the corresponding N-oxides, and secondary amines (i.e. amines of group Ib and IIb, fig. 8) to their corresponding hydroxylamines. Gorrod (72) pointed out that despite a lack of definitive studies, it can be concluded from available reports that the pKa of the nitrogen function in a given molecule is an indication of whether P-450 or FAD-amine oxidase is the enzyme involved in metabolic N-oxidation. P-450 is not involved, for example, in the N-oxidation of primary and secondary aliphatic amines. Recently, Beckett and Bélanger (73) summarized the mechanism of flavine-mediated N-oxidation of primary (fig. 9) and secondary (fig. 10) aliphatic amines and concluded that there is a common metabolic N-oxidative pathway for primary and secondary aliphatic amines which involves an N-hydroperoxide intermediate.

1. In Vitro Metabolic N-Oxidation

In vitro metabolic N-oxidation of primary and secondary aliphatic amines produces a variety of N-oxygenated products. Some of these are true metabolites while others are decomposition products

	PRIMARY (a)	SECONDARY (b)	TERTIARY (c)
GROUP I (pKa 8-11)	RCH_2NH_2	RCH_2NHR'	$RCH_2NR'R''$
PRODUCT	RCH_2NHOH	$RCH_2N(OH)R'$	$RCH_2\overset{+}{N}(O)R'R''$
GROUP II (pKa 1-7)	$ArNH_2$	$ArNHR$	$ArNR'R''$
PRODUCT	$ArNHOH$	$[ArN(OH)R]$	$Ar\overset{+}{N}(O)R'R''$
GROUP III (pKa<1)	$RC(=O)NH_2$	$RC(=O)NHR'$	$RC(=O)NR'R''$
PRODUCT	$RC(=O)NHOH$	$RC(=O)N(OH)R'$	--

Fig. 8. Different types of organic nitrogen compounds and their metabolic oxidation products (from Ref. 72).

(Explanation Fig. 9). Metabolic alpha-C-oxidation of (a) yields the unstable carbinolamine (b) which readily eliminates ammonia to give the ketone (c) (cf. scheme 9). Metabolic N-oxidation may proceed via an initial complex (d) between the amine, flavoprotein and molecular oxygen. The rate of dissociation of this complex dictates whether a ketone or oxime or hydroxylamine is formed.

Rapid dissociation produces the zwitterion (e) and free flavoprotein; proton rearrangement then leads to the N-hydroperoxide (f). The N-hydroperoxide will decompose readily in aqueous neutral solution by three different routes. One route yields the imine (g) (cf. scheme 9) with hydrogen peroxide also being formed. The imine subsequently hydrolyzes to the ketone (c). A second route which proceeds via the unstable intermediate (h) produces the oxime (i). A third route yields the nitroso compound (j) which rearranges to the oxime.

If rapid dissociation of the complex (d) does not occur, the flavoprotein in the complex can be reduced by transfer of two electrons from NADPH to form the radical complex (k) containing FpH_2. The N and O free radicals will interact to give the zwitterion as a complex (l) with FpH_2; the latter can then reduce the zwitterion and dissociation follows to yield flavoprotein, one molecule of water and the N-oxide (m) which undergoes proton rearrangement to yield the hydroxylamine (n).

Scheme was adapted from Beckett and Bélanger (67, 73).

R=$ArCh_2$, Fp=flavoprotein, heavy arrows=metabolic steps, light arrows=chemical changes.

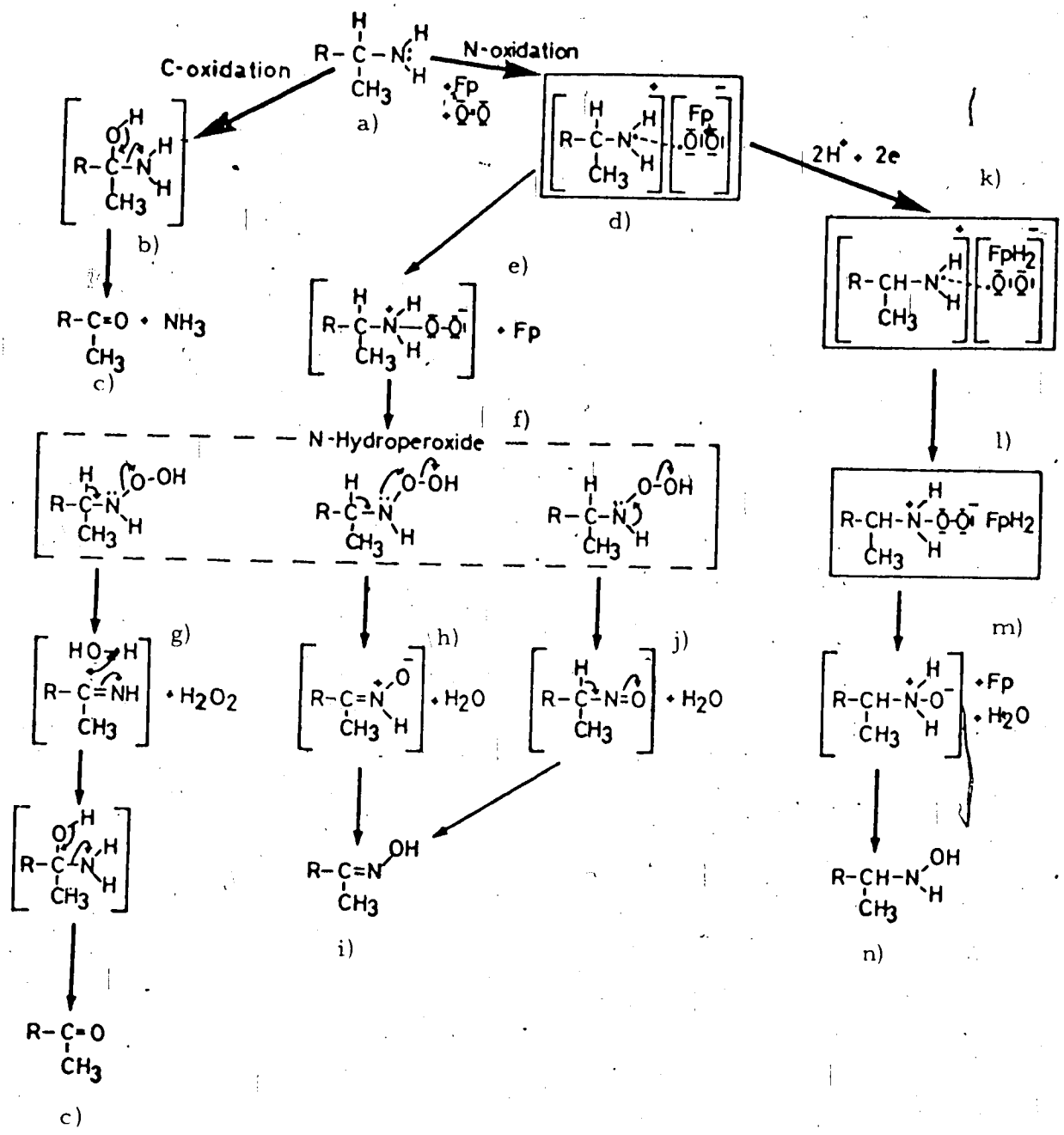


Fig. 9.- Schematic representation of the enzymatic and non-enzymatic steps in the metabolism of primary 'amphetamines'. See opposite page for explanation.

(Explanation Fig. 10). Metabolic alpha-C-oxidation of (a) can occur via two routes. One route yields the unstable alkylcarbinolamine (b) which rearranges under neutral aqueous conditions to the ketone (c) and a primary amine (d). The other route of alpha-C-oxidation will yield (e) which will rapidly eliminate the aldehyde (f) to give the primary amine (g).

N-oxidation will give the free radical ion complex (h) which will either dissociate to i or be reduced metabolically to j which proceeds via k and l to the secondary hydroxylamine (m). (cf. fig. 9). The dissociation of h to i will yield the unstable N-hydroperoxide (n), which can decompose in neutral aqueous solution in three different ways. One route will give hydrogen peroxide plus the unstable N-alkylimine (o) (cf. scheme 9) which readily hydrolyses to the ketone (c) and a primary amine (d). The N-hydroperoxide (n) may also decompose to the nitrone (p) which is readily converted by hydrolysis to the ketone (c) and a primary hydroxylamine (q). The third route leads to another nitrone (r) which is reasonably stable under neutral aqueous conditions if R' is other than H. If R' = H, then hydrolysis will occur in water to yield the primary hydroxylamine (s) and the aldehyde (f).

Scheme was adapted from Beckett and Bélanger (67, 73).

R=$ArCH_2$, R'=H, alkyl, aralkyl, Fp=flavoprotein, heavy arrows=metabolic steps, light arrows=chemical changes

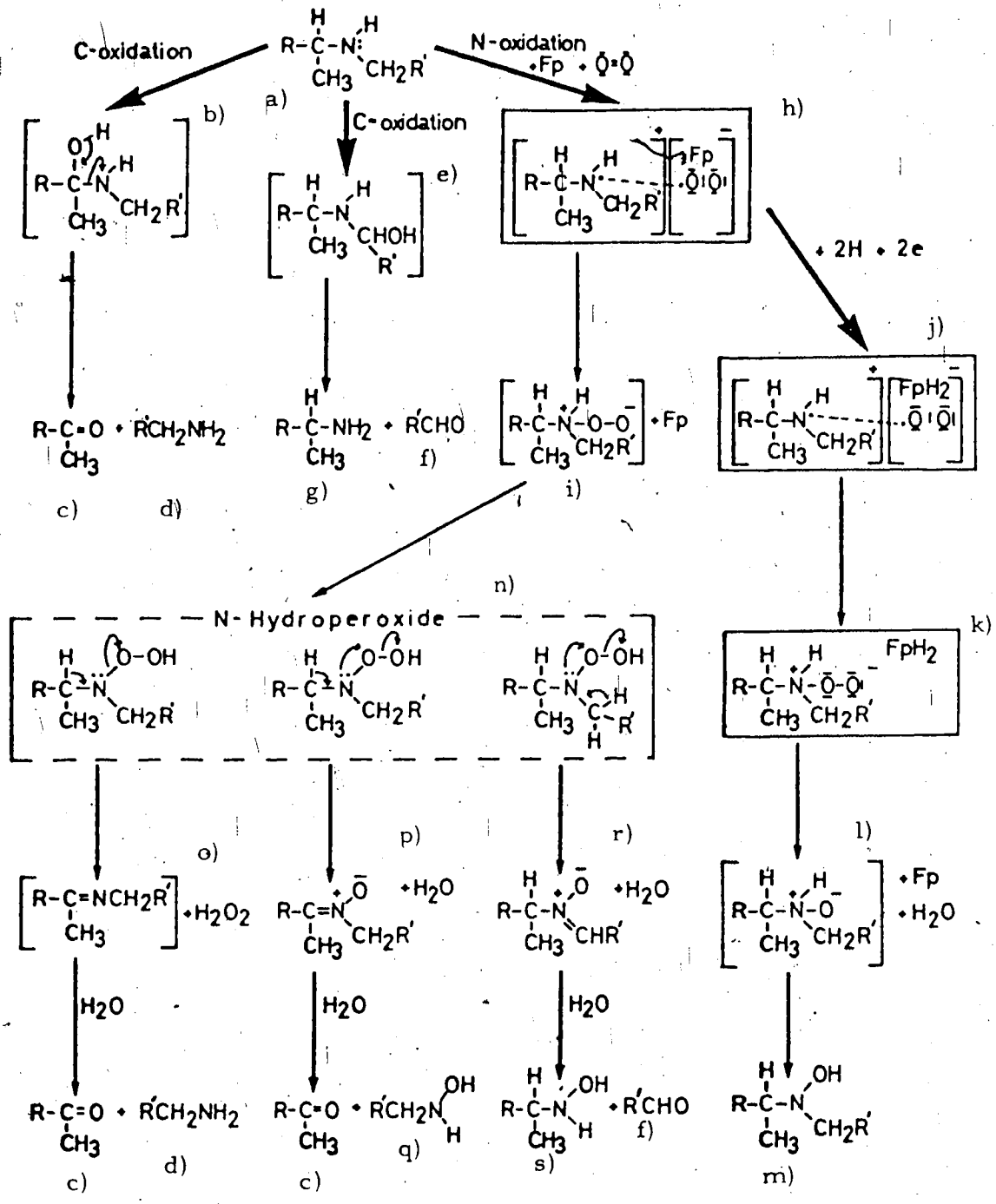
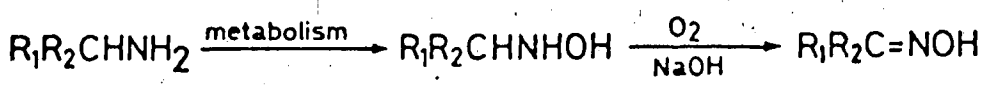


Fig. 10. Schematic representation of the enzymatic and non-enzymatic steps in the metabolism of secondary 'amphetamines'. See opposite page for explanation.

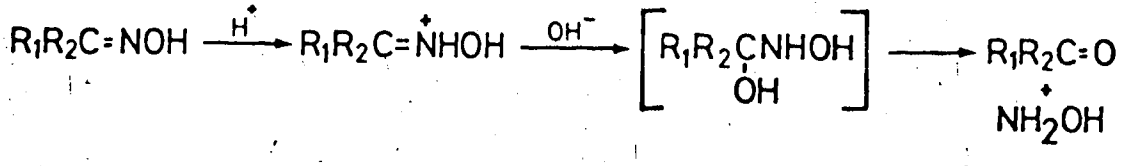
of the initial metabolites. Most frequently this decomposition is a result of the extraction or separation procedure employed (62-64).

Primary aliphatic amines are metabolized initially to the corresponding hydroxylamine (fig. 9). This metabolite is unstable especially when solutions are basified and shaken as is often the case during extraction procedures. This treatment converts the hydroxylamine to an oxime if the carbon atom alpha to the hydroxylamino group possesses a hydrogen atom (scheme 4). Oximes are sufficiently stable in



Scheme 4

alkaline solution to permit quantitative analysis. In acidic solutions they hydrolyze to the corresponding ketones (scheme 5; 62). If

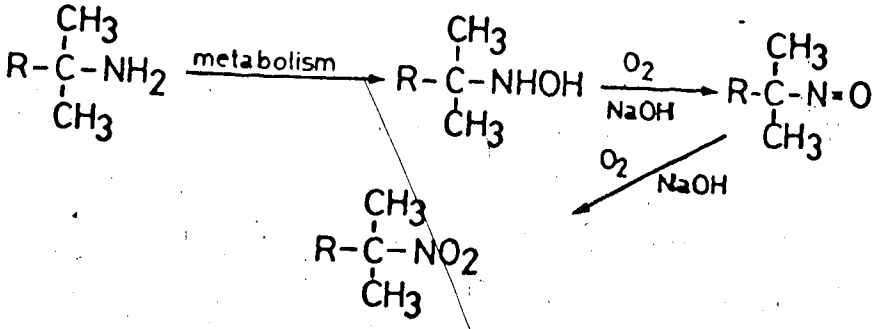


Scheme 5

the primary amine does not have a hydrogen atom on the alpha-carbon, e.g. phentermine (VII) or chlorphentermine (VIII), the initial N-oxygenated metabolic product, the hydroxylamine (65, 74, 75), is further oxidized to a nitroso compound and eventually to a nitro derivative when base is added (scheme 6; 66, 67).

$PhCH_2C(CH_3)_2NH_2$ VII

p-Cl$-PhCH_2C(CH_3)_2NH_2$ VIII



Scheme 6

When secondary aliphatic amines are used as substrates in vitro, the N-oxygenated product which may be isolated could be a secondary hydroxylamine, a nitrone, an oxime, or a primary hydroxylamine (62,63,68). The secondary hydroxylamine is the only true metabolite. The others are formed during the extraction and isolation procedures. A rationalization for their formation may be seen in fig. 10.

Many primary and secondary aliphatic amines have been shown to undergo in vitro metabolic N-oxidation to hydroxylamines (table 1). Extracts of incubation mixtures where these substrates have been used

TABLE 1. SOME PRIMARY AND SECONDARY ALIPHATIC AMINES KNOWN TO UNDERGO *IN VITRO* METABOLIC N-OXIDATION TO HYDROXYLAMINES.

Substrate	Metabolite	Species[1]	Ref.
N-Methyl-n-Octylamine	N-Hydroxy-N-Methyl-n-Octylamine	Rat	91, 92
(+)-Amphetamine	(+)-N-Hydroxyamphetamine	Rat, G.P., R	79
(-)-Amphetamine	(-)-N-Hydroxyamphetamine	Rat, G.P., R	79
Phenmetrazine	N-Hydroxyphenmetrazine	not stated	76
Phendimetrazine	N-Hydroxyphenmetrazine	not stated	76
Fenfluramine	N-Hydroxyfenfluramine	G.P., Rat	68
Phentermine	N-Hydroxyphentermine	R	65
Chlorphentermine	N-Hydroxychlorphentermine	R	64, 93
Phenethylamine	N-Hydroxyphenethylamine	R	64, 93
Propylhexedrine	N-Hydroxypropylhexedrine N-Hydroxynorpropylhexedrine	R, G.P.	54
Ephedrine	N-Hydroxyephedrine N-Hydroxynorephedrine	R, G.P.	94
Amitriptyline	N-Hydroxynortriptyline	R, G.P.	95

[1] R = rabbit, G.P. = guinea pig

can be shown to contain hydroxylamines using a combination of physical and chemical techniques including thin layer chromatography (t. l. c.), gas-liquid chromatography (g. l. c.), mass spectrometry (m. s.), and mild chemical oxidation and reduction (64, 69, 70, 76, 77). Figure 11 is a summary of the various methods used to indicate the presence of a secondary hydroxylamine metabolite in an extract. Similar methods may be used to identify primary hydroxylamines, but they form oximes rather than nitrones on mild oxidation.

2. In vivo metabolic N-oxidation

Although hydroxylamines are readily formed in vitro from medicinal primary and secondary aliphatic amines, conclusive evidence is lacking that N-oxidation is a general metabolic route in vivo. Lindeke et al. (65) have pointed out that in vivo, hydroxylamines are reduced to the parent amine. In addition, they may be chemically or metabolically converted to other products which escape detection. Reports have appeared which support in vivo N-hydroxylation of primary and secondary aliphatic amines (table 2). Caldwell et al. (78) have recently reported that as much as 22% of the dose of chlorphentermine (VIII) is excreted as the N-hydroxy metabolite in man. The apparent difficulty in determining the importance of N-oxidation in vivo stems from two points: (1) substrates utilized in earlier studies possessed a hydrogen on the carbon alpha to the nitrogen whereas chlorphentermine does not, thus affording greater stability to N-hydroxychlorphentermine, and (2) the significant species variation

TABLE 2. SOME PRIMARY AND SECONDARY ALIPHATIC AMINES CLAIMED TO UNDERGO *IN VIVO* METABOLIC N-OXIDATION TO HYDROXYLAMINES

Substrate	Metabolite	Species	Reference
Amphetamine Phentermine Chlorphentermine	N-Hydroxyamphetamine N-Hydroxyphentermine N-Hydroxychlorphentermine	Various	96
Phentermine	N-Hydroxyphentermine 2-Nitroso-1-Phenylpropane 2-Nitro-1-Phenylpropane	Man	66
Chlorphentermine	N-Hydroxychlorphentermine 1-(*p*-Chlorophenyl)-2-Nitroso-propane 1-(*p*-Chlorophenyl)-2-Nitropropane	Man	66, 78
(-)-N-Methylamphetamine	N-Hydroxy-N-Methylamphetamine N-Hydroxyamphetamine		97
Chlorpromazine	N-Hydroxy*nor*chlorpromazine	Man	69

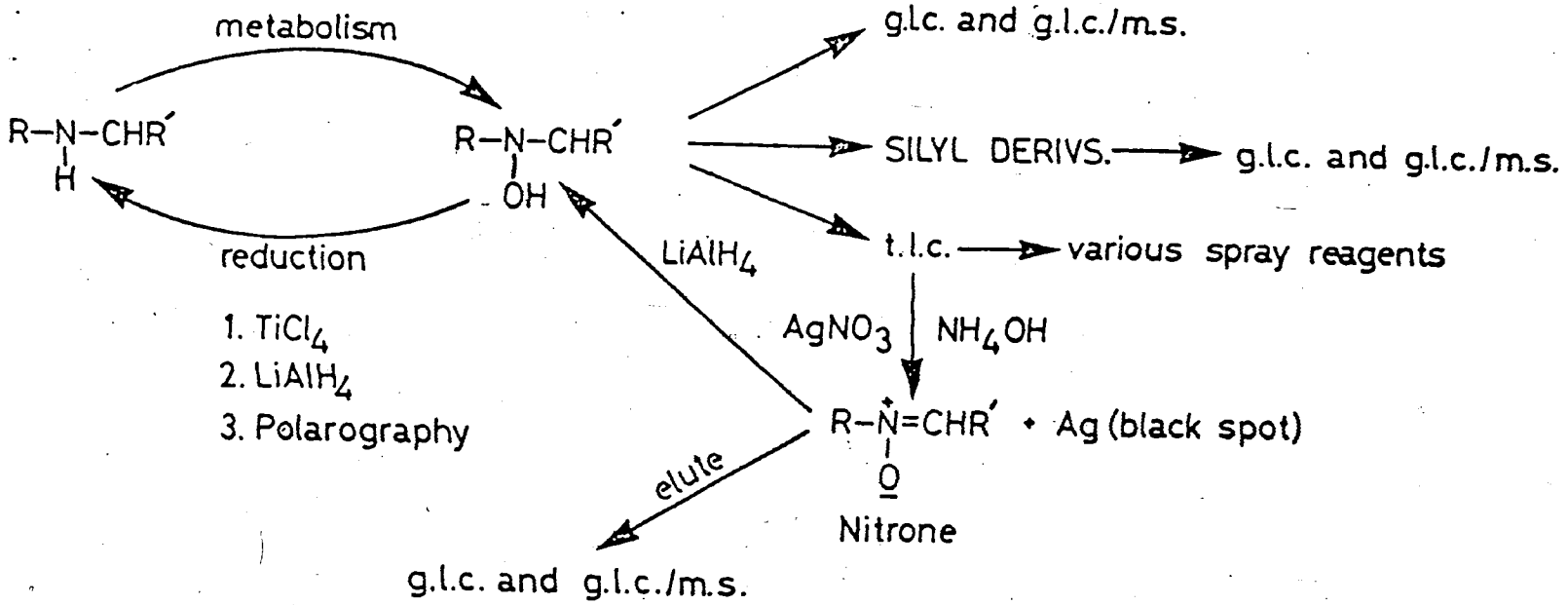
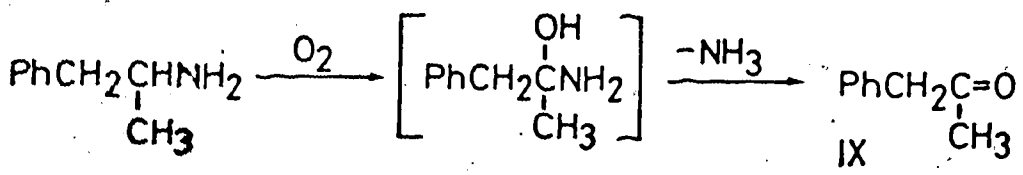


Fig. 11. Physical and chemical treatment of extracts to indicate the presence of secondary hydroxylamine metabolites.

in the extent of N-oxidation. It is also important to note that para-hydroxylation is a significant in vivo metabolic route for the "amphetamines" and this route is blocked in chlorphentermine by the presence of the chlorine in the para position.

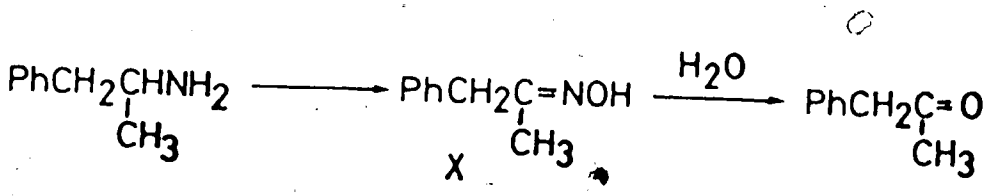
C. Metabolic Deamination of Amphetamine - Proposed Mechanisms

Phenylacetone (1-phenyl-2-propanone; IX) is an in vitro and in vivo metabolite of amphetamine (80-84). Much speculation has arisen on the mechanism of formation of this ketone. Brodie et al. (85) proposed that deamination of amphetamine proceeded via hydroxylation on the alpha-carbon and subsequent loss of NH_3 (scheme 7). This proposal remained popular until Hucker et al. (86)



Scheme 7

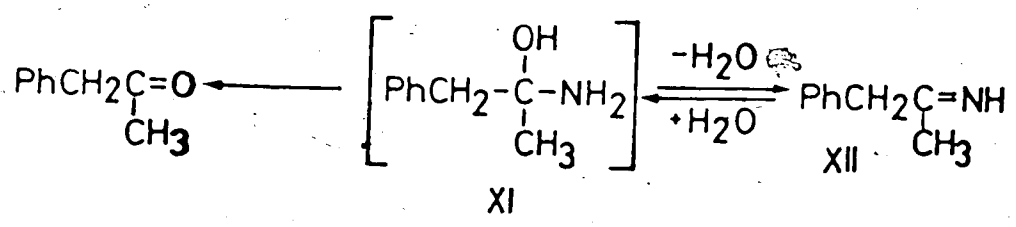
reported the isolation of phenylacetone oxime (X) from in vitro incubation mixtures containing amphetamine and rabbit liver microsomes. Hucker proposed that the oxime was the usual metabolic product of deamination of alpha-branched primary amines which subsequently hydrolyzed to phenylacetone (scheme 8). They did not dismiss the possibility that a hydroxylamine could be a precursor to the oxime but offered no proof that supported a hydroxylamine intermediate.



Scheme 8

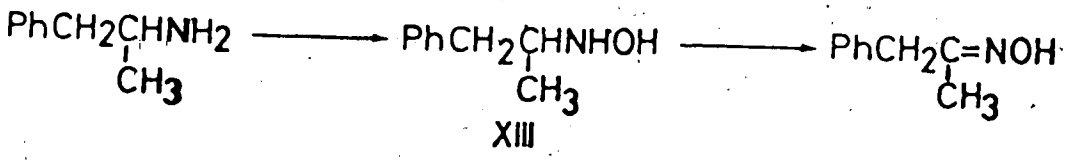
To resolve this discrepancy, Parli et al. (87, 88) performed experiments identical to those of Hucker et al. but utilizing ^{18}O in place of atmosphere. If the mechanism in scheme 8 was correct, the oxygen incorporated into phenylacetone would come from water and thus no ^{18}O would be present. If Brodie's theory (scheme 7) was correct, the ^{18}O would appear in phenylacetone.

Their results showed that indeed Brodie's theory was correct in that alpha-hydroxylation to the unstable carbinolamine (XI) preceeded deamination. However, more ^{16}O appeared in phenylacetone than could be explained on the basis of simple exchange between ^{18}O and ^{16}O. As a result, Parli and McMahon proposed a simple hydrolytic route to ketone via the reversible formation of imine (XII) from the initial hydroxylated intermediate (scheme 9).



Scheme 9

Studies by others (79) using (+)- and (-)-amphetamine appeared to confirm the alternative conclusion that it was N-hydroxyamphetamine which was formed initially from amphetamine and in turn oxidized to a mixture of syn- and anti-oximes (scheme 10). Beckett (63) considers oximes to be metabonates which he defines as metabolites



XIII

Scheme 10

which have undergone chemical or physical (not enzymatic) modification. The controversy over the mechanism of formation of the oxime has not yet been resolved (70, 77, 89) but it is now accepted by some authorities (78) that the oxime (X) is the metabolic precursor of the phenylacetone metabolite of amphetamine and its simple N-alkylated derivatives.

Oximes have been detected as in vivo metabolic products of amphetamines and related compounds (table 3). It seems reasonable to conclude that the oximes in vivo are formed in an analogous manner to that seen in vitro, i.e. via hydroxylamine formation. As a result, oxime formation in vivo could be construed to be circumstantial evidence in favor of in vivo metabolic hydroxylamine formation. However, quantitation of the oxime in vitro and possibly in vivo may be complicated in view of the fact that the oxime itself is further metabolized in vitro to a nitro compound (vide infra).

TABLE 3. SOME PRIMARY AND SECONDARY ALIPHATIC AMINES WHICH DEMONSTRATE IN VITRO AND IN VIVO OXIME FORMATION.

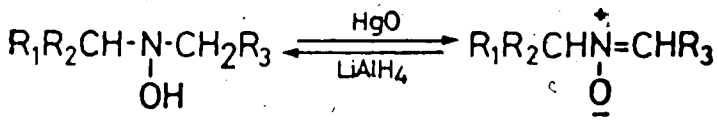
Substrate	Species[1] In Vivo	In Vitro	Reference
(+)-Amphetamine	R	R	86
(+)-Amphetamine (-)-Amphetamine (±)-Amphetamine	R, G. P.	R, G. P., Rat	62, 64, 79
p-Methoxyamphetamine		R, G. P.	70
Fenfluramine		Rat, G. P.	63, 68
(+)-N-Methylamphetamine (-)-N-Methylamphetamine (±)-N-Methylamphetamine	R, G. P.		62, 63, 97
(+)-N-Ethylamphetamine (-)-N-Ethylamphetamine (±)-N-Ethylamphetamine	R, G. P.		62, 63
Propylhexedrine	Man	R, G. P.	77

[1] R = rabbit, G. P. = guinea pig

D. Identification of products of metabolic N-oxidation

Nitrones have been identified as in vitro metabolic products of aliphatic secondary amines. The nitrone is the major in vitro metabolite of fenfluramine (table 4). It is believed that the nitrone is derived chemically from the hydroxylamine and is not a true metabolite.

Nitrones are easily identified by t.l.c., g.l.c., and by their chemical behavior (fig. 11). Their ease of formation from secondary hydroxylamines and their simple reduction back to secondary hydroxylamines (scheme 11) makes preparation and identification relatively simple (68,90).



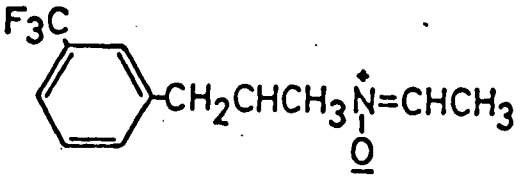
Scheme 11

1. t.l.c.

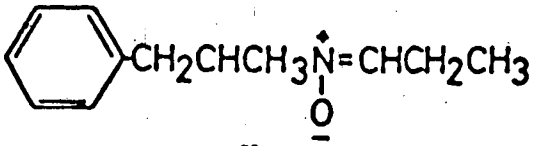
N-oxygenated metabolites of aliphatic amines can be separated by t.l.c. on silica gel G and identified by various detection methods (tables 5 and 6). Plates which incorporate a substance that fluoresces under uv light (254 nm) can be used to locate compounds which quench fluorescence. This method gives no indication of the chemical nature of the metabolite and in cases where two-dimensional chromatography is employed, "ghost" spots may result if the plate is exposed to uv

TABLE 4. SOME EXAMPLES OF IN VITRO NITRONE FORMATION.

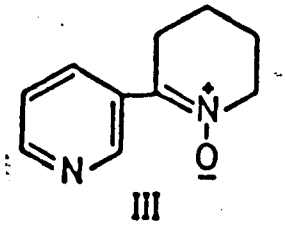
Substrate	Nitrone	Species	Ref.
Fenfluramine	I	guinea pig, rat	68,109
N-propylamphetamine	II	--	98
(-)-Anabasine	III	guinea pig, rabbit, rat	74
Phenmetrazine	IV	various	76,110
(+)-Methylamphetamine	V	guinea pig, rat	111



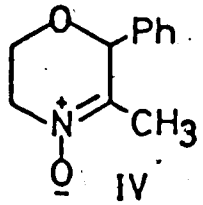
I



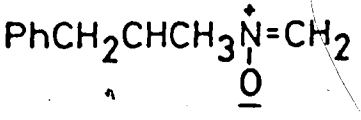
II



III



IV



V

TABLE 5. SOLVENT SYSTEMS AND DETECTION METHODS USED TO SEPARATE AND IDENTIFY N-OXYGENATED METABOLITES OF AMPHETAMINES AND RELATED COMPOUNDS BY THIN-LAYER CHROMATOGRAPHY (t.l.c.)

Substrate	Metabolites	Solvent System[a]	Detection Reagent	Ref.
Amphetamine	Oxime	C	Radioactivity	86
Amphetamine	1° Hydroxylamine	C80, M20	$AgNO_3$, NH_4OH; TTC[b]; iodine vapor	79
Phenmetrazine, Phendimetrazine	2° Hydroxylamine	B70, D15, M15	$AgNO_3$, NH_4OH	76
Fenfluramine	2° Hydroxylamine, Nitrone, Oxime	C90, A20	Dragendorff; $AgNO_3$, NH_4OH; TTC[b]	68
p-Methoxyamphetamine	1° Hydroxylamine Oxime	C80, M100; A40, EA35; H150, D0.5	$AgNO_3$, NH_4OH; 5% $CuCl_2$	70
Propylhexedrine	1° and 2° Hydroxylamine, Oxime	C80, M100; A40, EA35; H150, D0.5	$AgNO_3$, NH_4OH 5% $CuCl_2$	77

[a] A=acetone; B=benzene; C=chloroform; D=diethylamine; EA=ethyl acetate; H=n-hexane; M=methanol.

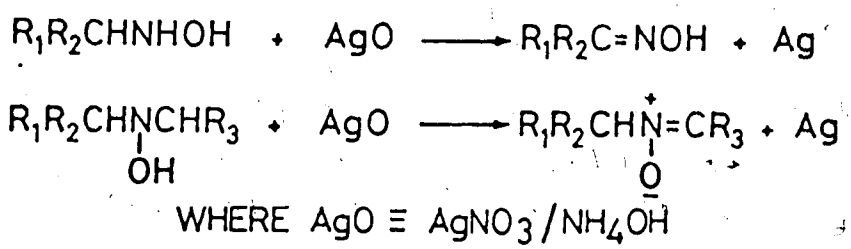
[b] Triphenyltetrazolium chloride

TABLE 6. EXAMPLES OF REAGENTS USED FOR DETECTION OF HYDROXYLAMINES ON THIN-LAYER CHROMATOGRAPHY (t. l. c.) PLATES.

Reagent	Compounds Detected	Color	Ref.
Picryl Chloride/Ammonia	Hydroxylamine	Orange	113
Csaky Reagent	Hydroxylamine, Oxime	Red	114
Modified Csaky Reagent	Hydroxylamine, Oxime	Red	113
Ammoniacol Silver Nitrate (Tollen's Reagent)	Reducing Substances	Black	112
2,3,5-Triphenyltetrazolium Chloride (TTC)	Reducing Substances	Red	112
Ferric Chloride/Potassium Ferricyanide	Reducing Substances	Blue	115
Ferric Chloride/4,7-Diphenyl-1,10-Phenanthroline	Reducing Substances	Red	91

light prior to the development of the plate in the second direction.

Hydroxylamine metabolites can be visualized through the use of spray reagents (table 6). These reagents generally detect reducing agents and are therefore non-specific. Ammoniacal silver nitrate, for example, is reduced to elemental silver by primary and secondary hydroxylamines (scheme 12). The result is a rapidly developing black spot which can be scraped from the plate and further analyzed (fig. 11). Oximes, amines and nitrones do not give immediate black spots with this reagent though such spots may develop slowly with the passage of time. Other reagents listed in table 6 can be used to identify metabolites in addition to hydroxylamines.

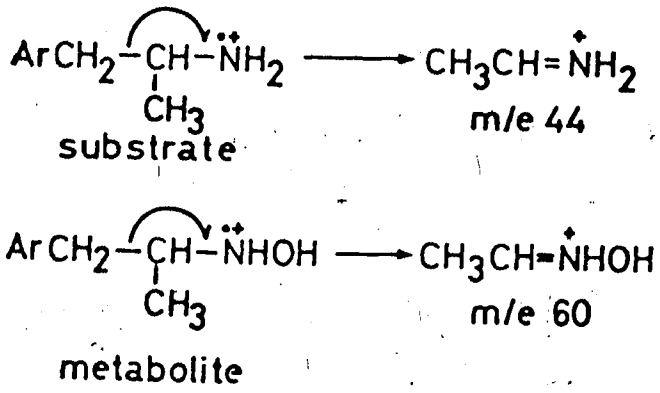


Scheme 12

2. g.l.c./m.s.

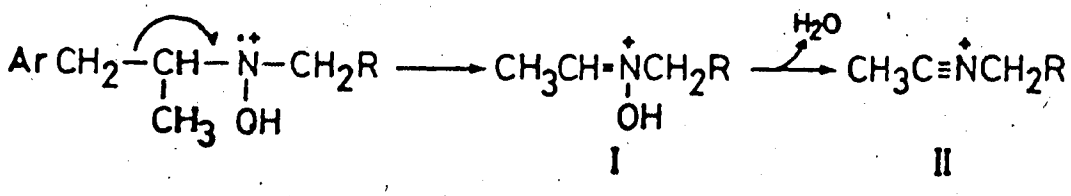
Secondary hydroxylamines, oximes, and nitrones of amphetamines give rise to diagnostic mass spectra without prior derivatization (98). Primary hydroxylamines have proven to be too unstable to allow g.l.c./m.s. investigation. They can, however, be analyzed by direct insertion into the mass spectrometer.

A diagnostic ion in the mass spectra of primary hydroxylamines is that of m/e 60 which arises as shown in scheme 13. It is absent in the spectra of the parent amine which instead contains an ion at m/e 44. Accurate mass measurement of this m/e 60 fragment has confirmed its composition. The hydroxylamines, like the parent amines, do not give abundant molecular ions.

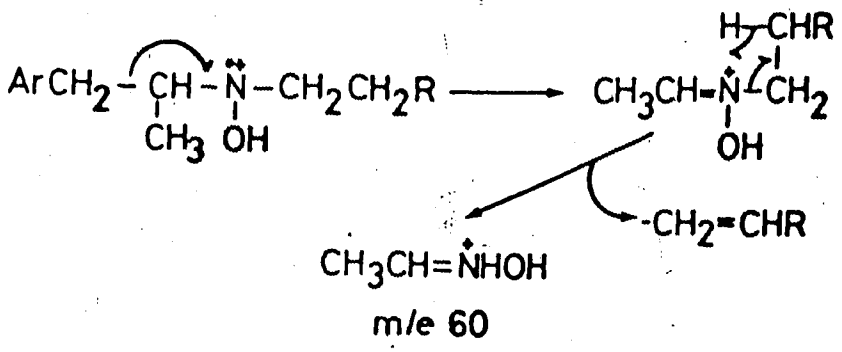


Scheme 13

G.l.c./m.s. examination of secondary hydroxylamines reveals the presence of diagnostic ions which permit identification of the metabolites. Secondary hydroxylamines give diagnostic ions (I) and (II) as shown in scheme 14. If the N-alkyl group is larger than methyl, the ion of m/e 60 (cf. seheme 13) is again formed as a result of the expulsion of the N-alkyl group as shown in scheme 15.

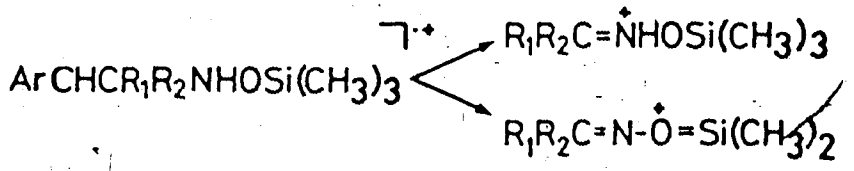


Scheme 14



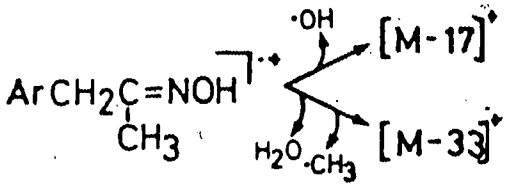
Scheme 15

Primary hydroxylamines can be characterized on g. l. c. /m. s. as their trimethylsilyl (TMS) derivatives. Lindeke, et al. (64) studied the g. l. c. /m. s. behavior of the TMS derivatives of the N-hydroxy metabolites of amphetamine, phenethylamine, phentermine, and chlorphentermine. Diagnostic ions (scheme 16) were observed which corresponded to the N-hydroxylated fragment ions in previous schemes (vide supra).



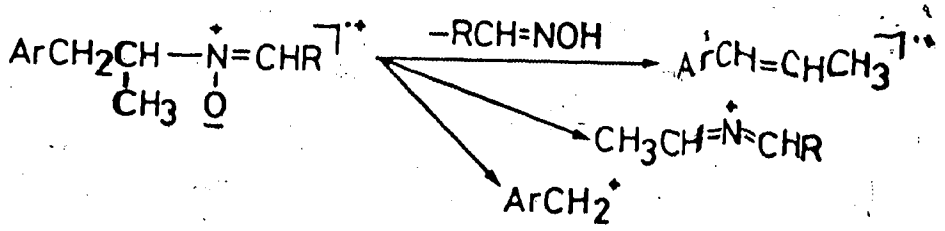
Scheme 16

Oximes, in contrast to the hydroxylamines and the parent amines, give abundant molecular ions along with ions of $(M-17)^+$ and $(M-33)^+$ which arise as shown in scheme 17.



Scheme 17

Nitrones give molecular ions of low abundance but produce three abundant fragment ions as shown in scheme 18 (68, 98).



Scheme 18

E. In vivo metabolism of amphetamines

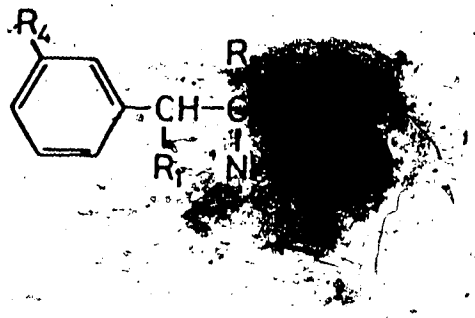
In vivo metabolism of amphetamines and related compounds has been the subject of a number of papers (80, 81, 83, 99-104). The amphetamines undergo several routes of biotransformation in vivo including

(a) aromatic hydroxylation followed by conjugation;

(b) N-dealkylation (compounds 2, 3, 4, 6, 7, 8; Table 7);

(c) deamination (may follow dealkylation) and subsequent further oxidation to benzoic acid; and

TABLE 7. THE STRUCTURE AND SOME PHYSICOCHEMICAL PROPERTIES OF THE AMPHETAMINES.



No.	Drug	R_1	R_2	R_3	R_4	pK_a	Partition Coefficient*
1	Amphetamine	H	H	H	H	9.9	1.00
2	Methylamphetamine	H	Me	H	H	10.11	2.31
3	Ethylamphetamine	H	Et	H	H	10.23	5.56
4	Pondinil	H	C_3H_6Cl	H	H	--	--
5	Norephedrine	OH	H	H	H	9.55	0.002
6	Ephedrine	OH	Me	H	H	9.6	0.031
7	Fenfluramine	H	Et	H	CF_3	9.1	67.4
8	Mephentermine	H	Me	Me	H	10.25	2.54

*relative to amphetamine

Data adapted from Williams, et al. (103).

(d) aliphatic hydroxylation on the side chain especially the benzylic carbon (except 6 and 7; Table 7).

The amount of unchanged drug excreted varies from species to species (Table 8) and little if any metabolites appear in the feces. Aromatic hydroxylation is encountered more frequently than the other metabolic routes. This is especially true of the rat. Also, considerable species variation is encountered (Table 9).

In the rat, the extent of in vivo aromatic hydroxylation seems to be correlated with the lipid solubility of the substrate and as the aromatic hydroxylation of many compounds occurs in the lipid containing liver microsomes, this reaction would be expected to occur in rat liver microsomes in vitro. An early report (105) indicated that amphetamine was not para-hydroxylated by rat liver microsomal preparations. However, more recent reports indicate that indeed para-hydroxylation of amphetamine and phentermine does occur in vitro in the presence of rat liver microsomes (106-108), but only to a very small extent and is dependent upon substrate concentration.

TABLE 8. EXTENT TO WHICH SOME AMPHETAMINES ARE EXCRETED UNCHANGED IN VARIOUS SPECIES.

Drug	Species*				
	Rat	Rabbit	Guinea Pig	Dog	Man
Amphetamine	12	4	19	30	34
Methamphetamine	11(14)	2(2)	1-3(4-16)	20(35)	23(26)
Ethylamphetamine	--	--	--	--	17(24)
Pondinil	1	1-8	3	17	1
Norephedrine	48	8	--	--	86
Ephedrine	42(45)	0·5(2·5)	2(41)	6(64)	1(73)
Fenfluramine	--	--	--	--	7(10)
Mephentermine	0(13)	0(22)	--	0(16)	--

*values in brackets are the sum of the N-alkylamine and the metabolically produced primary amine. Yields are given in per cent.

Data adapted from Williams, et al. (103).

TABLE 9. THE EXTENT OF AROMATIC HYDROXYLATION OF VARIOUS AMPHETAMINES IN DIFFERENT SPECIES.

Drug	Species				
	Rat	Rabbit	Guinea Pig	Dog	Man
Amphetamine	60	6	0	6	2
Methamphetamine	53	--	0	30	18
Norephedrine	28	3	--	--	0-1
Ephedrine	14	11	1	1	0
Pondinil	58	6	15	22	37
Mephentermine	32	5	--	28	--
Fenfluramine	--	--	--	0	0

Yields are given in per cent.

Data adapted from Williams, et al. (103)

III. AIMS AND OBJECTIVES OF EXPERIMENTS

A perusal of the literature will show that current research has established that many aliphatic amines are metabolized *in vitro* to hydroxylamines. These metabolites are extremely reactive and can be metabolically, chemically or physically converted to other N-oxygenated products including oximes, nitrones, nitroso and nitro compounds. In most instances, C-oxygenated metabolites are also formed concomitantly. It is also apparent that, whereas microsomal preparations of guinea pig and rabbit liver possess significant N-oxidative properties, similar preparations of rat liver have been reported to be much less active.

An examination of the available literature also shows that much effort has been directed towards studies of the *in vitro* N-oxidation of tertiary aliphatic amines which form N-oxides, and amphetamine and other related primary aliphatic amines which form primary hydroxylamines. However, considerably less has been reported on *in vitro* N-oxidation of secondary aliphatic amines.

In comparison to rabbit and guinea pig liver preparations, rat liver homogenate has generally been regarded as a poor model for studies of *in vitro* metabolic N-oxidation. However, there are no reports in the literature to indicate that attempts have been made to optimize the *in vitro* assay conditions for metabolic N-oxidation in rat liver preparations. In view of this, it was considered desirable to determine whether *in vitro* conditions might be found that would significantly improve metabolic N-oxidation in rat liver homogenates.

A detailed study of the metabolism of aliphatic secondary amines using rat liver preparations was undertaken for the following reasons: (a) to see if rat liver homogenates were generally low in N-oxidation activity; (b) to determine quantitatively the extent of metabolic N-oxidation; (c) to see whether rat liver could be induced to N-oxidize the chosen substrate; (d) to determine whether the presence of nicotinamide in the incubation mixture had an activating or inhibiting effect on metabolic N-oxidation of secondary amines; (e) to determine the extent of further metabolism of the primary metabolites and their decomposition products.

A preliminary objective of the study was to select an appropriate secondary amine substrate for the in vitro investigations. Considered among the compounds were N-methylamphetamine, N-ethylamphetamine, N-propylamphetamine, and fenfluramine. N-propylamphetamine was eventually chosen because of its ready availability, i.e. it was easily prepared and it was not a controlled substance, and because of the relative stability of its N-oxidized metabolites.

The in vivo metabolism of amphetamine and some of its cogeners has been studied in detail. The in vivo C-oxidative pathways are generally well known but the significance of in vivo N-oxidation is controversial. To further elucidate the importance of the metabolic N-oxidative route in rats, it seemed appropriate to determine whether

N-oxidation of amphetamines did occur in vivo, and, if so, to attempt to compare quantitatively the amounts of N- and C-oxidation.

IV. METHODS AND MATERIALS

A. Chemicals and Reagents

Materials obtained from commercial sources were: glucose-6-phosphate (G-6-P), nicotinamide adenine dinucleotide phosphate ($NADP^+$), reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide, flavine adenine dinucleotide (FAD), 3-methylcholanthrene (3-MC), and β-glucuronidase type H-1 (Helix pomatia) with sulfatase activity (Sigma Chemical Co., St. Louis, Mo., USA); phenylacetone (1-phenyl-2-propanone), Diazald®, and trifluoroacetic acid (Aldrich Chemical Co.); SKF 525-A, (±)-amphetamine sulfate, (±)-N-hydroxyamphetamine succinate, and p-hydroxyamphetamine hydrobromide (gifts from Smith, Kline and French Laboratories, Montreal); crystalline bovine serum albumin, fraction V (Mann Research Laboratories, New York); TRIS (base) Ultra Pure (Schwarz/Mann, Orangeburg, N.J.); and Phenol Reagent 2N Solution (Fisher Scientific, Fair Lawn, N.J.). Other solvents and chemicals were of reagent grade and used without further purification except for diethyl ether which was distilled fresh daily. Substrates employed which required custom synthesis are described below. Custom synthesis was provided by Dr. C.W. Kazakoff and Dr. R. Dawe.

B. Animals

Male Wistar rats weighing 200-250 g were obtained from Woodlyn Farms (Guelph, Ontario) and maintained in wire suspension cages and

allowed food and water ad. lib. Rats employed in in vivo studies were maintained individually in metabolism cages (Model E1000, Maryland Plastics) with food and water ad lib. Uremic rats used in the in vivo study were kindly supplied by Dr. U. K. Terner.

C. Gas-liquid Chromatography (g. l. c.)

G. l. c. columns and usual operating conditions are shown in table 10. A Perkin-Elmer model 990 or Hewlett-Packard model 5700A gas chromatograph each with dual flame ionization detectors was used. Retention times (Tr) and peak areas were determined with either an Infotronics model CRS-208 or Hewlett-Packard model 3380A electronic peak integrator. p-Chloropropiophenone (PCP) was used as internal standard for in vitro studies and benzylamphetamine (BZA) as internal standard for in vivo studies.

D. Gas-liquid Chromatography-Mass Spectrometry (g. l. c./m. s.)

Mass spectra of authentic reference compounds and samples were obtained by combined g. l. c./m. s. using columns as described in the text and table 10 in a Hewlett-Packard model 5710A gas chromatograph coupled to a Hewlett-Packard model 5981A mass spectrometer at an ionizing potential of 70 eV.

E. Thin-layer Chromatography (t. l. c.)

Chromatograms of extracts from in vitro experiments were run on glass plates (20 X 20 cm) spread to a thickness of 0. 5 mm with

TABLE 10. GAS-LIQUID CHROMATOGRAPHY COLUMNS AND OPERATING CONDITIONS[1,2]

No.	Length[3] (m)	Liquid Phase	Support[4]	Injection Port (°C)	Detector (°C)
A	1.8	0.5% OV-101	Chromosorb 750	250	250
B	1.8	7 1/2% Carbowax 20M	Chromosorb W	250	250
C	1.8	5% OV-101	Chromosorb 750	250	250
D	1.2	7 1/2% Carbowax 20M	Chromosorb 750	210	210
E	1.2	10% Apiezon L, 5% KOH	Chromosorb 750	210	210

1. Column temperatures are given where compound is described in text.
2. Gas flow rates: He = 60 ml min^{-1}; H_2 = 25 psi; air = 30 psi
3. All columns were 6 mm O.D. glass tubing.
4. All solid supports were acid-washed, DMCS treated, 80-100 mesh

a slurry of silica gel G-PF254 (Brinkman) and activated at 100°C for 1 h. The solvent system used was chloroform-acetone (9:2). Spots were detected by short-wave uv light (254 nm), iodine vapor, or ammoniacal silver nitrate spray (Tollen's reagent).

F. Nuclear Magnetic Resonance (n.m.r.) and Infrared (i.r.) Spectrometry

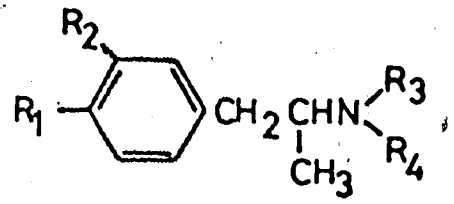
N.m.r. spectra were recorded using a Varian A-60D spectrometer with deuterochloroform as solvent and tetramethylsilane (TMS) as the internal standard. I.r. spectra were recorded on a Unicam SP1000 spectrophotometer.

G. Substrates and Reference Compounds

Most of the compounds listed below were prepared in cooperation with, or by, Dr. C.W. Kazakoff. Data is included mainly for reference purposes.

1. N-n-Propylamphetamine and related compounds

(+)- and (-)-NPA (XIV) were prepared from (+)- and (-)-amphetamine by the method described by Leonard et al. (116) except that propionic anhydride was substituted for acetic anhydride. (±)-NPA was obtained from 1-phenyl-2-propanone and n-propylamine according to Temmler (117), using ethanol as solvent. The free bases were converted to hydrochloride salts and recrystallized from



Compound	R_1	R_2	R_3	R_4
XIV	H	H	n-Pr	H
XV	H	H	n-Pr	OH
XVI	H	H	$=CHCH_2CH_3$	O^-
XVII	H	H	$CH_2CHOHCH_3$	H
XVIII	H	H	Et	H
XIX	H	H	n-Bu	H
XXII	MeO	H	n-Pr	H
XXIII	HO	H	n-Pr	H
XXIV	$PhCH_2O$	MeO	n-Pr	H
XXV	HO	MeO	n-Pr	H
XXVI	MeO	H	Me	H
XXVII	HO	H	Me	H
XXVIII	HO	MeO	Me	H
XXIX	MeO	H	Et	H
XXX	HO	H	Et	H
XXXI	HO	MeO	Et	H
XXXII	MeO	H	n-Bu	H
XXXIII	HO	H	n-Bu	H
XXXIV	HO	MeO	n-Bu	H

ethanol-ether. The hydrochlorides were colorless solids. Each gave i.r., n.m.r. and m.s. consistent with their structure.

(±)-NPA hydrochloride had m.p. 155.5-157°. Anal. Calcd. for $C_{12}H_{20}ClN$: C, 67.43; H, 9.43; N, 6.55. Found: C, 67.38; H, 9.59; N, 6.35.

(+)-NPA hydrochloride, $[\alpha]_D^{25} = +19.0°$ (C=2, H_2O), had m.p. 178-180°. Anal. Calcd. for $C_{12}H_{20}ClN$: C, 67.43; H, 9.43; N, 6.55. Found: C, 67.29; H, 9.55; N, 6.63.

(-)-NPA hydrochloride, $[\alpha]_D^{25} = -16.5°$ (C=2, H_2O) had m.p. 176.5-178.5°. Anal. Calcd. for $C_{12}H_{20}ClN$: C, 67.43; H, 9.43; N, 6.55. Found: C, 67.48; H, 9.31; N, 6.41.

Each of the three free bases separately chromatographed as a single peak (Tr = 2.2, 155°) on column D.

Syntheses of N-hydroxy-1-phenyl-2-(n-propylamino)propane (XV), N-[(1-methyl-2-phenyl)ethyl]-1-propanimine N-oxide (XVI) and 1-phenyl-2-propanone oxime (X) have been described previously (90,98,109).

(±)-N-(2-Hydroxypropyl)amphetamine (XVII) was prepared using the method described for (±)-NPA except that 1-amino-2-propanol was used instead of n-propylamine. It was converted to a hydrochloride and recrystallized from ethanol-ether. XVII hydrochloride was a colorless solid, m.p. 135-136°. The free

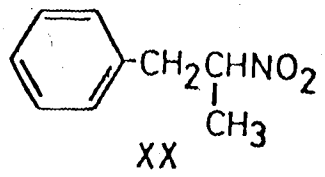
base chromatographed as a single peak (Tr = 13.0, 155°) on column D. It gave i.r., n.m.r., m.s. consistent with its structure.

Anal. Calcd. for $C_{12}H_{20}ClNO$: C, 62.73; H, 8.77; N, 6.10. Found: C, 62.74; H, 9.14; N, 6.08.

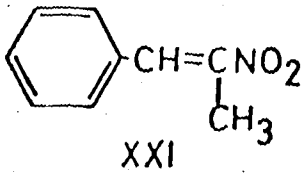
(±)-N-Ethylamphetamine (NEA; XVIII) and (±)-N-(n-butyl)-amphetamine (NBA; XIX) were prepared from 1-phenyl-2-propanone and ethylamine or n-butylamine, respectively, in a manner similar to that used to prepare (±)-NPA. Both were converted to their respective hydrochlorides and recrystallized from ethanol-ether. (±)-NEA hydrochloride was a colorless solid, m.p 143-145°, lit. 145-146° (116). The free base chromatographed as a single peak (Tr = 2.6, 155°) on column D. (±)-NBA hydrochloride was a colorless solid, m.p. 167-169°, lit. 168-169° (118). The free base chromatographed as a single peak (Tr = 3.2, 155°) on column D. Both (±)-NEA and (±)-NBA gave i.r., n.m.r., m.s., and C, H, and N analysis consistent with their structures.

2. 2-Nitro-1-Phenylpropane (XX)

A solution of benzaldehyde (15.5 ml), ammonium acetate (10 g), and nitroethane (16.5 ml) in acetic acid (125 ml) was



XX



XXI

heated on a steam bath for 3h, cooled, then partitioned between benzene and water. The organic layer was washed with portions of 2% aqueous sodium hydroxide then water, dried ($MgSO_4$) and evaporated to give a yellow solid which was identified by n. m. r. as 2-nitro-1-phenylpropene (XXI), m. p. 57-58°, lit. 64° (119).

A portion of XXI was dissolved in dioxane and slowly added dropwise to a stirred solution of sodium borohydride (304 mg) in dioxane-water (35 ml, 6:1) at room temperature. The solution was acidified to pH 3.0 with dilute hydrochloric acid and extracted with ether. The ether solution was washed with brine, dried ($MgSO_4$) and evaporated to a yellow oil, which was shown by g.l.c. (155°, column D) and g.l.c./m.s. to consist mainly of

2-nitro-1-phenylpropane (XX) and a small quantity of 1-phenyl-2-propanone. Column chromatography (silica gel G) of this oil with benzene as eluent produced a yellow oil which was shown by g.l.c. (Tr = 6.4, 155°) on a column D to be pure XX. N.m.r., i.r., and m.s. analysis was consistent with the proposed structure.

Anal. Calcd. for $C_9H_{11}NO_2$: C, 65.45; H, 6.67; N, 8.48. Found: C, 65.34; H, 6.93; N, 8.48.

3. 1-(4-Methoxyphenyl)-2-(n-propylamino)propane (XXII) Hydrochloride

A suspension of platinum dioxide (0.10 g) in dry ethanol (25 ml) was hydrogenated for 0.5 h at 30 psi in a Parr hydrogenator. A solution of 1-(p-methoxyphenyl)-2-propanone (120) (3 g) and n-propylamine (0.8 g) in dry ethanol (25 ml) was added and hydrogenation at 30 psi was continued for 24 h. The mixture was filtered and the filtrate evaporated in vacuo to an oil which was dissolved in ether and treated with a solution of hydrogen chloride in ether. The white solid which formed was crystallized from ethanol-ether and gave the title compound (2.13 g), m.p. 154-155°. I.r., n.m.r., and m.s. were consistent with this compound. The free base gave a single peak on both g.l.c. columns A (Tr = 2.4, 140°) and B (Tr = 8.2, 180°).

Anal. Calcd. for $C_{13}H_{22}ClNO$: C, 64.05; H, 9.10; N, 5.75. Found: C, 64.22; H, 9.05; N, 5.64.

4. 1-(4-Hydroxyphenyl)-2-(n-propylamino)propane (XXIII) Hydrochloride

The free base was liberated from XXII hydrochloride (0.3 g) and dissolved in benzene (10 ml) to which aluminum bromide (0.65 g) was added. After heating under reflux for 2.5 h, this solution was poured into an aqueous pH 8.5 buffer (50 ml) and extracted with ether (3 x 50 ml). Basic material was precipitated from the ether extract by the addition of an ethereal solution of hydrogen chloride and crystallized from ethanol-ether to give the title compound (0.107 g), m.p. 164-165°, as a white solid. Its m.s., i.r., and n.m.r. spectra were consistent with the proposed structure. The free base gave a single peak on g.l.c. column A (Tr = 1.9, 160°).

Anal. Calcd. for $C_{12}H_{20}ClNO$: C, 62.73; H, 8.77; N, 6.10. Found: C, 62.69; H, 8.92; N, 5.88.

5. 1-(4-Benzyloxy-3-methoxyphenyl)-2-(n-propylamino)propane (XXIV) Oxalate

The title compound as the free base was prepared from 1-(4-benzyloxy-3-methoxyphenyl)-2-propanone (121) (0.45 g), n-propylamine (1 ml), platinum oxide (0.1 g) in absolute ethanol (50 ml), in the same manner described for XXII free base. A solution of anhydrous oxalic acid in ether was added to an ether solution of

XXIV base. The resultant oxalate was crystallized from methanol-ether as a colorless monohydrate (0.37 g), m.p. 145-146°. I.r., n.m.r., and m.s. were in agreement with this structure. The free base gave a single peak on g.l.c. column A (Tr = 4.6, 180°).

Anal. Calcd. for $(C_{20}H_{27}NO_2)_2 \cdot (COOH)_2 \cdot H_2O$: C, 68.65; H, 7.95; N, 3.81. Found: C, 68.46; H, 7.89; N, 4.05.

6. 1-(4-Hydroxy-3-methoxyphenyl)-2-(n-propylamino)propane (XXV)

The free base was liberated from XXIV oxalate (0.2 g) and dissolved in ethanol (30 ml) containing 10% palladium-on-charcoal (0.1 g). The mixture was hydrogenated at 30 psi for 24 h, filtered, then evaporated to give an oil (0.11 g) which could not be converted to a solid derivative (hydrochloride, oxalate, maleate). The oil gave i.r., n.m.r., and m.s. consistent with the proposed structure. It gave a single peak on both g.l.c. columns A (Tr = 0.[illegible] 180°) and C (Tr = 13.3, 155°). It was used as an oil in the quantitative analysis experiment.

7. 1-(4-Methoxyphenyl)-2-(methylamino)propane (XXVI) Hydrochloride

This compound was prepared in a manner analogous to XXII except that methylamine was used instead of n-propylamine. The hydrochloride was recrystallized from ethanol-ether and gave the title compound, m.p. 174-175°. I.r., n.m.r., and m.s. were

consistent with the proposed structure. The free base gave a single peak on g. l. c. (Tr = 3. 7, 155°), column C.

Anal. Calcd. for $C_{11}H_{18}ClNO$: C, 61. 25; H, 8. 40; N, 6. 49. Found: C, 61. 28; H, 8. 37; N, 6. 61.

8. 1-(4-Hydroxyphenyl)-2-(methylamino)propane (XXVII) Hydrochloride

The free base was liberated from XXVI and treated in a manner analogous to the preparation of XXIII. The resultant hydrochloride was recrystallized from ethanol-ether and gave the title compound, m. p. 134-135°. I. r., n. m. r. and m. s. were consistent with the proposed structure. The free base gave a single peak on g. l. c. (Tr = 4. 5, 155°), column C.

Anal. Calcd. for $C_{10}H_{16}ClNO$: C, 59. 55; H, 7. 99; N, 6. 94. Found: C, 59. 73; H, 7. 62; N, 6. 83.

9. 1-(4-Hydroxy-3-methoxyphenyl)-2-(methylamino)propane (XXVIII)

This compound was prepared in a manner analogous to XXV except that methylamine was used instead of n-propylamine. Attempts to obtain a crystalline solid (hydrochloride, oxalate, maleate) were not successful. G. l. c. /m. s. confirmed the structure of XXVIII which was the major component on g. l. c. (Tr = 6. 8, 155°) column C. Elemental analysis was not attempted.

10. 1-(4-Methoxyphenyl)-2-(ethylamino)propane (XXIX) Hydrochloride

This compound was prepared in a manner analogous to XXII except that ethylamine was used instead of n-propylamine. The hydrochloride was recrystallized from ethanol-ether and gave the title compound, m.p. 154-156°, I.r., n.m.r., and m.s. were consistent with the proposed structure. The free base gave a single peak on g.l.c. (Tr = 4.6, 155°) column C.

Anal. Calcd. for $C_{12}H_{20}ClNO$: C, 62.74; H, 8.77; N, 6.10. Found: C, 62.79; H, 8.65; N, 5.84.

11. 1-(4-Hydroxyphenyl)-2-(ethylamino)propane (XXX) Hydrochloride

The free base was liberated from XXIX and treated in a manner analogous to the preparation of XXIII. The resultant hydrochloride was recrystallized from ethanol-ether and gave the title compound, m.p. 159-160°. I.r., n.m.r., and m.s. were consistent with the proposed structure. The free base gave a single peak on g.l.c. (Tr = 5.6, 155°) column C.

Anal. Calcd. for $C_{11}H_{18}ClNO$: C, 61.25; H, 8.40; N, 6.49. Found: C, 61.21; H, 8.24; N, 6.36.

12. 1-(4-Hydroxy-3-methoxyphenyl)-2-(ethylamino)propane (XXXI)

This compound was prepared in a manner analogous to XXV except that ethylamine was used instead of n-propylamine. Attempts to

obtain a crystalline solid (hydrochloride, oxalate, maleate) were not successful. G. l. c./m. s. confirmed the structure of XXXI which was the major component on g. l. c. (Tr = 8.3, 155°) column C. Elemental analysis was not attempted.

13. 1-(4-Methoxyphenyl)-2-(n-butylamino)propane (XXXII) Hydrochloride

This compound was prepared in a manner analogous to XXII except that n-butylamine was used instead of n-propylamine. The hydrochloride was recrystallized from ethanol-ether and gave the title compound, m. p. 176-177°. I. r., n. m. r., and m. s. were consistent with the proposed structure. The free base gave a single peak on g. l. c. (Tr = 10.1, 155°) column C.

Anal. Calcd. for $C_{14}H_{24}ClNO$: C, 65.21; H, 9.38; N, 5.43. Found: C, 65.41; H, 9.24; N, 5.28.

14. 1-(4-Hydroxyphenyl)-2-(n-butylamino)propane (XXXIII) Hydrochloride

The free base was liberated from XXXII and treated in a manner analogous to the preparation of XXIII. The resultant hydrochloride was recrystallized from ethanol-ether and gave the title compound, m. p. 158-159°. I. r., n. m. r., and m. s. were consistent with the proposed structure. The free base gave a single peak on g. l. c. (Tr = 14.8, 155°) column C.

Anal.Calcd. for $C_{13}H_{22}ClNO$: C, 64.06; H, 9.09; N, 5.75.
Found: C, 64.24; H, 9.18; N, 5.91.

15. 1-(4-Hydroxy-3-methoxyphenyl)-2-(n-butylamino)propane (XXXIV)

This compound was prepared in a manner analogous to XXV except that n-butylamine was used instead of n-propylamine. Attempts to obtain a crystalline solid (hydrochloride, oxalate, maleate) were not successful. G.l.c./m.s. confirmed the structure of XXXIV which was the major component on g.l.c (Tr = 20.6, 155°) column C. Elemental analysis was not attempted.

H. Pretreatment of Animals

The drugs, dosages and routes of administration are listed in table 11.

I. Preparation of Liver Microsomal Fraction for In Vitro Studies

Animals were fasted overnight and sacrificed by cervical dislocation. Livers were removed and immediately placed in ice cold 1.15% KCl. Subsequent operations were performed at 4°C. A 20% w/v homogenate corresponding to 200 mg wet weight liver ml^{-1} in isotonic KCl was prepared in a Potter homogenizer equipped with a teflon pestle. The microsomal supernatant was obtained by centrifugation of the liver homogenate at 12,000 x g for 20 minutes (0-3°C).

TABLE 11. PRETREATMENT SCHEDULE FOR IN VITRO AND IN VIVO EXPERIMENTS

No.	Drug[1]	Route	Dose[2] (mg kg^{-1})	Frequency	Vehicle
1	(±)-N-(n-propyl)amphetamine	i. p.	[redacted]	daily x 14 d	A
2	(±)-N-(n-propyl)amphetamine	p. o.	[redacted]	14 d	B
3	3-Methylcholanthrene	i. p.	20	once	C
4	Phenobarbital	i. p.	75	daily x 5 d	A
5	SKF 525-A	i. p.	50	one h before sacrifice	A
6	(±)-N-(n-propyl)amphetamine	p. o.	100	once	B
7	(±)-N-(n-propyl)amphetamine	i. p.	20	daily x 5 d	A
8	(±)-Amphetamine	p. o.	45	once	B
9	(±)-N-methylamphetamine	p. o.	10	once	B
10	(±)-N-Ethylamphetamine	p. o.	12.5	once	B
11	(±)-N-(n-butyl)amphetamine	p. o.	100	once	B

1. Control animals received the appropriate vehicle in the same manner as the treated group.
2. Total volume: i. p. = 1 ml kg^{-1}; p. o. = 5 ml kg-1
3. Drug was administered in the drinking water. Total dose varied and is stated in text.

A = 0.1 M phosphate buffer, pH 7.4; B = tap water; C = corn oil.

J. Determination of Cytochrome P-450 and Microsomal Protein

For these determinations, 3.5 ml and 1 ml respectively of the 12,000 x g supernatant were transferred to separate 10 ml ultracentrifuge tubes and brought to volume with 1.15% KCl. After centrifugation at 105,000 x g for 1 h (0-5°C), the supernatant was discarded and the remaining pellet resuspended either in distilled water for estimation of protein, or in 50 mM phosphate buffer pH 7.4 containing 10^{-3} µM EDTA for cytochrome P-450 determination. Microsomal protein, for standardization of enzymatic activity as defined by Fouts (11) and Mazel (136), was determined colorimetrically according to Lowry et al. (122) as modified by Miller (123). The method of Omura and Sato (124) was used for cytochrome P-450. All uv measurements were carried out on a Pye-Unicam model SP1800 spectrophotometer with strip chart recorder.

K. Standard In Vitro Incubation Method

One ml of the 12,000 x g supernatant was added to 25 ml Erlenmyer flasks containing G-6-P (20 µmol), $NADP^+$ (4.4 µmol), $MgCl_2$ (20 µmol), substrate (10 µmol) and sufficient 0.1 M phosphate or TRIS·HCl buffer, pH 7.4, to make 6 ml. Samples were incubated for 60 min at 37°C in a Dubnoff shaking incubator (120 oscillations min^{-1}) under atmosphere. At the end of the incubation period, the flasks were immersed in ice, internal standard was added and the contents of each flask were extracted three times with freshly distilled ether. The ether extracts were combined, a few drops of

iso-butanol added, and the extracts were concentrated on a 45°C water bath to a final value of 50 µl. Three µl of this concentrated extract was injected into the gas chromatograph.

L. Treatment of Urine From In Vivo Studies

Urine samples from each group of treated animals and controls were divided into three sets. One set was adjusted to pH 9.5 with solid sodium carbonate and extracted either three times with equal volumes of ether-methylene chloride (14:11) or for 24 h with ether alone in a continuous liquid-liquid extractor. A second set was buffered to pH 7 with phosphate buffer and hydrolyzed for 24 h at 37°C using β-glucuronidase type H-1 (30,000 units ml^{-1} urine) then extracted as above after buffering to pH 9.5 with sodium carbonate. The third set was acidified to pH <1 with an equal volume of 6 N HCl and placed in a boiling water bath for 1 h. After the acid hydrolysis was complete, a volume of 6 N NaOH, equal to the volume of acid, was added, the pH was adjusted to 9.5 with solid sodium carbonate and the resulting solution was extracted as described above. In all cases internal standard was added prior to extraction.

M. Quantitative analysis

1. In vitro metabolism studies

Known but varying quantities (0.1-10 µmol) of each substrate and each suspected metabolite were added to separate portions of

aged microsomal suspensions containing 2 µmol PCP as internal standard and each of the mixtures obtained was extracted three times with equal volumes of freshly distilled ether at either pH 7.4 or 12. A few drops of iso-butanol were added to the combined extracts which were then individually concentrated to 50 µl on a water bath. These extracts were gas chromatographed on column D (155°) for quantitation of the N-oxygenated metabolites and on column E (155°) for the deaminated and N-dealkylated metabolites. Calibration curves were constructed of the ratio of peak areas of the compound to internal standard versus the amount of compound added. These calibration curves were linear over the concentration ranges encountered in the metabolism studies.

2. In vivo studies

Known but varying quantities (1-16 mg) of substrates and metabolites were added to portions of urine containing 4 mg BZA as internal standard and extracted at pH 9.5 either three times with equal volumes of an ether:methylene chloride (14:11) mixture or for 24 h with ether alone in a continuous liquid-liquid extractor. The extracts were evaporated to dryness in a rotary evaporator and redissolved in absolute ethanol (200 µl) prior to g.l.c. analysis. Calibration curves were constructed as described in procedure M.1 above except that g.l.c. was performed on column C (155°). The curves were found to be linear throughout the concentration ranges encountered in the in vivo experiments.

V. RESULTS AND DISCUSSION

A. In Vitro Metabolism of (±)-NPA In Rat Liver Homogenates

1. Observed Metabolites

Metabolism of (±)-NPA at pH 7.4 using the 12,000 x g supernatant of homogenized rat liver fortified with G-6-P, $NADP^+$, and $MgCl_2$ but not nicotinamide, resulted in the formation of various products (table 12) including two N-oxygenated compounds identified as N-hydroxy-1-phenyl-2-(n-propylamino)propane, i.e. N-OH-NPA (XV), and N-[(1-methyl-2-phenyl)ethyl]-1-propanimine N-oxide (XVI), i.e. NPA-nitrone, by a comparison of their g.l.c., g.l.c./m.s. (fig. 12,13), and t.l.c. properties with those of authentic samples of XV and XVI. Other components in the in vitro incubation mixture identified in the same manner were: the unchanged substrate (XIV; fig. 14), 1-phenyl-2-propanone (IX; fig. 15), amphetamine (VI; fig. 16), 1-phenyl-2-propanone oxime (X; fig. 17), and a previously unreported metabolite, 2-(2-hydroxypropylamino)-1-phenylpropane, i.e. 2-OH-NPA (XVII; fig. 18). This last metabolite will be discussed in detail later.

2. G.l.c./m.s. Identification of N-Oxygenated In Vitro Metabolites of (±)-NPA

Secondary hydroxylamines are more stable to g.l.c./m.s. analysis than the corresponding primary hydroxylamines (98). Primary hydroxylamines, without prior derivatization, may decompose to their corresponding oximes in the g.l.c./m.s. system before

TABLE 12. GAS-LIQUID CHROMATOGRAPHY RETENTION TIMES (Tr), THIN-LAYER CHROMATOGRAPHY R_f VALUES, AND YIELDS OF RECOVERED (±)-N-PROPYLAMPHETAMINE AND ITS *IN VITRO* METABOLITES FROM THE FORTIFIED 12,000 x g SUPERNATANT OF HOMOGENIZED RAT LIVER[1].

Compound		Yield (%)[2]	Tr (Min)[3]		R_f[4]
			Column D	Column E	
$PhCH_2CH(CH_3)NH(CH_2)_2CH_3$	(XIV)	91.9-94.5	1.9	9.7	0.2
$PhCH_2CH(CH_3)N(CH_2)_2CH_3$ OH	(XV)	0.3-0.6	13.2	-	0.65
$PhCH_2CH(CH_3)N{=}CHCH_2CH_3$ O	(XVI)	1.0-2.0	22.1	-	3
$PhCH_2COCH_3$	(IX)	0.2-0.5	1.8	3.2	0.08
$PhCH_2CH(CH_3)NH_2$	(VI)	2.5-3.0	1.8	3.8	0.15
$PhCH_2C(CH_3){=}NOH$	(X)	0.1-0.2	16.5	-	0.8
$PhCH_2CH(CH_3)NHCH_2CHOHCH_3$	(XVII)	0.2-0.4	13.0	-	0.1

[redacted]

[1] Standard *in vitro* incubation mixtures incubated for 1h at 37°

[2] Total recovery of (±)-NPA and metabolites was 98.8±0.4%, where (±) is the standard error of the mean

[3] Both columns at 155°

[4] T.l.c. performed on silica gel G-PF254 in chloroform:acetone (9:2).

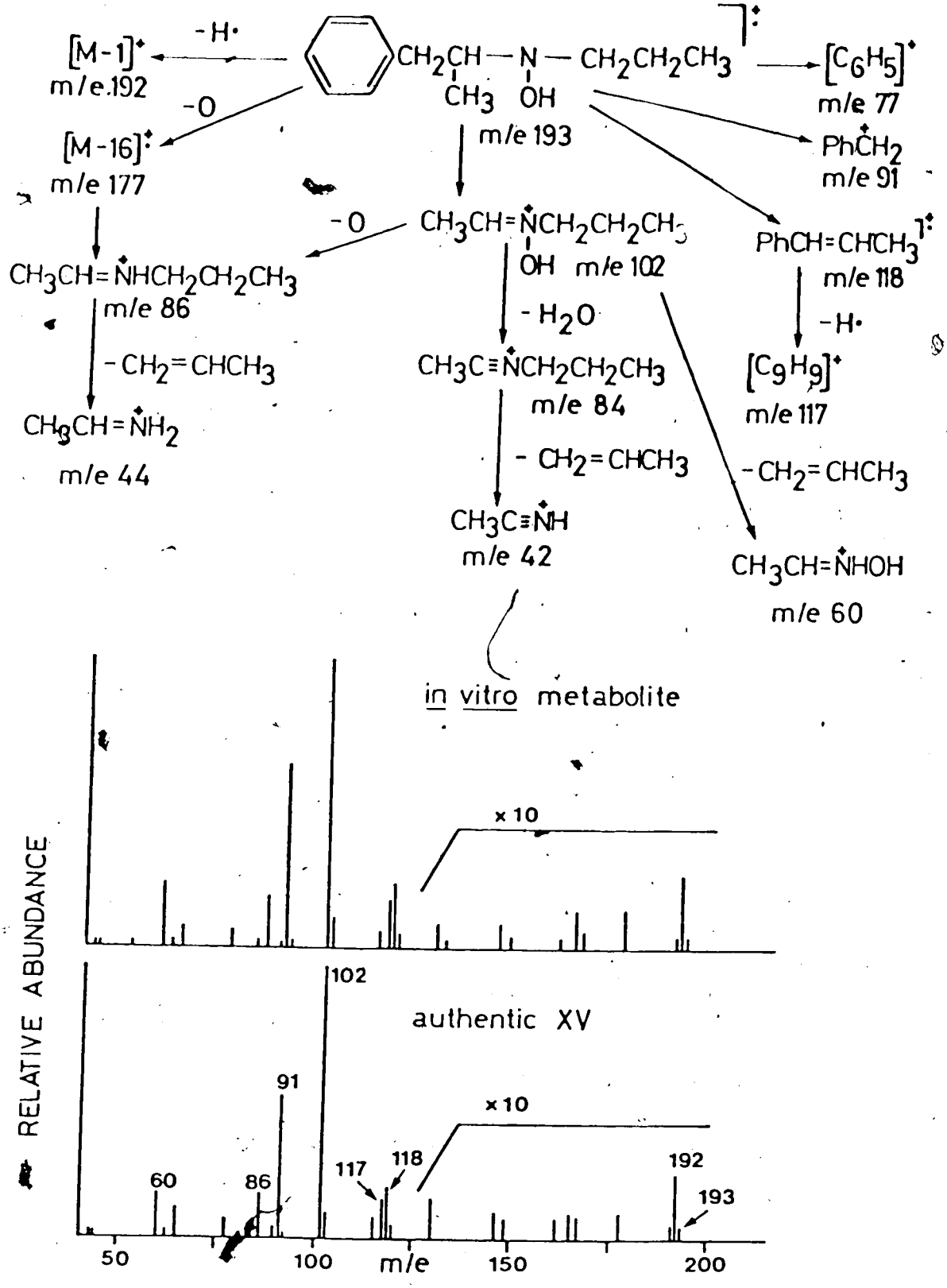
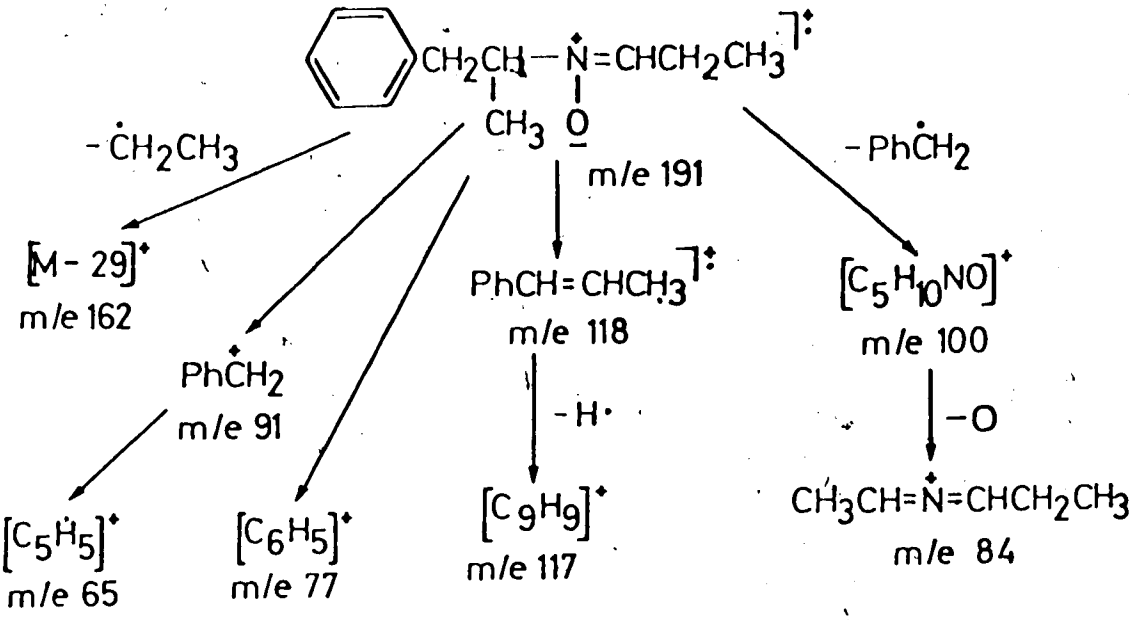


Fig. 12. G.l.c./m.s. identification of N-hydroxy-1-phenyl-2-(n-propylamino)propane (XV) as an in vitro metabolite of (±)-N-propylamphetamine.



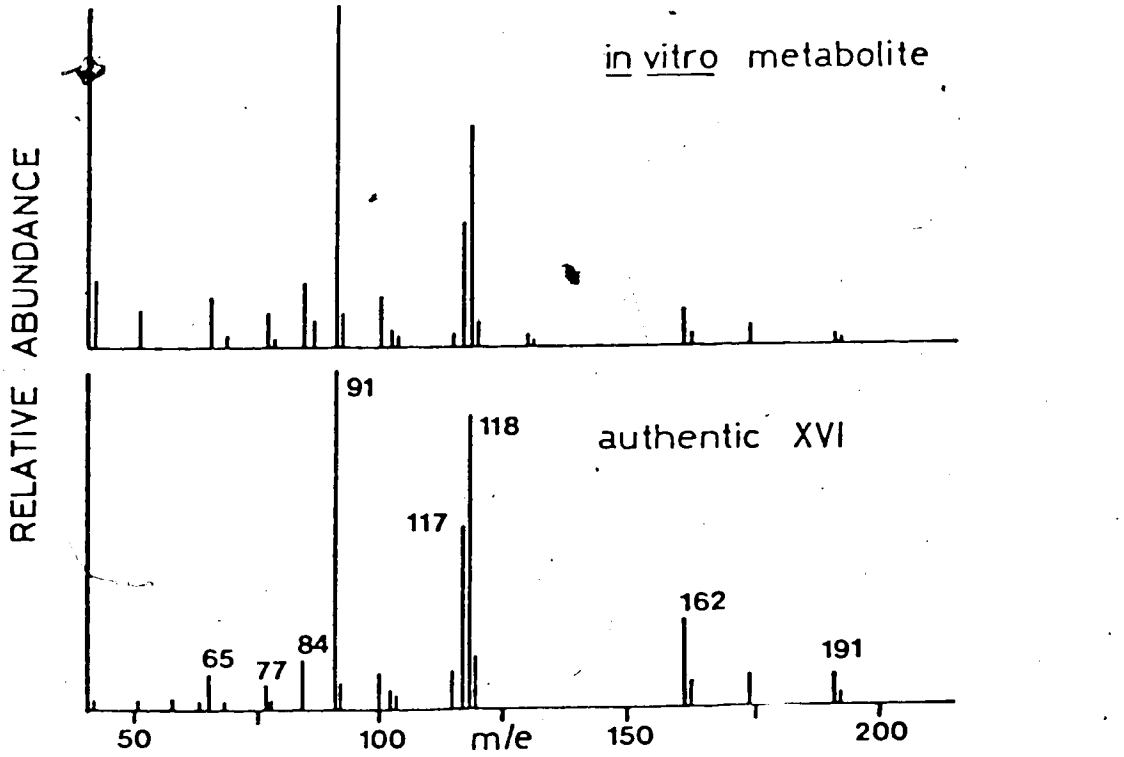
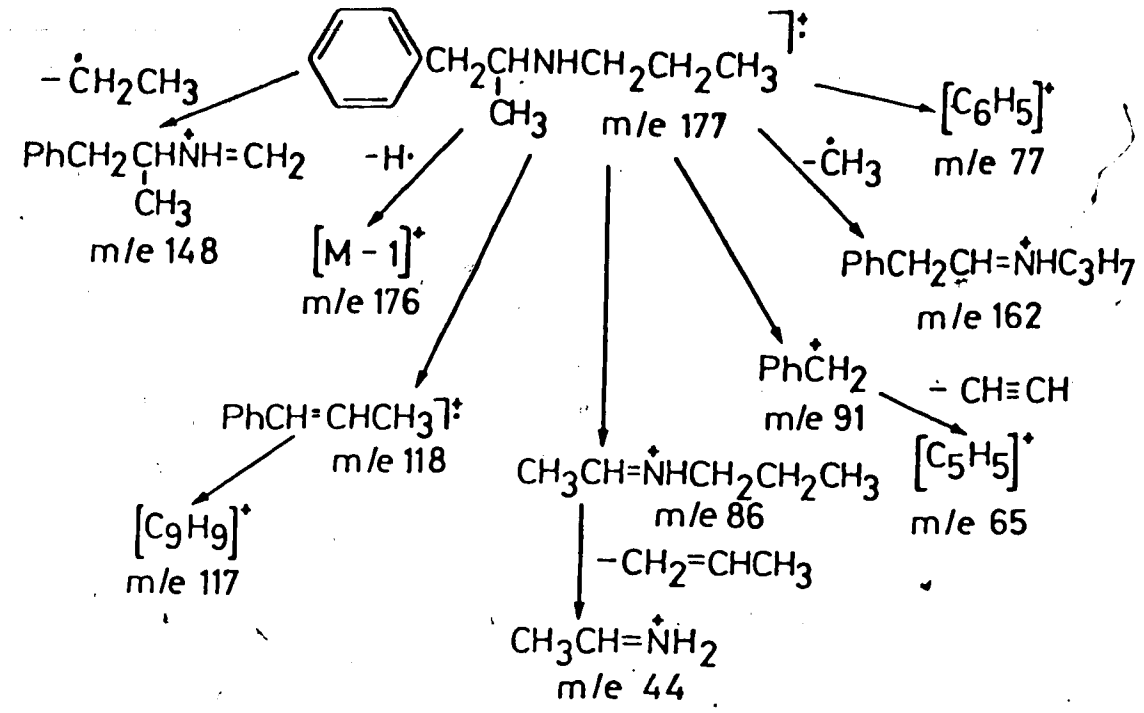


Fig. 13. G.l.c./m.s. identification of N-[(1-methyl-2-phenyl)ethyl]-1-propanimine N-oxide (XVI) as an in vitro metabolite of (±)-N-propylamphetamine.



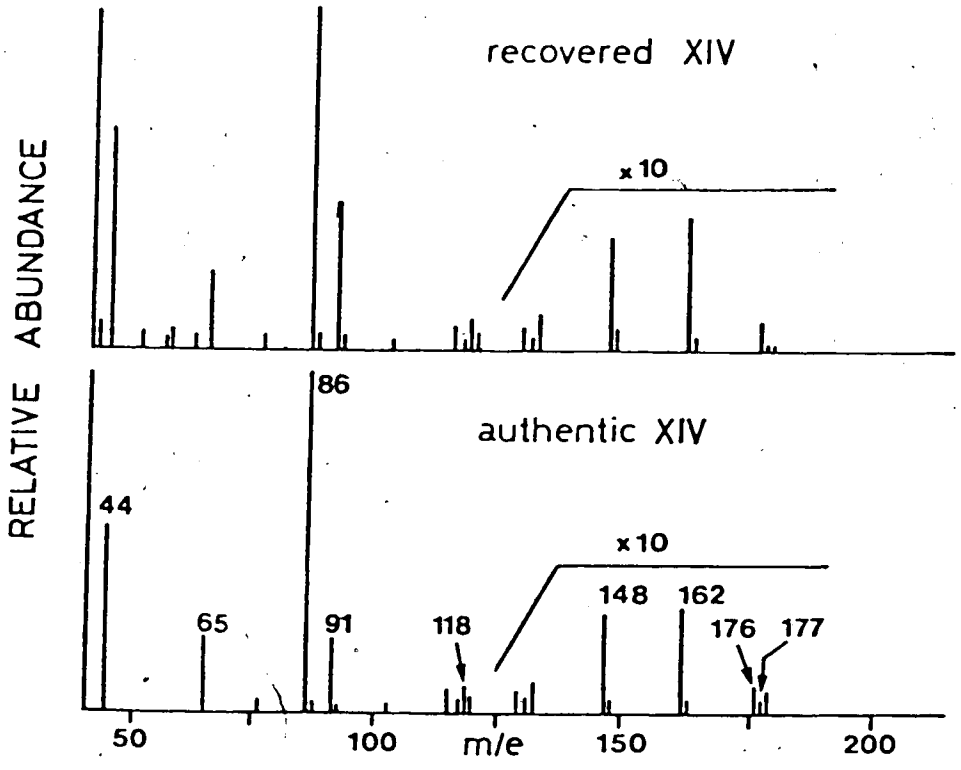
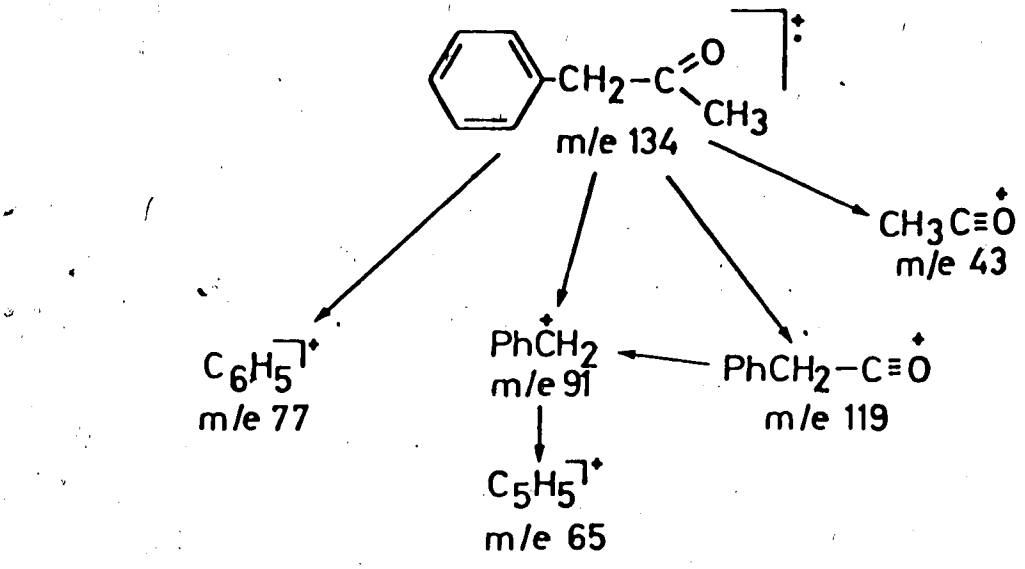


Fig. 14. G.l.c./m.s. of authentic (±)-N-propylamphetamine compared to g.l.c./m.s. of (±)-N-propylamphetamine recovered from in vitro incubation mixtures.



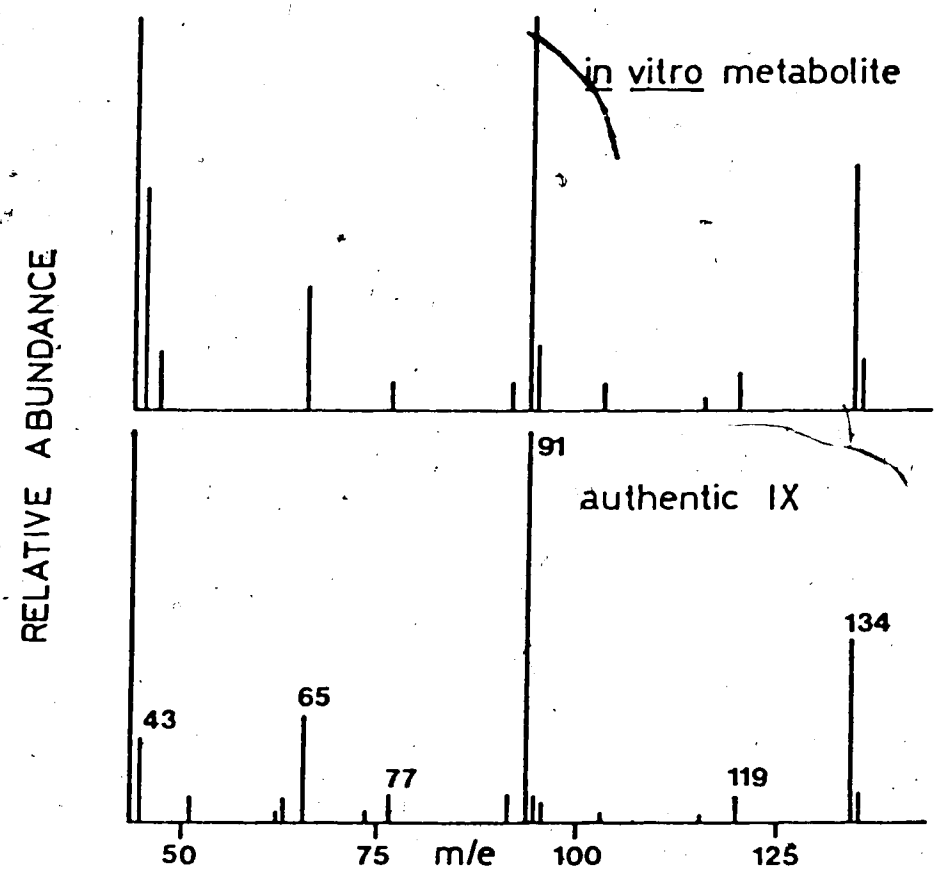
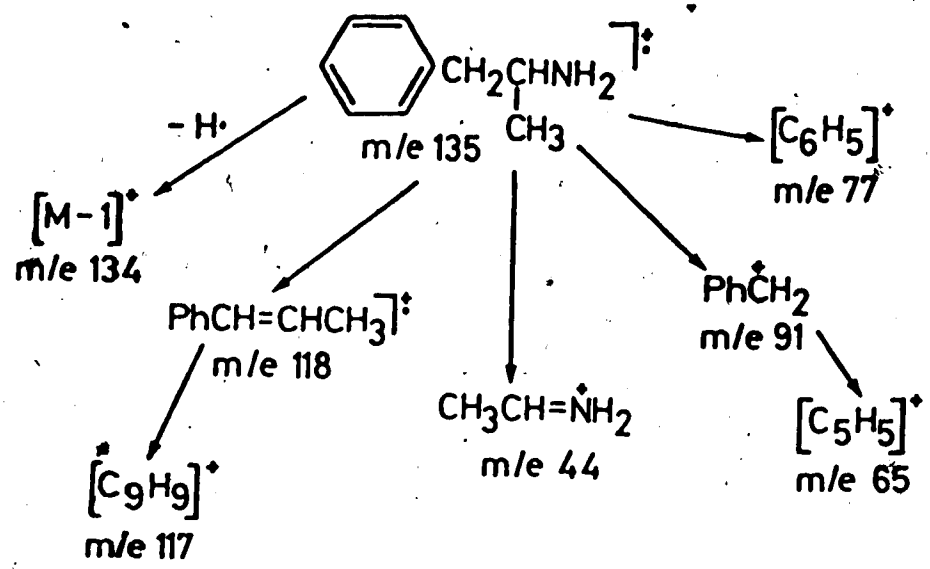


Fig. 15. G.l.c./m.s. identification of 1-phenyl-2-propanone (IX) as an in vitro metabolite of (±)-N-propylamphetamine.



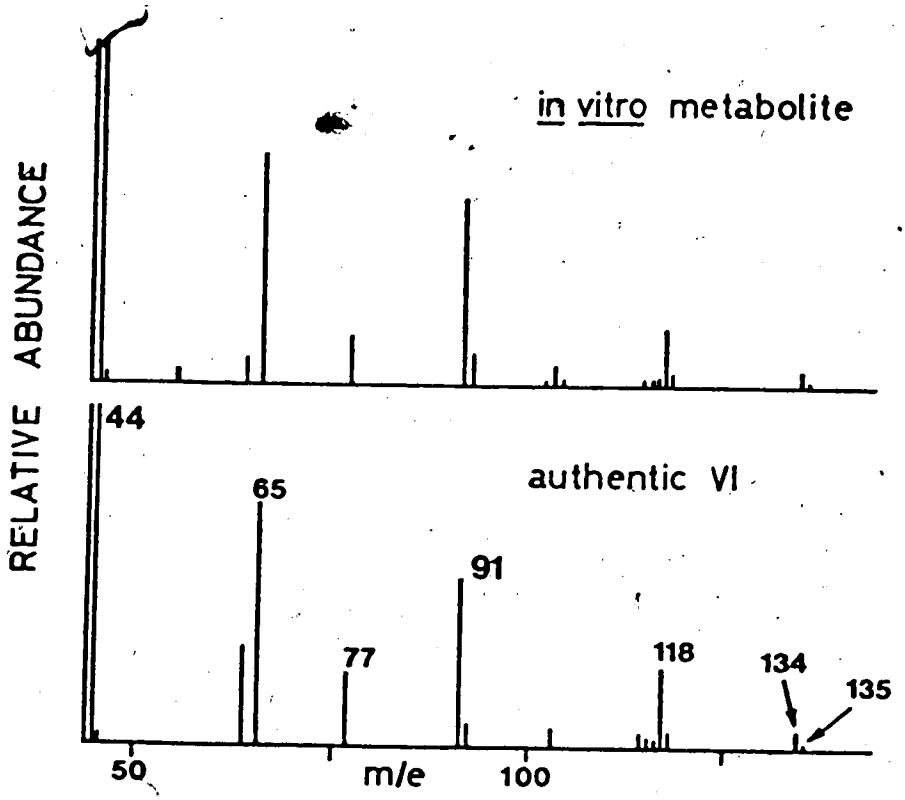
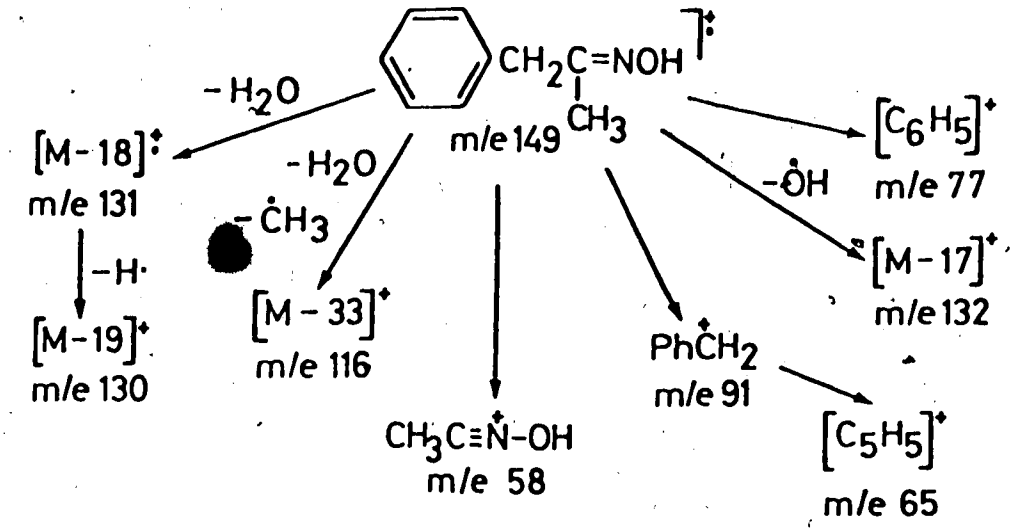


Fig. 16. G.l.c./m.s. identification of amphetamine (VI) as an in vitro metabolite of (±)-N-propylamphetamine.



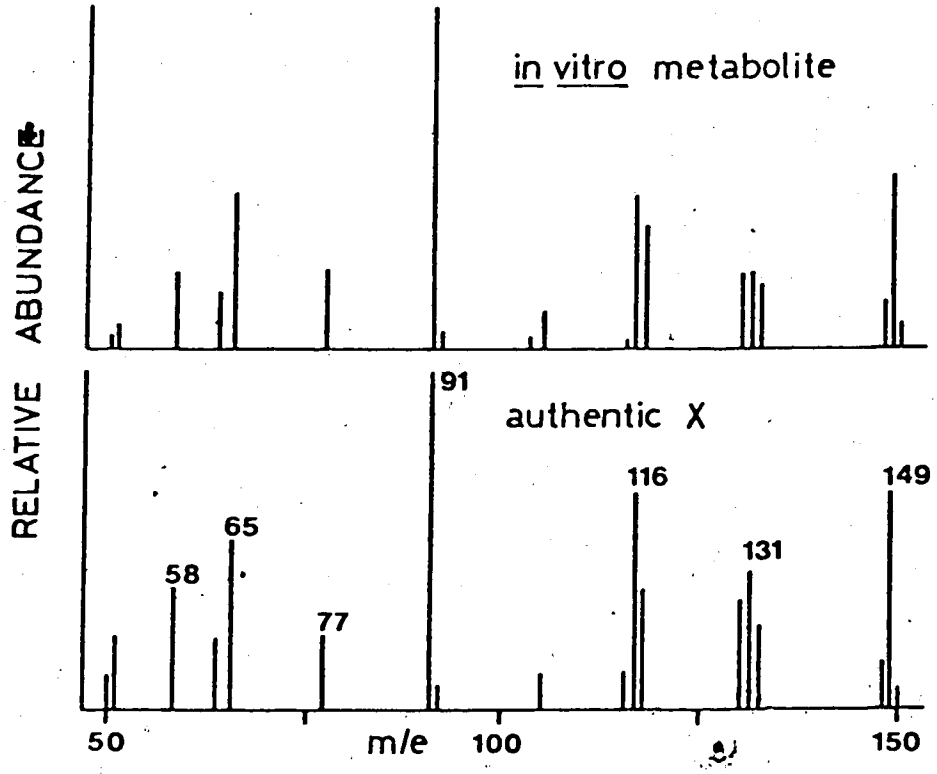
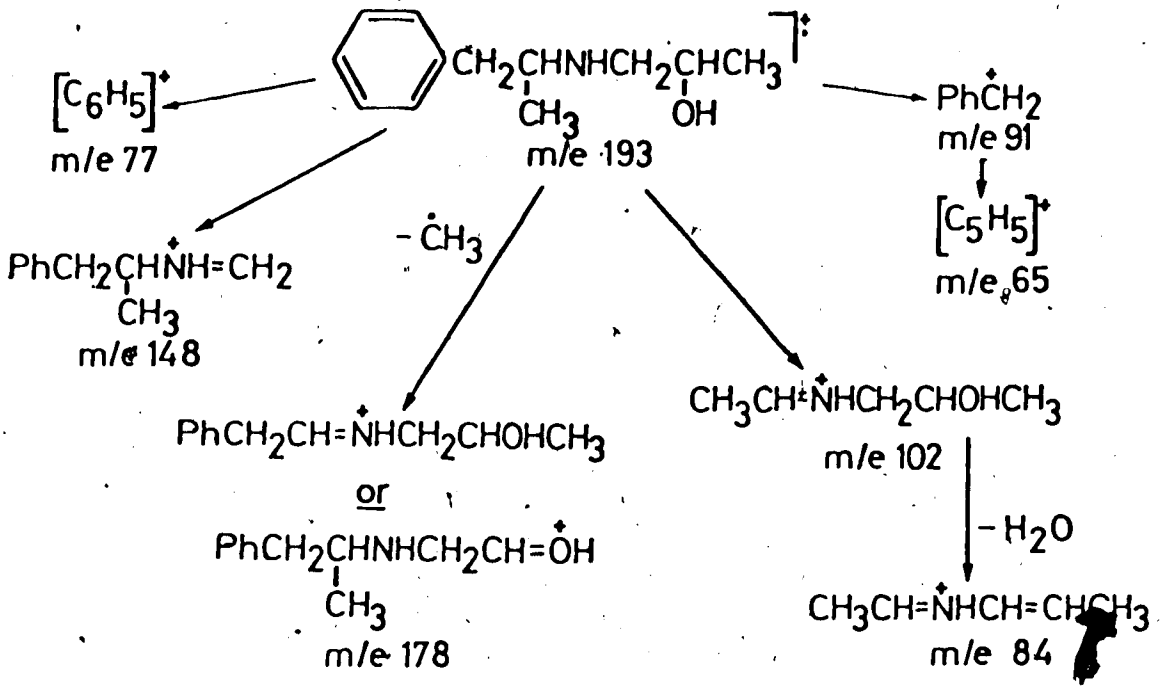


Fig. 17. G.l.c./m.s. identification of 1-phenyl-2-propanone oxime (X) as an in vitro metabolite of (±)-N-propylamphetamine.



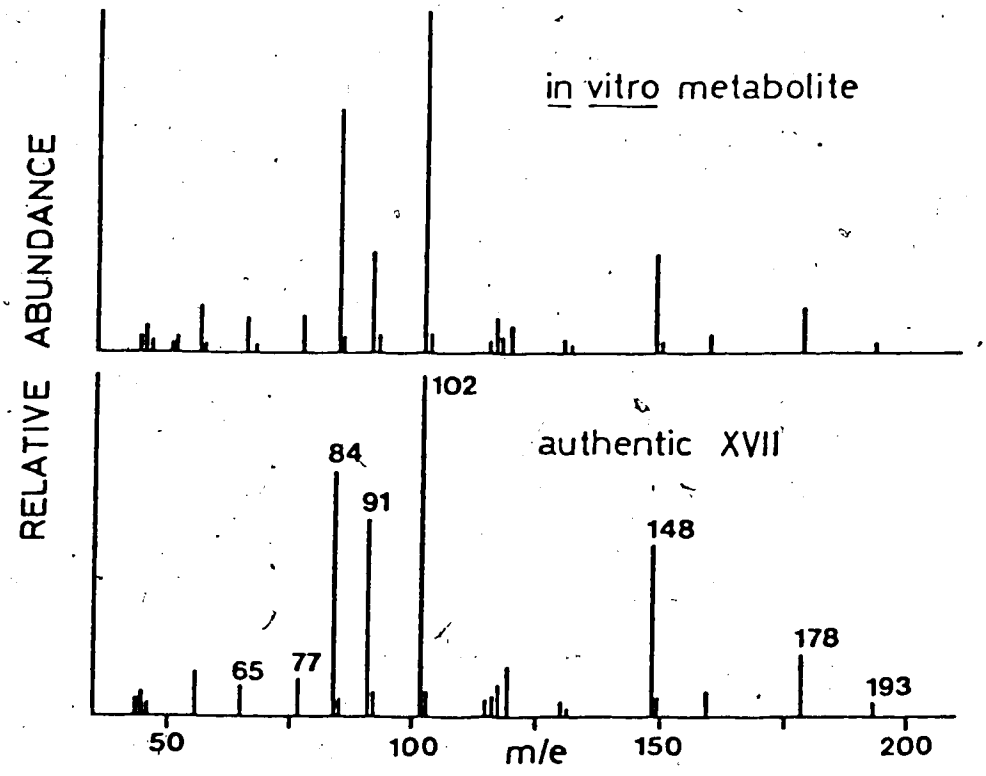


Fig. 18. G.l.c./m.s. identification of 2-(2-hydroxypropylamino)-1-phenylpropane (XVII) as an in vitro metabolite of (±)-N-propylamphetamine.

characteristic mass spectra can be obtained. If combined g.l.c./m.s. is to be used to identify primary hydroxylamines, they can be converted to their trimethylsilyl (TMS) derivatives (64) prior to analysis. Subsequent mass spectra of these silyl derivatives contain ions which have been shown to be diagnostic of N-hydroxylated compounds (cf. scheme 16).

The most reliable method of obtaining characteristic mass spectra of primary hydroxylamines is by direct-inlet or direct-insertion mass spectrometry in which the sample is introduced directly into the ion source and the spectrum recorded. Beckett, Coutts and Ogunbona (98) used this method exclusively for the m.s. characterization of primary hydroxylamines which would otherwise decompose.

G.l.c./m.s. analysis and interpretation of the fragmentation patterns of N-OH-NPA and its related nitrone have been reported previously (98). These compounds may also decompose to some extent in the g.l.c./m.s. system, but in comparison to the primary hydroxylamines, this decomposition is negligible. In the present study, steps were taken to obviate any loss of metabolite through on-column decomposition (vide infra).

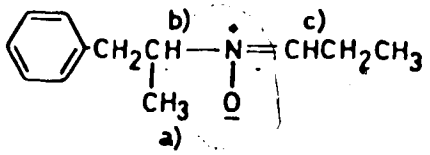
The g.l.c./m.s. information shown in fig. 12 was obtained from synthetic N-OH-NPA and from N-OH-NPA produced metabolically by, and isolated from, incubation mixtures where (±)-NPA was

used as substrate. These spectra were identical to those reported previously (98). Like the parent amine, N-OH-NPA did not give an abundant molecular ion (m/e 193) but had a low abundance $(M-1)^+$ ion of m/e 192. Other abundant ions present in the mass spectrum of N-OH-NPA were of m/e 118, 117, 102 (base peak), 91, 86, 84, 77, 65, and 60. Of particular diagnostic importance were the ions of m/e 102, 91, 86 and 60. The composition of each of these ions is shown in fig. 12 which indicates that the oxidation has occurred on the $-CH(CH_3)NHCH_2CH_2CH_3$ side-chain and not on the benzyl group (cf. fig. 14). The presence of the abundant fragment ion m/e 60 was particularly diagnostic. This fragment ion is observed in the mass fragmentation of primary and secondary (if the N-alkyl group is larger than $-CH_3$) N-hydroxy amphetamines (cf. scheme 14). The m/e 60 fragment ion is considered to be virtually indisputable evidence that the oxygen atom is located on the nitrogen atom. Beckett et al. (98) reported that the mass spectrum of N-OH-NPA was similar regardless of whether combined g.l.c./m.s. or direct-inlet m.s. was used.

The nitrone (XVI) derived from (±)-NPA also fragments in the mass spectrometer in a fashion which can be used for identification (fig. 13). The mass spectrum of the nitrone, like that of N-OH-NPA, contained a molecular ion of very low abundance. Major diagnostic ions in the mass spectrum of the nitrone were of m/e 162, 118,

117, 100, 91 (base peak), and 84. A single outstanding feature of the mass spectrum of XVI was the relative abundance of the ions of m/e 117 and 118. Beckett et al. (98) showed the m/e 118 ion as the base peak in their mass spectrum of nitrone XVI whereas the m/e 91 ion is the base peak in fig. 13. This may reflect either a difference in scanning technique or a difference in m.s. instruments. Never-the-less, the m/e 117 and 118 ions appear in mass spectra of other amphetamines (cf. fig. 14) but their relative abundances are quite small in comparison to those seen in the mass spectrum of XVI.

Beckett, Coutts and Ogunbona (98) pointed out that from mass spectral data, the position of the double bond attached to the nitrogen atom of XVI could not be determined. They stated that the double bond could be either on the N-alkyl side-chain or on the benzylic side of the N atom. In a subsequent report (109), these same authors examined purified XVI and obtained n.m.r. data indicating the position of the double bond to be as shown in fig. 13. An n.m.r. spectrum of XVI prepared in the course of the present studies substantiated their assignment of the position of the double bond. The n.m.r. spectrum of XVI (in $CDCl_3$) was: δ 0.89 (t, 3H, J=6.5, $CH_2\underline{CH}_3$); 1.46 (d, 3H, J=6.5, $CH\underline{CH}_3$); 2.00-3.55 (m, 4H, $\underline{CH}_2$ groups); 3.60-4.36 (m, 1H, $\underline{CH}CH_3$); 6.27 (t, 1H, J=6.5, N=$\underline{CH}$); 7.14 (s, 5H, $C_6\underline{H}_5$). Of particular diagnostic value



were the chemical shifts and multiplicity of the signals produced by the three groups identified as (a), (b), and (c) in the structure above of XVI. The protons of the methyl group (a) came into resonance at δ 1.46; the signal was a 3-proton doublet. One methine proton (b) was a downfield multiplet (δ 3.60-4.36) and the other methine proton (c) gave a signal which was particularly diagnostic. It was a far-downfield (δ 6.27) triplet as would be expected if it were directly attached to a $C=\overset{+}{N}-O^-$ carbon atom.

The g.l.c./m.s. behavior of various oximes has been reported (98). Whether combined g.l.c./m.s. or direct-inlet m.s. is performed, oximes generally produce abundant molecular ions. In the case of 1-phenyl-2-propanone oxime (X), in addition to an abundant molecular ion of m/e 149 (fig. 17), there are diagnostic ions of m/e 132, 131, 130, 116, 91 (base peak), 77, 65, and 58. The m/e 116 is considered to be of greatest importance (98) and its formation is thought to be the result of the sequential loss of H_2O and a methyl radical from the molecular ion (fig. 17).

3. Quantitation of In Vitro Metabolites

As outlined previously, the quantitation of N-OH-NPA was based on the direct g.l.c. analysis immediately after ether extraction of the incubated in vitro metabolism mixtures at pH 7.4. It has been shown that N-hydroxy metabolites of the amphetamines are sensitive to work-up procedures employed during analysis (62, 98). This

is especially true in g. l. c. examinations where the choice of column and geometry of column fit in the gas chromatograph oven are important. The Perkin-Elmer model 990 and Hewlett-Packard models 5700A and 5710A gas chromatographs used in the present study are designed in such a manner as to permit positioning of a glass column within a few millimeters of the injection port septum. This allows direct on-column injection of sample and eliminates exposure of labile metabolites to the heated metal surfaces of the injection port where decomposition of metabolites can occur.

To determine the stability of N-OH-NPA in the g. l. c. system to be used for quantitative analysis, N-OH-NPA was liberated from its oxalate salt in pH 7.4 phosphate buffer and extracted immediately into freshly distilled ether. Three µl of the ether extract was then analyzed by g. l. c. on 7.5% Carbowax 20M on Chromosorb W under the conditions listed in Table 10 for column D (155°). Decomposition of N-OH-NPA to the related nitrone (XVI) and oxime (X) was observed and accounted for up to 20-25% of the total peak area integrated.

The possibility existed that the oxime and nitrone were formed in the aqueous solution prior to extraction, but this was ruled out as a major source of oxime and nitrone when serial samples were prepared. In these samples, aliquots of a solution of N-OH-NPA oxalate dissolved in pH 7.4 buffer were extracted with ether as

before, at intervals over a several hour period, and analyzed by g.l.c. The rate of conversion of N-OH-NPA to oxime and nitrone was quite slow ($<5\%\ h^{-1}$). The conclusion was that on-column decomposition of N-OH-NPA was the major source of oxime and nitrone.

Calibration curves for N-OH-NPA constructed with data obtained from g.l.c. columns in which Chromosorb W had been used were difficult to reproduce and particularly at low concentrations (0.1 - 1.0 μmol), the calibrations were unreliable.

Several other types of liquid phases for g.l.c. analysis of N-OH-NPA were screened, i.e. OV-101, OV-3, OV-25, XE-60, and OV-225. For the most part, these columns were found to be unsuitable either because of poor peak resolution or extensive on-column destruction of N-OH-NPA.

Up to this point, g.l.c. column packing material had been prepared in the standard manner; the selected liquid phase was dissolved in the appropriate solvent and added to a slurry of stationary phase in the same solvent used for the liquid phase. The solvent was then removed under reduced pressure in a rotary evaporator. This standard method has been criticized (125) as being grossly inappropriate for the preparation of high efficiency g.l.c. columns because of the tendency of fine particles of Chromosorb W to fracture in a rotary evaporator. This fracturing process

results in exposure of active (i.e. uncoated) sites which reduce column efficiency, performance, and, as in the case of N-hydroxy compounds, these sites might act as catalysts for on-column decomposition. The authors of this critical paper (125) recommended the use of a new solid support, Chromosorb 750, which is made of harder and more uniform particles than Chromosorb W and, as a result, was less likely to fracture and subsequently develop undesired active sites. The ultimate benefit was greater column efficiency and peak resolution.

When the column packing was prepared according to Leibrand and Dunham (125) and Chromosorb 750 was substituted for Chromosorb W, on-column decomposition of N-OH-NPA was considerably reduced and overall peak resolution for all compounds was improved. This method of column preparation was adopted and used exclusively thereafter.

Calibration curves for the quantitation of metabolically produced N-OH-NPA by means of g.l.c. analysis on this new column were linear and reproducible in the concentration ranges encountered in the in vitro experiments.

The slope of the calibration curves for N-OH-NPA, and the other compounds quantitated, were calculated on a Digital computer model PDP-8L, using a custom program for linear regression and correlation (appendix 1). The computer program was designed to calculate quantities of drug or metabolite from the peak areas of drug

or metabolites and internal standard, and the slope of the calibration curve. Subsequent modifications of this program permitted the calculation of the amount of metabolite in μmol of product formed mg^{-1} protein h^{-1} (appendix 1).

In the absence of adequate amounts of the authentic nitrone (XVI) standard curves for this product were not available initially and detector response for the nitrone, for purposes of quantitation, was considered to be comparable to that for N-OH-NPA in view of their structural similarity. However, after several in vitro experiments, it became apparent that the sum of substrate and metabolites recovered accounted for only 96-97% of the starting material. Numerous possibilities were considered which might account for less than 100% recovery including the presence of undetected metabolites.

The obvious approach seemed to be construction of a standard curve for the nitrone in view of the fact that this was the only compound for which a standard curve had not been prepared. Comparison of the detector response of nitrone XVI and N-OH-NPA would determine if in fact a quantitative difference existed.

Authentic nitrone was eventually prepared from N-OH-NPA by means of a mild oxidation with yellow mercuric oxide (cf. scheme 11; 90, 109). After purification, g.l.c., g.l.c./m.s., and n.m.r. characterization, a standard curve for the pure nitrone (0.1 -

0.5 μmol) on g.l.c. column D (155°) revealed that there was indeed a difference in the detector sensitivity to the nitrone when compared to N-OH-NPA and this difference accounted for much of the "missing" metabolites. Total recovery from *in vitro* incubation mixtures using NPA as substrate was then established at 98.8±0.4%.

Quantitative analysis of the oxime (X) and phenylacetone (IX) were uncomplicated. Both of these compounds were easily and completely extracted with ether from both neutral (pH 7.4) and basic (pH 12 - 13) solutions. The oxime may be routinely quantitated on g.l.c. column D (155°) where there were no interfering peaks. Phenylacetone elutes from column D (155°) coincident with NPA and must be quantitated on column E (155°), on which these two compounds separate completely (table 12) and on which no interfering peaks are observed. Calibration curves for these two compounds were linear and reproducible in the concentration ranges encountered in the *in vitro* experiments (0.05 - 0.5 μ mol). Final calculation of the quantities of oxime and phenylacetone present in the *in vitro* incubation mixtures was determined by the computer program previously described.

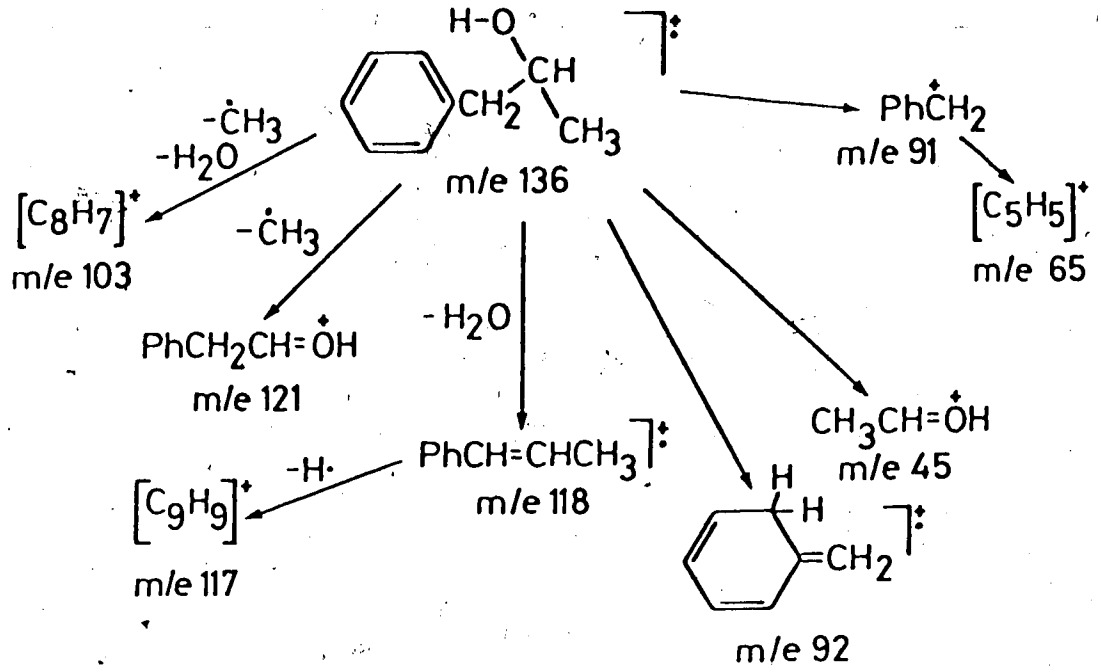
Hucker (89) has reported that phenylacetone (IX) was metabolically reduced to the corresponding alcohol, 1-phenyl-2-propanol, by rabbit liver microsomes *in vitro*. On g.l.c. column E (155°), the Tr of 1-phenyl-2-propanol was observed to be 3.4 min. This places it exactly in between phenylacetone (IX) and amphetamine (VI), both of which are observed as *in vitro* metabolites of NPA.

G. l. c. traces of ether extracts of in vitro metabolism mixtures where NPA was used as substrate, did not contain any detectable amounts of 1-phenyl-2-propanol. If any was prese , it must have been in very small quantities. A final attempt to qualitatively identify 1-phenyl-2-propanol in the in vitro mixtures involved the use of g. l. c./m. s.

The alcohol, although structurally related to phenylacetone, produces a distinctly different mass spectrum (fig. 19) in which the base peak is of m/e 92, rather than of m/e 91, as is the case for phenylacetone and amphetamine.

Repeated m. s. scanning of the tail of the phenylacetone peak and of the region where 1-phenyl-2-propanol would be expected in the gas chromatogram of an ether extract of an in vitro incubation mixture did not produce any m. s. fragments suggestive of the presence of 1-phenyl-2-propanol. If the ketone metabolite (IX) of NPA was metabolized to the related alcohol, the amounts involved were inconsequential.

Recovery of unchanged NPA from the in vitro incubation mixtures could be quantitated by examining either neutral (pH 7.4) or basic (pH 12-13) ether extracts. As would be expected of a basic amine, NPA was more efficiently extracted from aqueous solutions at pH 12-13 than at pH 7.4, but on both column D (155^{o}) and E (155^{o}) linear and reproducible cal ation curves were obtained at both



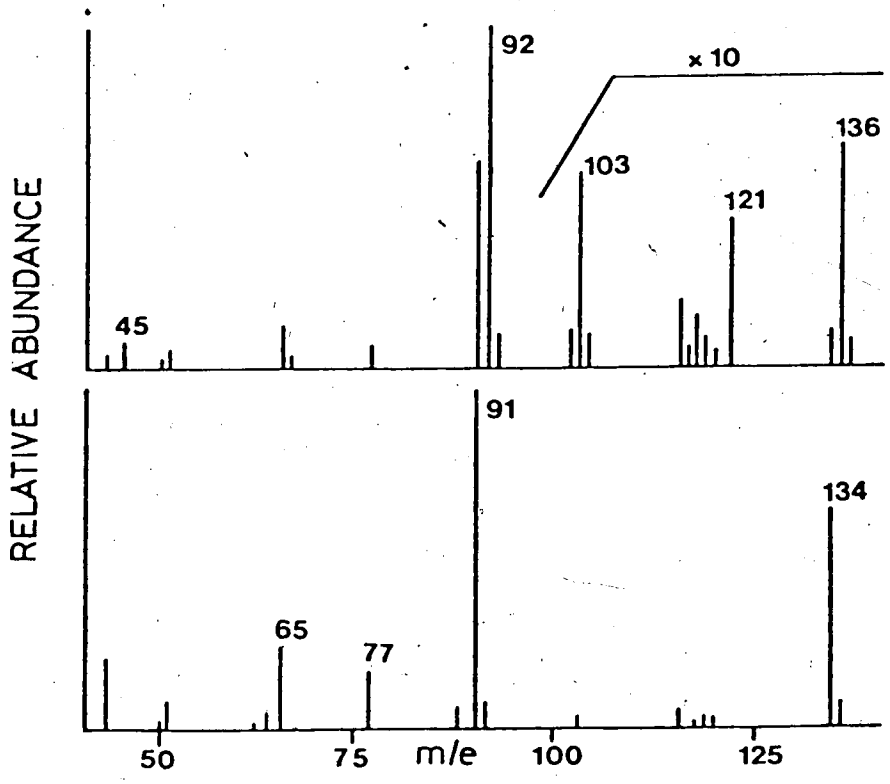


Fig. 19. G.l.c./m.s. behavior of 1-phenyl-2-propanol (cf. fig.15) and a comparison of the g.l.c./m.s. line diagrams for 1-phenyl-2-propanol (top) and 1-phenyl-2-propanone (bottom).

pH values. NPA present in ether extracts of in vitro incubation mixtures at pH 7.4 and pH 12-13 was routinely quantitated on column E (155°).

NPA can also be quantitated on column D (155°). However, when in vitro incubation mixtures are extracted and analyzed on column D, phenylacetone and amphetamine which have virtually identical g.l.c. retention times (table 12), elute from this column simultaneously with NPA. Therefore, to quantitate NPA on column D, the amounts of phenylacetone and amphetamine must first be determined on column E and then these values subtracted from the amount of "NPA" observed on column D.

Recovery of amphetamine, the N-dealkylated metabolite of NPA, from aqueous solutions at pH 7.4 was erratic and non-reproducible. G.l.c. calibration curves of ether extracts of amphetamine from aqueous solutions at pH 7.4 were non-linear and subject to significant errors, especially in the concentration range of 0.1 - 0.5 μmol. Accurate and reproducible quantitation of amphetamine could only be obtained by ether extraction of the aqueous samples at pH 12 - 13.

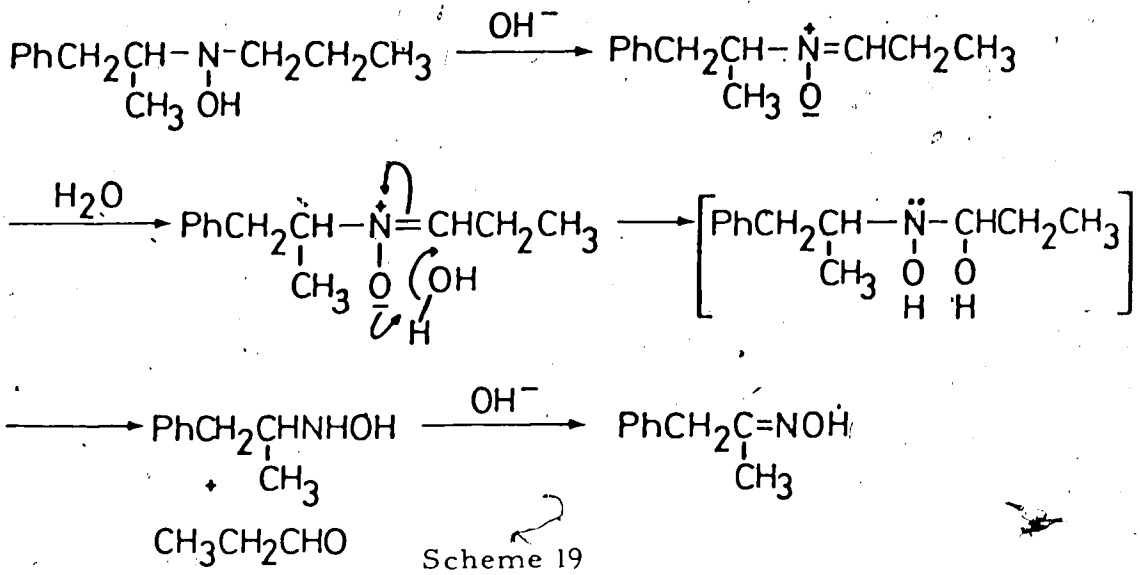
Beckett et al. (68) pointed out that primary, and to some extent, secondary hydroxylamines were susceptible to decomposition in aqueous solutions of NaOH. The rate of decomposition of secondary hydroxylamines was not reported but it was claimed that the disappearance of the secondary hydroxylamine was inversely proportional to

the length of the N-alkyl substituent. In view of the necessity to basify the *in vitro* incubation solutions prior to quantitative analysis of amphetamine, it was necessary to determine the effects of adjusting the pH to 12-13 on the stability of the N-oxygenated metabolites of NPA.

Standard *in vitro* incubation mixtures at pH 7.4 were prepared with (±)-NPA as substrate and incubated at 37° for 1 h. At the end of the incubation period, duplicate samples were extracted and analyzed as follows: one group of samples was extracted with ether immediately at pH 7.4 and analyzed on g.l.c. column D (155°) for N-OH-NPA (XV), the related nitrone (XVI) and the oxime (X), and on column E (155°) for phenylacetone (IX) and NPA; a second group of samples was adjusted to pH 12-13 and immediately extracted and analyzed as above on column D for the N-oxygenated compounds and on column E for phenylacetone, NPA and amphetamine.

Quantitation and comparison of these two sets of samples revealed that exposure to aqueous NaOH for a short period of time (2-5 min) resulted in only a minor (5-10%) decomposition of N-OH-NPA to the related nitrone and oxime. However, when similar samples, or aqueous samples containing only small amounts (0.1-0.5 μmol) of pure N-OH-NPA or nitrone, were left for extended periods (*e.g.* 12-24 h) at pH 12-13, complete conversion of N-OH-NPA and nitrone to oxime was observed by g.l.c. analysis and could be shown to be quantitative by reference to calibration curves.

A rationalization for the conversion of N-OH-NPA and its nitrone to oxime in the presence of base can be seen in scheme 19. The <u>in vitro</u> metabolite, N-OH-NPA, is converted in basic



Scheme 19

solution to the nitrone which subsequently hydrolyzes to N-hydroxyamphetamine. Under basic conditions, N-hydroxyamphetamine is extremely unstable and instantly decomposes to the oxime (112).

The oxime (X), formed from N-OH-NPA and its nitrone in the presence of aqueous NaOH, is relatively stable to basic conditions. If the presence of this oxime in metabolism extracts was indicative of metabolic N-oxidation, it follows then, that quantitation of total N-oxidation of NPA could be accomplished by converting all N-oxygenated products to the relatively stable oxime.

To test this approach to the quantitation, incubated metabolism mixtures containing (±)-NPA, microsc and cofactors were adjusted to pH 12-13 with NaOH and left ding for 24h. Upon g.l.c. analysis it first appeared that no al of the N-OH-NPA had been converted to oxime since a g. c pea se Tr corresponded

to N-OH-NPA persisted. Adding more base and heating to 37° for 24h did not further affect this peak. A g.l.c./m.s. analysis of the peak was performed and the fragments observed are shown in fig. 18. The absence of fragments at m/e 60 and m/e 86 was strong evidence that the unknown compound was not N-OH-NPA (cf. fig.12), but was, instead, an additional alkali-stable metabolite. The ions of major diagnostic importance were of m/e 148, 102, 91, 84, 77 and 65. These fragments confirmed that metabolic oxidation had not occurred on the benzyl group but rather on the

$$-CH(CH_3)NHCH_2CH_2CH_3$$

side-chain of NPA. The presence of strong ions at m/e 148 and m/e 84 were of particular diagnostic value in locating the position of the hydroxyl group in this metabolite on the N-alkyl side-chain. Comparison of the g.l.c./m.s. of an authentic sample of XVII with that of the metabolite confirmed the structure to be that of XVII.

In view of the discovery of this new metabolite whose g.l.c. retention time corresponded to N-OH-NPA, changes had to be made in the analytical schemes employed. To analyze for XVII, all of the N-OH-NPA present in the in vitro incubation mixture had to be converted either to nitrone and/or oxime (vide supra). As a result, quantitation using the pH 12-13 extract had to be based on

"total N-oxidized" products. This was defined as the sum of the components of the incubation mixture isolated and identified as containing an N-O bond (in this case, oxime and nitrone). Similarly, if quantitation of N-OH-NPA was desired, the amount of XVII contributing to the N-OH-NPA peak in the neutral extract had to be determined and subtracted from the amount integrated as N-OH-NPA. Repeated observations showed, however, that the amount of XVII in the neutral extract was relatively constant and contributed little to the N-OH-NPA peak. Thus, these involved mathematical manipulations were discontinued. Furthermore, once the metabolic pattern of N-OH-NPA had been established, quantitation of N-OH-NPA itself was discontinued in favor of reporting N-oxidation on the basis of the sum of oxime and nitrone after NaOH treatment of the incubation mixtures.

Although p-hydroxyamphetamine is a major in vivo metabolic product of amphetamine in the rat (81, 83, 103) and has been identified in trace quantities in vitro (106, 108), neither its presence nor that of p-hydroxy-NPA could be confirmed in this in vitro study. However, a significant amount of administered (±)-NPA is converted to p-hydroxy-NPA in vivo in rats (vide infra). Studies to be reported later have revealed that the efficiency of ether extraction of p-hydroxyamphetamine from aqueous solutions was extremely low, i.e. 20-25%, even at pH 9.5. It is possible that small amounts of

p-hydroxyamphetamine were formed in vitro from NPA and simply not detected. The extraction efficiency of p-hydroxy-NPA on the other hand, was shown to be greater from doped solutions, i.e., 60-70%, at pH 9.5, but none could be detected in the in vitro incubation mixtures.

4. Establishment of Optimal Conditions For In Vitro Metabolic N-Oxidation In Rat Liver Homogenates

In order to obtain precision between experiments, standardized in vitro conditions must be determined. The enzymatic assay should be performed under conditions in which the concentration of substrate and cofactors are not rate-limiting, and product formation should be linear with respect to time during the incubation period. Thus, various factors, among them, the concentrations of enzyme, substrate, cofactors, and period of incubation, must be evaluated to establish optimal conditions.

These studies were undertaken only after acceptable methods of extraction, identification and quantitation of in vitro metabolites of NPA had been established.

a. Cofactor Concentration

The cofactors of interest for initial study were G-6-P, $NADP^+$, and Mg^{++}. In each of these experiments, the volume and concentration of the components of the incubation mixture, other than the particular cofactor under study, were held constant. By varying

the concentration of each cofactor individually over a range of concentrations followed by incubation, at 37° for 1h, and extraction and g. l. c. analysis of the metabolism solution, information was readily obtained pertinent to the requirements for each individual cofactor for N-oxidation of NPA.

In incubation mixtures where only G-6-P had been omitted, it was not surprising to see a certain amount of metabolism of (±)-NPA occur in 1h in view of the presence of endogenous G-6-P in the soluble fraction of the liver homogenate (fig. 20). The addition of 10 µmol of G-6-P however, increased metabolism 2-3 fold when compared to samples in which G-6-P had not been added. A marked departure from linearity was observed over the 60 minute period in this sample, indicating an early depletion of G-6-P. Addition of 20 µmol G-6-P significantly increased the amount of N-oxidation at 30 minutes when compared to the 10 µmol sample and improved linearity through the 60 minute period. Inclusion of 40 µmol G-6-P did not significantly improve the rate of N-oxidation when compared to samples in which 20 µmol had been added, indicating that 40 µmol G-6-P was supramaximal.

That $NADP^+$ was an absolute requirement for metabolic N-oxidation to occur can be seen in fig. 21. Omitting $NADP^+$ from the incubation mixture completely inhibited the N-oxidation of (±)-NPA. Addition of 2.2 µmol $NADP^+$ catalyzed N-oxidation

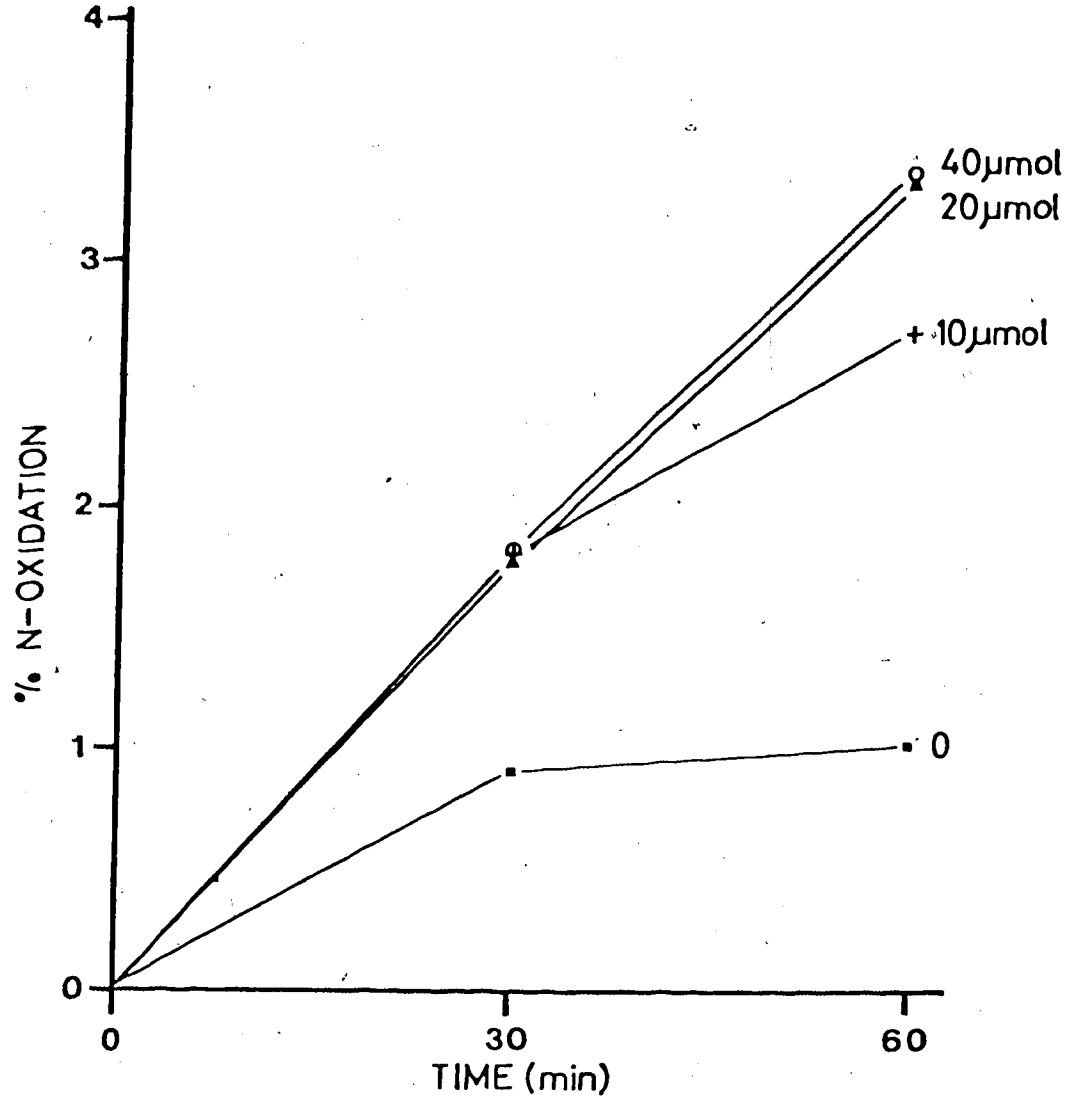


Fig. 20. The effect of concentration of glucose-6-phosphate on the in vitro N-oxidation of (±)-N-propylamphetamine.

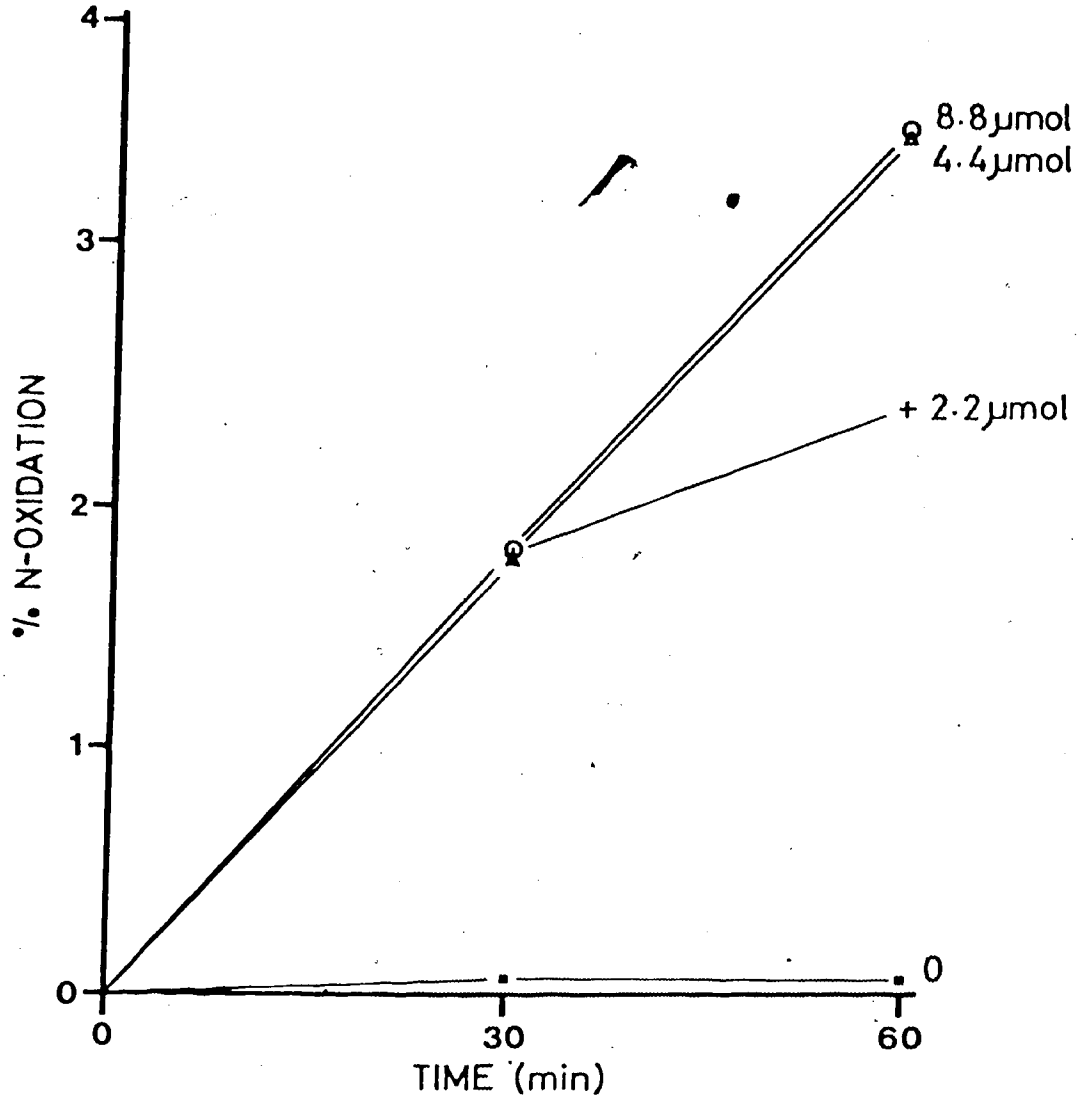


Fig. 21. The effect of concentration of nicotinamide adenine dinucleotide phosphate on the in vitro N-oxidation of (±)-N-propylamphetamine.

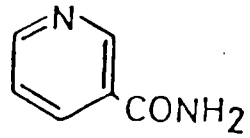
but departure from linearity in the ensuing 30 min indicated that the $NADP^+$ was either consumed or destroyed and little further metabolism occurred. Addition of 4.4 or 8.8 μmol $NADP^+$ improved the rate of N-oxidation when compared to samples containing 2.2 μmol but adding more that 4.4 μmol did not further increase metabolism. This indicated that more than 4.4 μmol $NADP^+$ was not needed for maximal N-oxidation of NPA.

When NADPH (4 μmol) instead of $NADP^+$ was added in aliquots to a standard incubation mixture over a 60 min incubation period, nearly identical amounts of metabolites were observed as when $NADP^+$ was used. Addition of NADPH and $NADP^+$ to the incubation mixture did not increase metabolism over the amount seen for $NADP^+$ alone. This information indicated that sufficient enzymes were present to carry out the required conversion of $NADP^+$ to NADPH and that additional NADPH was not necessary.

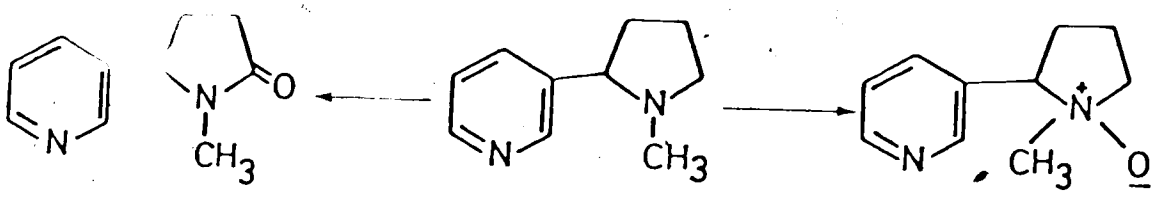
An absolute requirement for Mg^{++} could not be demonstrated despite that fact that Mg^{++} is thought to be involved with G-6-P dehydrogenase and generation of NADPH (11).

The results of varying the concentration of G-6-P and $NADP^+$ and the subsequent effects on N-oxidation of NPA indicate that both are required for maximal metabolic activity but neither of the two appear to be the rate-limiting step in metabolic N-oxidation under the conditions employed in this assay.

Nicotinamide is often added to incubation mixtures as a cofactor in the belief that its presence prevents the loss of $NADP^+$ by inhibiting nucleotidase, an enzyme responsible for destruction of $NADP^+$. Schenkman et al. (126) and Parli and Mannering (127) refute this and claim that its presence inhibits metabolic C-oxidation of certain substrates. Since nicotinamide is itself metabolically N-oxidized to its N-oxide (128,129), it may compete with substrate in metabolic N-oxidation studies and it would therefore, seem advisable to omit nicotinamide from all in vitro metabolism reactions. However, conflicting evidence is available. In vitro metabolic C-oxidation of nicotine to cotinine is not adversely affected by the presence of nicotinamide and in fact the rate of metabolic N-oxidation is actually increased (scheme 20; 130,131). The presence of nicotinamide in in vitro incubation mixtures also



nicotinamide



cotinine nicotine nicotine-N-oxide

Scheme 20

increases the rate of phentermine N-oxidation (93). Since these results were obtained from a tertiary (nicotine) and a primary (phentermine) amine substrates, it was of interest to see what

the effects would be of including nicotinamide in the (±)-NPA (a secondary amine) incubation mixture.

The results (table 13; fig. 22) indicate that the presence of nicotinamide in the incubation mixture reduced significantly the amount of metabolic N-oxidation of (±)-NPA. Since nicotinamide is extracted during in vitro metabolism studies and is an interfering peak in qualitative and quantitative g.l.c. studies, its presence was undesirable in incubation mixtures. For this reason, and because of the results shown in Table 13 and fig. 22, it was apparent that the inclusion of nicotinamide in NPA incubation mixtures was unnecessary. This may also be true for the in vitro metabolism of other medicinal secondary amine derivatives of amphetamine.

FAD is involved in N-oxidation of some aliphatic amines but it has not been demonstrated whether exogenous FAD has any effect on the in vitro N-oxidation of secondary aliphatic amines. Inclusion of 50-100 µmol FAD in standard in vitro incubation mixtures may slightly reduce N-oxidation of (±)-NPA (table 14). With the small amount of information available, it is impossible to make conclusions about the involvement of exogenous FAD in metabolic N-oxidation. There may also be a factor or factors, as yet unidentified, that are required in addition to FAD. Further research in this area is required.

TABLE 13. THE EFFECT OF NICOTINAMIDE ON THE IN VITRO N-OXIDATION OF (±)-N-PROPYLAMPHETAMINE IN FORITFIED INCUBATION MIXTURES PREPARED WITH THE 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATE.

Conc. of nicotinamide[1] (μ mol)	Percentage of total N-oxidation[2,3,4] after an incubation of	
	30 min	60 min
0	1.83 ± 0.24	3.12 ± 0.32
30	1.74 ± 0.20	3.09 ± 0.35
60	1.30 ± 0.29	2.66 ± 0.32
120	1.22 ± 0.13	2.34 ± 0.46

[1] per 6 ml of incubate

[2] Sum of metabolites XV, XVI, and X

[3] Average of 4 determinations; values are ± standard deviation

[4] See footnote to table 15

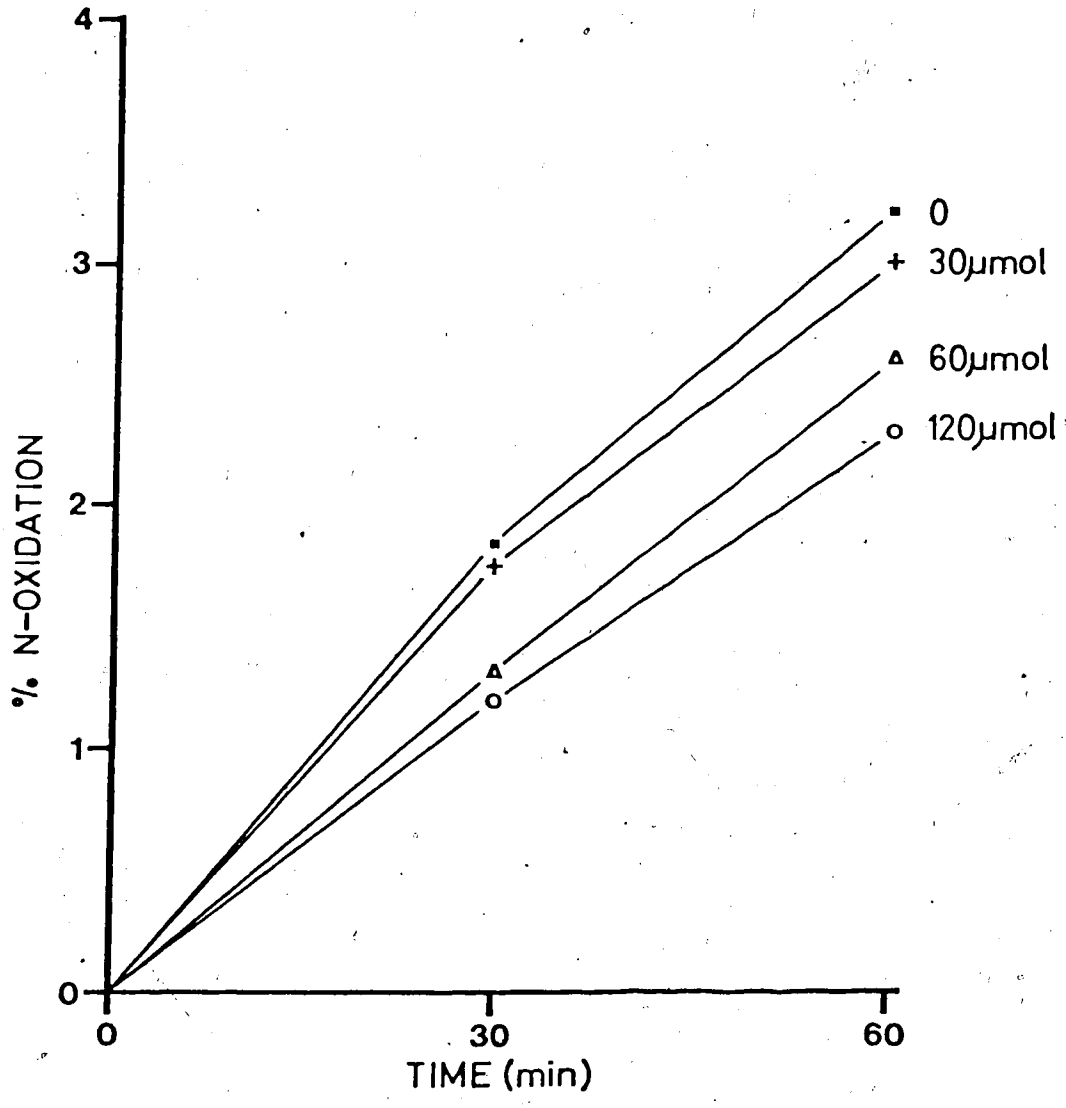


Fig. 22. Effect of nicotinamide on the in vitro N-oxidation of (±)-N-propylamphetamine.

TABLE 14. EFFECT OF FLAVINE ADENINE DINUCLEOTIDE ON THE IN VITRO N-OXIDATION OF (±)-N-PROPYLAMPHETAMINE BY FORTIFIED 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATE[1]

Concentration FAD[2] (μmol)	μmol N-oxidized[3] product mg^{-1} protein h^{-1}
0	0.15±0.02
50	0.14±0.02
100	0.14±0.02

[1] one hour incubation at 37°. Values are (±) S.E.M. (n=2).

[2] per 6 ml of incubate

[3] sum of metabolites X and XVI

b. Buffer Type and pH

Gorrod (72) pointed out that the optimum pH for *in vitro* metabolic N-oxidation varies considerably with the substrate involved. It was readily apparent (72) that the work published to date on the *in vitro* metabolism of the amphetamines generally employed phosphate buffer pH 7.4. It follows that to establish optimum *in vitro* conditions for N-oxidation, the effect of pH must be considered.

Standard incubation mixtures containing (±)-NPA, in which the pH of 0.1 M phosphate buffer ranged from 7 to 9, were incubated for 1h at 37° and extracted with ether and analyzed on g.l.c. column D (155°) and E (155°). From the results depicted in fig. 23, and Table 15, it was abundantly clear that the optimum pH for metabolic N-oxidation of (±)-NPA was not 7.4 but rather near 8.4. A 2-3 fold increase in total N-oxidation was observed as a result of this pH change. Above pH 8.4 the amount of metabolism began to decrease, presumably as a result of enzyme inactivation.

This observed change in metabolism with change in pH of incubation mixtures might be explained at least in part by considering the pKa of the amphetamines (*i.e.* pKa 9-10). By increasing the pH from 7.4 to 8.4 the amount of unionized NPA will also increase. It is the unionized moity which is able to penetrate the lipoidal barrier

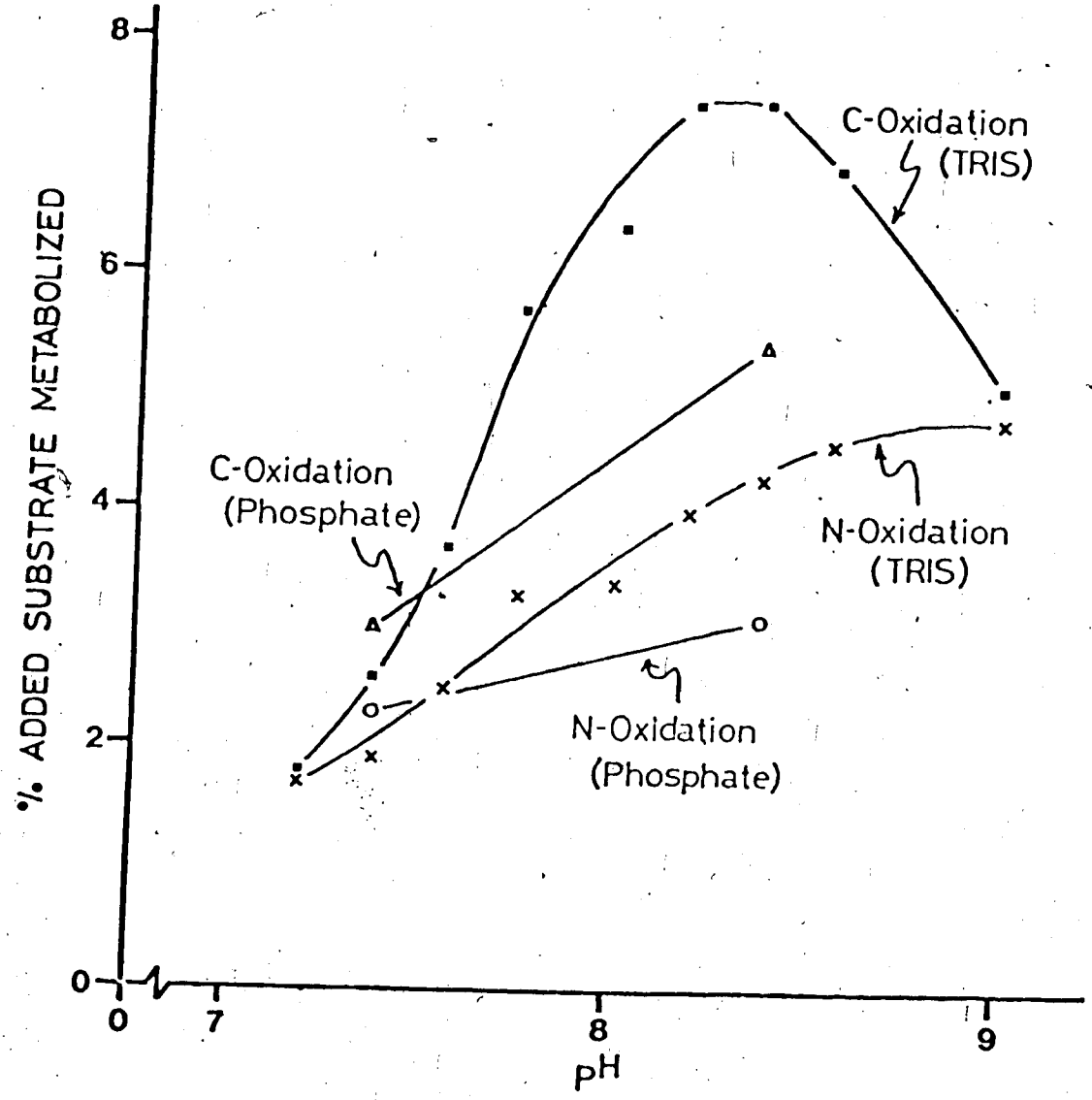


Fig. 23. Effect of pH and buffer type on the in vitro N- and C-oxidation of (±)-N-propylamphetamine.

TABLE 15. EFFECT OF pH ON THE IN VITRO N- AND C-OXIDATION OF N-PROPYLAMPHETAMINE IN FORTIFIED INCUBATION MIXTURES PREPARED FROM THE 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATES[1]

Substrate	pH	N-oxidized Products[2]		C-oxidized Products[2]	
		µmol	µ mol mg^{-1} protein h^{-1}	µmol	µ mol mg^{-1} protein h^{-1}
(+)-NPA	7.4	0.11±0.02	0.04±0.004	0.77±0.07	0.31±0.02
	8.4	0.26±0.04	0.1 ±0.001	1.05±0.1	0.42±0.03
(-)-NPA	7.4	0.26±0.03	0.10±0.01	0.70±0.07	0.28±0.02
	8.4	0.53±0.14	0.2 ±0.03	0.96±0.09	0.39±0.05
(±)-NPA	7.4	0.19±0.01	0.07±0.005	0.62±0.04	0.23±0.01
	8.4	0.40±0.02	0.15±0.006	0.89±0.07	0.33±0.02

[1] Values are ± standard deviation representing 3 experiments, each of which was done in duplicate. Samples incubated for 1h at 37°.

[2] The term 'C-oxidized products' refers to compounds isolated and identified as possessing a C-O function introduced during either the metabolism reaction or the subsequent analytical procedure. Similarly the term 'N-oxidized products' refers to isolated compounds shown to possess an N-O group. It is not implied that the total C-oxidized products are formed by metabolic C-oxidation reactions; C-oxidized compounds can be formed as a result of an initial metabolic N-oxidation (Beckett and Bélanger, 67,73).

surrounding the suspended microsome and subsequently interact with the enzymes contained therein. The result is increased metabolism if the amount of enzyme present is sufficient to react with the increased amount of drug.

A second possibility could conceivably involve pH dependent effects on the tertiary structure of the enzymes involved where stereochemical fit of NPA to the enzyme active site might become optimized at a given pH, which in this case would be 8.4.

A third possibility, also related to pKa, is an increased extraction efficiency of the metabolites at higher pH values being reflected as false increases in metabolism.

This last possibility can be dismissed, however, in view of the fact that the standard curves for g.l.c. analysis in this experiment were constructed from extracts done at the appropriate pH values and thus accounting for changes in extraction efficiency.

The effect that the pH of the incubation mixture might have on the stereospecificity of the enzyme active site cannot be ascertained on the basis of the information available. However, the theory cannot be dismissed entirely in view of the fact that the ultimate downward trend in C-oxidation as pH increased, occurred sooner than trend reversal for N-oxidation. This may reflect a pH effect on stereospecificity but more likely is a reflection of different substrate specificity of NPA for P-450 (C-oxidation) and FAD-amine oxidase (N-oxidation).

The most logical conclusion then, would seem to be that as pH increased from 7.4 to 8.4, the amount of unionized drug available to pass the lipoidal barrier around the microsomes increased and the greater effective concentration of NPA at the enzyme active sites favored more extensive metabolism and that as pH increased, the enzymes, especially those responsible for C-oxidation, were inactivated. It should be pointed out that one experiment was performed where the pH was adjusted to 8.4 and (+)-N-methylamphetamine was used as the substrate instead of NPA. The results indicated that this increase in the pH of the incubation mixture also increases the N-oxidation of N-methylamphetamine.

During the course of the studies on the effect of pH on N-oxidation, it was realized (132) that phosphate buffer might not have the buffer capacity (i.e., ability to maintain a constant pH) that would be desirable at higher pH values (i.e. pH 9). A more versatile buffer was necessary and as a result, TRIS·HCl buffer was selected. Subsequent experiments involving metabolism of (±)-NPA at pH values ranging from 7-9 revealed that there was an increased N- and C-oxidation of (±)-NPA in the presence of 0.1 M TRIS·HCl or TRIS·Acetate buffers at pH values which were compared to phosphate buffer (fig. 23). It is not clear what role phosphate buffer plays in metabolism in this system but similar inhibitory effects have been demonstrated elsewhere (133) when aminopyrine was the substrate.

These findings resulted in the adoption of TRIS·HCl as the buffer of choice in subsequent metabolism studies involving N-oxidation of NPA at both pH 7.4 and 8.4. It is noted that pH 8.4 is somewhat of a departure from physiological pH but the object was to determine those factors which affect in vitro N-oxidation of NPA, especially in the rat, whose reputation for metabolic N-oxidation of amphetamines is poor (79). Much of the in vitro work reported involving metabolism of aliphatic amine substrates via N-oxidation in rat liver homogenates will now have to be re-examined.

c. Substrate Concentration and Stereoisomerism

The quantity of substrate employed in an in vitro incubation may effect an interpretation of the amount of metabolism observed. If, for example, too small a quantity of substrate is employed and it is subsequently completely metabolized within a short period of time, enzyme induction may increase the rate of metabolism but obviously not the total amount of metabolites produced. If complete metabolism of a substrate is desired, serial sampling must be performed to detect changes in rates of metabolism in the presence of increased enzyme levels.

Excessive concentrations of substrate could also effect interpretation of data. For example, when performing studies on inhibition of in vitro metabolism, the effect of a competitive inhibitor like

SKF 525-A may not be seen if the concentration of the substrate in the study is so high that it either blocks access of the inhibitor to the enzyme active site or displaces the inhibitor from that site. The result would be the impression that little or no inhibition occurred in the presence of that particular inhibitor.

To determine the optimum concentration of NPA that should be employed in routine in vitro studies of N-oxidation, standard incubation mixtures in TRIS·HCl buffer, pH 8.4, were prepared in which the amount of protein in each flask was held constant at 2 mg, and the amount of (±)-NPA per flask varied from 1 to 20 μ mol. Incubation at 37° for 1h followed by extraction and g.l.c. analysis produced the data shown in fig. 24.

The rate of production of both N- and C-oxidized products was virtually linear until the high concentrations of substrate were reached. The fastest rates observed were, as expected, with the lowest concentrations of substrate. The decision to adopt 10 μmol as the concentration of substrate to be used in subsequent studies was based upon two observations: (1) metabolism of (±)-NPA at this concentration was virtually linear with respect to time so that changes in protein concentration of liver homogenate would be reflected as changes in the rate of metabolism and (2) in view of the small amounts of some metabolites, higher concentrations of substrate and consequently larger amounts of metabolites, was desirable for quantitation and identification.

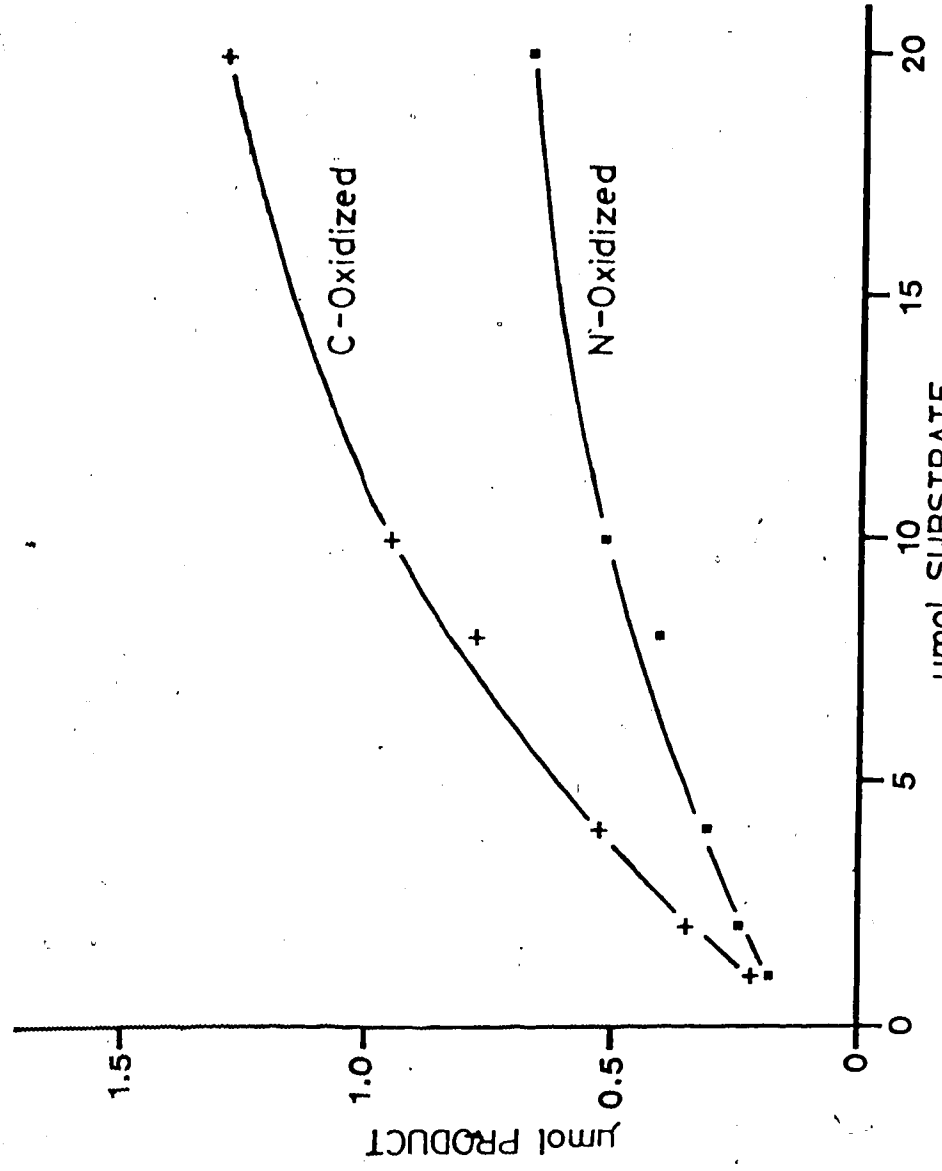


Fig. 24. Effect of substrate concentration on the in vitro N- and C-oxidation of (±)-N-propylamphetamine.

It has been reported (134, 135) that the rate of C-oxidation (i.e., deamination and N-dealkylation) of (+)-isomers of some amphetamines is greater than that for the corresponding (-)-isomer, whereas the rate of N-oxidation of the (-)-isomer is greater than that for the (+)-isomer.

The early results reported here were obtained with (±)-NPA as substrate. To determine the relative contributions of the (+)- and (-)-isomers to the overall amount of in vitro metabolism of NPA, pure samples of (+)- and (-)-NPA were prepared (see Methods and Materials, sec. G.1) and used as substrates for in vitro metabolism by rat liver 12,000 x g supernatant, and the results compared to those obtained with (±)-NPA.

These experiments revealed that C-oxidation was favored in the case of (+)-NPA whereas N-oxidation was favored with (-)-NPA (table 15).

The selective metabolism of the stereoisomers might in part be explained by considering differences in substrate configuration. Binding of NPA with P-450 is apparently sterically more favorable in the case of the (+)-isomer than the (-)-isomer whereas the contrary might apply for the binding of NPA with FAD-amine oxidase.

d. Optimizing Enzyme Preparation and Concentration

Improper technique or lack of standardization of the technique employed in the preparation of microsomal enzymes are

the most frequently encountered causes of questionable results in in vitro metabolic studies. Some of the techniques outlined below are a result of personal experience while others have been adapted from established procedures (11,136).

As mentioned previously, all animals were sacrificed by cervical dislocation. On no occasion were any animals that were to be used in in vitro studies ever sacrificed by the use of any anesthetic.

After sacrifice, the liver may or may not be perfused. Perfusion, usually with isotonic KCl or NaCl, is sometimes recommended when studies involve determination of P-450. Perfusion reduces the amount of hemoglobin (blood) remaining in the liver which can interfere with the P-450 absorption spectrum (11,136). As an alternative to perfusion, the animal may be exsanguinated via the abdominal aorta with a large bore needle and 10 ml syringe immediately after cervical dislocation. This technique, employed in these studies, was found to be acceptable. It should also be noted that perfusion carries with it the danger of lowering the amount of microsomal protein present in the liver tissue (11,136), with a subsequent decrease in in vitro metabolic activity.

Homogenization of liver tissue was the most difficult technique to standardize. Homogenizing for too short a period of time did not rupture liver cells sufficiently to release the enzymes while too vigorous homogenization released mitochondrial enzymes and

extraneous protein, i.e., protein detected by the Folin-Phenol reagent but which is not responsible for drug metabolism (vide infra). Sufficient heat may be generated by vigor homogenization and the result is denaturation of enzymes. To avoid heat-mediated autolysis, the homogenizing tube can be inserted in a beaker of ice during the homogenizing process. The objective is to obtain a smooth homogenate that possesses maximum enzymatic activity with a minimum amount of extraneous protein.

Two basic homogenizing methods were available: the Virtis blender and the Potter homogenizer. Much discussion has been published regarding both homogenizers and both have applications and disadvantages (11, 136).

Two criteria were utilized in determining the efficiency of the homogenizing process: (1) the amount of microsomal protein present in the 12,000 x g supernatant and (2) the extent of metabolism of substrate reported as μmol of product formed mg^{-1} microsomal protein h^{-1}.

It was generally observed that for the Virtis homogenizer, longer times and high speeds were required to obtain a smooth homogenate than were required for the Potter homogenizer. The protracted homogenization procedure resulted in greater amounts of metabolically inactive protein as measured by the criteria listed above. When the Potter homogenizer was used, consistent amounts of protein and

reproducible metabolism could be obtained with 7-8 excursions of the pestle, if the liver was minced prior to homogenization. The Potter homogenizer was adopted for preparation of liver homogenates and was considered to be superior to other methods.

It is worth noting that, within limits, it is difficult to over-homogenize liver tissue with a Potter homogenizer. The important factor is to standardize the procedure with respect to time and vigor of homogenization.

Methods of isolation of the microsomal supernatant from the crude homogenate are varied (11, 136). It is generally accepted that centrifugation of the crude homogenate at 9,000 x g for 15-20 min is sufficient to separate the microsomal fraction (supernatant) from the particulate fraction.

Two factors were of interest in the preparation of the microsomal fraction: (1) if the amount of metabolites produced in vitro was to be quantitated on the basis of mg protein (enzyme) ml^{-1} of homogenate, it would be desirable to have reproducible quantities of metabolically active protein in each sample, and (2) cross-contamination of the microsomal supernatant with mitochondria, which contain reductases, may have adverse effects on the qualitative and quantitative nature of the metabolism mediated by the microsomes. The criteria used to evaluate the effect of variations in centrifugal force applied to the liver homogenate were: (1) µmol product formed mg^{-1} protein h^{-1}

and (2) changes in patterns of metabolites as observed on g. l. c. analysis.

A pooled 20% homogenate of three rat livers in 1.15% KCl was divided into equal portions and subjected to one of the following centrifugation procedures: (1) 9,000 x g for 15 min; (2) 9,000 x g for 30 min; (3) 12,000 x g for 10 min; (4) 12,000 x g for 20 min; (5) 12,000 x g for 20 min followed by decanting the supernatant which was then centrifuged again at 12,000 x g for 20 min. Subsequent protein determination and *in vitro* metabolism using (±)-NPA as substrate revealed (table 16) that while there was little difference in metabolism, the amount of protein present varied from 1.5 mg ml^{-1} of homogenate in the sample centrifuged twice at 12,000 x g, to 2.8 mg ml^{-1} in the homogenate centrifuged at 9,000 x g for 15 min. The implications of these results are obvious. The higher amount of apparently inactive protein present in the latter would give inaccurate estimations of the extent of *in vitro* metabolism when expressed on the basis of µmol product formed mg^{-1} of microsomal protein. The g. l. c. results seemed to indicate that there was no cross-contamination of the microsomal supernatant with mitochondrial enzymes, that is, the relative amounts of metabolites in all samples were nearly identical.

For reproducible results, the method adopted was centrifugation at 12,000 x g for 20 min. This produced a clear, beige-colored

TABLE 16. EFFECT OF CENTRIFUGAL FORCE ON THE YIELD OF MICROSOMAL PROTEIN IN THE SUPERNATANT OF RAT LIVER HOMOGENATE AND SUBSEQUENT IN VITRO METABOLISM OF (±)-N-PROPYLAMPHETAMINE.

Centrifugal force	mg protein ml^{-1} supernatant	μmol product[1] h^{-1}	μmol product[1] mg^{-1} protein h^{-1}
9,000 x g, 15 min	2.8±0.14	0.80±0.06	0.29±0.01
9,000 x g, 30 min	2.2±0.12	0.76±0.05	0.35±0.02
12,000 x g, 10 min	2.1±0.11	0.77±0.04	0.37±0.02
12,000 x g, 20 min	1.8±0.12	0.81±0.05	0.45±0.02
12,000 x g, 20 min, decanted and repeated	1.5±0.08	0.77±0.04	0.51±0.03

[1] total of both N- and C-oxidized metabolites of (±)-NPA (see table 12). Samples incubated for 1h at 37°. Values are ±S.E.M. (n=2).

supernatant, which, when prepared from a 20% w/v homogenate of rat liver, contained 1.6 to 1.8 mg protein ml^{-1}.

e. Relationship Between Rate of Metabolism and Time of Incubation

For reproducible results, the rate of metabolism of a given substrate should be linear with time during the course of the experiment. To assess this parameter, (±)-NPA (10 μmol) was incubated in standard incubation mixtures for up to 90 min. Samples were taken at 0, 5, 10, 15, 30, 45, 60 and 90 min, extracted and analyzed on g.l.c. The results are illustrated in fig. 25.

N-OH-NPA was the metabolite of major interest in this study. This metabolite was detectable in the 5 minute samples and continued to increase in concentration for the first 15 min, after which little further increase in the amount of N-OH-NPA could be demonstrated. At the same time, the amount of nitrone and oxime continued to increase through the first 60 min. After 60 min, linearity of metabolism was lost. Adding additional cofactors at 60 min did not improve metabolism, indicating loss of microsomal enzyme activity. Under the assay conditions employed, N-oxidation was linear with respect to time from 15-60 minutes.

It had been demonstrated that both the oxime and nitrone could arise from N-OH-NPA purely by chemical means but nothing was known of the possibility of enzymatic conversion. To determine if any oxime or nitrone arose as a result of metabolism of N-OH-NPA, N-OH-NPA was used as the substrate in incubation mixtures

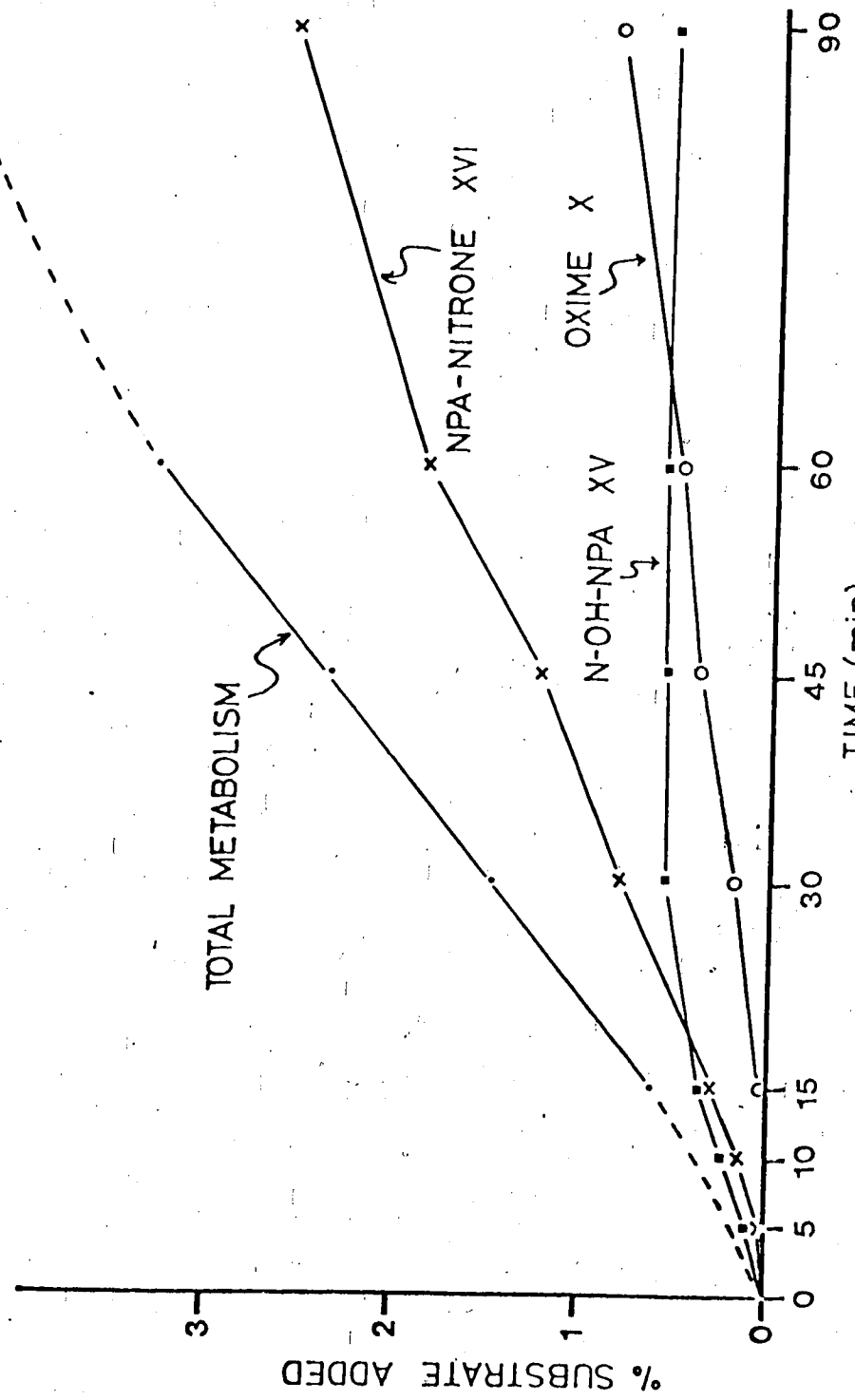


Fig. 25. Production of N-oxidized metabolites in vitro from (±)-N-propylamphetamine using the fortified 12,000 x g supernatant of rat liver homogenate.

containing microsomes but no cofactors, boiled microsomes with cofactors, or microsomes complete with cofactors. The results of this experiment are shown in fig. 26. The rate of conversion of N-OH-NPA to oxime and nitrone in the first two solutions was identical but slightly slower than that observed in the intact metabolism mixture. This indicates that one cannot rule out enzyme-mediated conversion of N-OH-NPA to oxime or nitrone. Beckett and Al-Sarraj (79) reported that N-hydroxyamphetamine was not metabolized by rabbit, guinea pig or rat liver homogenates but rather the conversion of N-hydroxyamphetamine to oxime in vitro was solely chemical. Beckett and Gibson (137) have reported that N-hydroxy-dibenzylamine was metabolized by rabbit liver microsomes in vitro to as yet unidentified metabolites. It appears then, that whether or not the N-hydroxy metabolites are further metabolized, apparently varies with the individual N-hydroxy compound.

5. In Vitro N-oxidation Following Pretreatment of Rats

It has been accepted that many drugs and chemicals increase the levels of hepatic drug metabolizing enzymes with subsequent increases in metabolic activity (vide supra). Gorrod (72) has pointed out that little information is available regarding induction of the N-oxidation of secondary aliphatic amines. Gorrod cites unpublished observations of Beckett and others that indicate phenobarbital or 3-MC pretreatments do not effect the level of in vitro

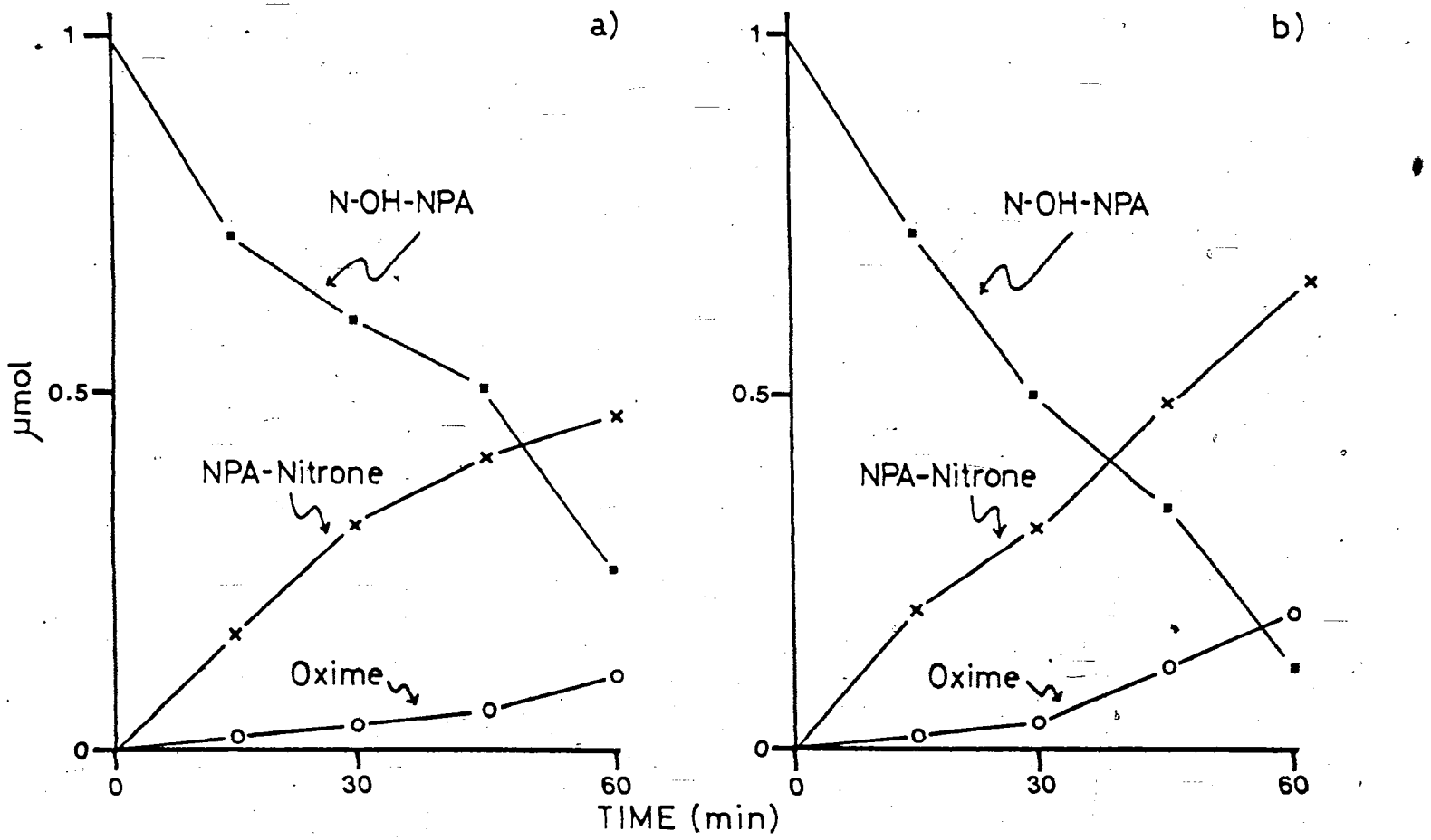


Fig. 26. Non-enzymatic (a) and enzymatic (b) conversion of N-OH-NPA to NPA-nitrone and oxime (n=2).

N-oxidation of amphetamine or N-methylamphetamine. The only clear evidence regarding increased N-oxidation after enzyme induction with phenobarbital or 3-MC was obtained from studies on amines which are N-oxidized by P-450, i.e. principally group II and III substrates (fig. 8).

a. Pretreatment with (±)-NPA

An early attempt to increase the in vitro metabolic N-oxidation of NPA involved injecting rats with (±)-NPA·HCl according to schedule 1 (table 11). The animals were fasted on the fourteenth day of treatment prior to preparation of the 12,000 x g supernatant on the following day. An ether extract of a portion of the liver homogenate was adjusted to pH 12 and extracted with ether. This extract was examined by g.l.c. on column C (155°) and confirmed that it contained no residual NPA or metabolites. When the 12,000 x g supernatant was used with normal cofactors, except nicotinamide, to metabolize (±)-NPA, (+)-NPA, or (-)-NPA, no significant differences were observed (table 17) from results obtained with non-pretreated animals.

There was a possibility that the metabolic activity of the microsomal enzymes was affected in the early stages of the NPA pretreatment. It has been shown (138) that chronic amphetamine treatment in vivo decreases protein synthesis in the liver and as a result decreases the rate of its own metabolism and possibly that of other substrates.

TABLE 17. IN VITRO METABOLISM OF (+), (-), AND (±)-N-PROPYLAMPHETAMINE IN FORTIFIED INCUBATION MIXTURES PREPARED WITH THE 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATE OBTAINED FROM RATS TREATED CHRONICALLY WITH INTRAPERITONEAL INJECTIONS OF (±)-N-PROPYLAMPHETAMINE[1]

Substrate	μmol product mg^{-1} protein h^{-1}	
	Control[2]	Pretreated[2]
(±)-NPA	0.41±0.04	0.42±0.06
(+)-NPA	0.44±0.05	0.43±0.05
(-)-NPA	0.47±0.05	0.48±0.06

[1]Rats received (±)-NPA (10 mg kg^{-1}) for 14 days

[2]Sum of all metabolites (see table 12) produced in vitro. Samples incubated for 1h at 37°. Values are (±) S.E.M. (n=2).

To determine if there were any changes in the in vitro metabolism of NPA in the early stages of chronic NPA treatment, rats (300-400 g) were prepared and submitted to the same procedure as above except that animals were sacrificed after 1, 2, 4, 6, 8, 10, 12, and 14 days of pretreatment with (±)-NPA. In vitro metabolism of (±)-NPA was studied in each animal and the results are shown in fig. 27. As Magour et al. (138) pointed out for amphetamine, metabolism of NPA under these conditions appeared to decrease. However, in this case, the metabolism gradually returned to near control levels. As concluded by Magour et al. (138), it seems that the tolerance reported as a consequence of chronic amphetamine treatment (139, 140) is not a result of increased metabolism of amphetamine but rather more likely a cellular effect producing tolerance within the CNS.

When the amount of metabolism of NPA was expressed as μmol product formed mg^{-1} protein h^{-1}, the results are virtually consistent throughout the duration of the pretreatment. This was a reflection of the relationship of the rate of metabolism with respect to protein concentration. Observations of the food intake of these animals showed a marked decrease in food consumed during the pretreatment period which would lead to the conclusion that the anorexiant effects of the amphetamine was in operation. The result may have been an increased gluconeogenesis from protein with subsequent decrease in enzymes reflected as decreased microsomal protein and lower amounts of metabolites produced.

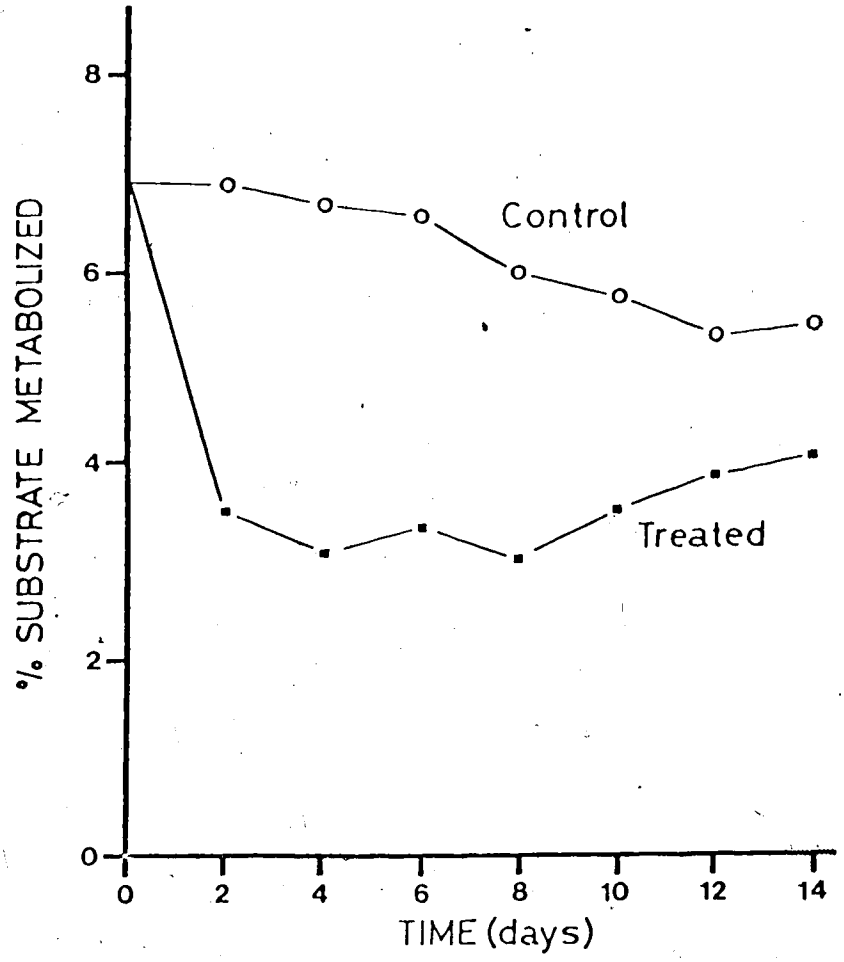


Fig. 27. Effect of chronic treatment of rats with (±)-N-propylamphetamine on the in vitro metabolism of (±)-N-propylamphetamine (n=2).

To determine the effects of chronic treatment with NPA for extended periods of time up to 4 weeks, (±)-NPA was administered in the drinking water (schedule 2, table 11) to eliminate the necessity of daily handling of animals and at the same time to keep doses low enough to obviate the decreases in microsomal protein seen with i.p. treatment.

Rats weighing 300-400 g were housed individually in wire suspension cages and (±)-NPA·HCl (0.01%) was dissolved in their drinking water. For the ensuing 4 weeks, food and water intake were measured daily. The total dose of NPA ingested ranged from 86.2 mg to 95.5 mg. At the end of the four-week treatment period, the animals were fasted overnight prior to preparation of the 12,000 x g supernatant as before. When (±)-NPA was used as the substrate in fortified in vitro incubation mixtures which were incubated at 37° for 1h followed by ether extraction and g.l.c. analysis, no differences were found in in vitro metabolism or in the levels of microsomal protein or P-450 (table 18). The conclusion was, therefore, that pretreatment of rats with NPA does not induce its own metabolism but instead, in high doses, leads to a decrease in microsomal protein and a subsequent decrease in its own metabolism.

b. Pretreatment of Rats With 3-MC

Pretreatment of rats (250-300 g) with 3-MC (schedule 3, table 11) resulted in a 39% increase in total N-oxidized products

TABLE 18. IN VITRO METABOLISM OF (±)-N-PROPYLAMPHETAMINE IN FORTIFIED INCUBATION MIXTURES PREPARED WITH 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATE OBTAINED FROM RATS TREATED CHRONICALLY WITH (±)-N-PROPYLAMPHETAMINE IN THE DRINKING WATER.[1]

μmol product mg^{-1} protein h^{-1}		mg protein[3]		m μmol Cyt P-450 gm^{-1} protein	
Control[2]	Pretreated[2]	Control	Pretreated	Control	Pretreated
0.42±0.05	0.41±0.06	2.1±0.21	2.2±0.25	0.40±0.04	0.37±0.04

[1] (±)-NPA·HCl (0.01%) was dissolved in drinking water and offered to rats for 4 weeks

[2] Sum of all in vitro metabolites after incubation of 1h at 37°. Values are (±) S.E.M. (n=2).

[3] mg protein per ml of 12,000 x g supernatant

and a 11% increase in C-oxidized products when (±)-NPA was used as substrate in vitro at pH 8.4 (table 19). It was noted that the increase in total C-oxidized products was due mainly to an increase in 1-phenyl-2-propanone (IX) and amphetamine (VI). No increase in the side-chain hydroxylated product (XVII) was noted.

The increase in IX and VI while there was no increase in XVII suggests that two enzymes were involved; one enzyme (i.e., for alpha-C-oxidation of NPA to IX and VI) which was susceptible to 3-MC induction, and a second (i.e., for penultimate-C-oxidation of NPA to XVII) which was not.

Beckett et al. (see ref. 72) have claimed that 3-MC pretreatment of rats in vivo does not stimulate in vitro metabolic N-oxidation of amphetamine or N-methylamphetamine. The results with NPA, showing an increased N-oxidation after 3-MC pretreatment, indicate that there may be more than one form of FAD-amine oxidase involved in metabolic N-oxidation of aliphatic amines, similar to the existence of multiple forms of P-450 for metabolic C-oxidation (34), each with different substrate specificities.

c. Pretreatment of Rats With Phenobarbital

Pretreatment of rats (250-300 g) with phenobarbital (schedule 4, table 11) and subsequent in vitro incubation of (±)-NPA using the fortified 9000 x g supernatant, resulted in a 30-40% increase in the in vitro metabolism of (±)-NPA to amphetamine and 1-phenyl-2-propanone at pH 8.4. A proportionally greater increase

TABLE 19. EFFECTS OF 3-METHYLCHOLANTHRENE PRE-TREATMENT[1] OF RATS ON THE IN VITRO METABOLISM[2] OF (±)-N-PROPYLAMPHETAMINE IN FORTIFIED 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATE.

Metabolite	μ mol h^{-1}		μ mol mg^{-1} protein h^{-1}	
	Control	Pretreated	Control	Pretreated
1-phenylacetone (IX)	0.08±0.001	0.10±0.01	0.04±0.004	0.042±0.003
amphetamine (VI)	0.37±0.05	0.49±0.06	0.21±0.04	0.25 ±0.02
2-OH-NPA (XVII)	0.037±0.004	0.04±0.004	0.02±0.003	0.02 ±0.003
oxime (X)	0.03±0.002	0.05±0.005	0.02±0.003	0.05 ±0.005
NPA-nitrone (XVI)	0.25±0.03	0.38±0.02	0.14±0.02	0.20 ±0.03

[1] 20 mg kg^{-1} single i.p. dose 24h before sacrifice

[2] incubated 1h at 37°. Values are (±) S.E.M. (n=2).

in amphetamine production than 1-phenyl-2-propanone production was observed in the phenobarbital pretreated animals. This more selective increase in amphetamine production suggests that the enzymes responsible for N-dealkylation of NPA are more susceptible to phenobarbital induction than the deamination enzymes.

Total N-oxidized products after phenobarbital treatment either stayed the same, or as in the case of one experiment actually declined slightly. Beckett and Gibson (137) observed a similar decrease in the amount of N-hydroxy metabolite formed from dibenzylamine and concluded that phenobarbital pretreatment increased the metabolism of the initial N-hydroxy metabolite to an as yet unknown compound. If N-OH-NPA was metabolized to something than what has been reported previously (vide supra), this new metabolite could not be observed on g.l.c. or g.l.c./m.s. analysis.

6. In Vitro N-Oxidation in the Presence of Enzyme Inhibitory Substances

The incorporation of many substances into the microsomal incubation medium has shown clear differences between the enzymes responsible for C-oxidation in organic compounds and those enzymes responsible for the N-oxidation of certain organic nitrogen compounds (72). Beckett et al. (62) claim that SKF 525-A does not affect N-hydroxylation of amphetamine while at the same time it blocks

formation of the deaminated metabolite, phenylacetone. In a subsequent report, Beckett, Gorrod and Lazarus (141) reported slight inhibition of the N-hydroxylation of N-methylamphetamine when SKF 525-A was present. Both of these substrates are of group I (fig. 8), and presumably are N-oxidized by the same enzyme and subject to the same types of inhibition.

To determine the effects of SKF 525-A on the in vitro N-oxidation of (±)-NPA, SKF 525-A (2 μmol) was included in standard incubation mixtures which were then incubated for 1h. Subsequent g.l.c. analysis revealed that only traces of amphetamine and 1-phenyl-2-propanone were formed indicating virtually complete inhibition of P-450 mediated metabolism. There was also a moderate (25%) reduction in total N-oxidation. Whether SKF 525-A inhibited FAD-amine oxidase or whether this tertiary (group Ic, fig. 8) amine can also serve as a substrate for the N-oxidative enzyme system and thus compete with NPA is not known. G.l.c./m.s. investigation of a new peak in the extracts of these in vitro samples suggested that SKF 525-A was metabolized to some extent but incomplete data made positive identification of the metabolite impossible.

Pretreatment of rats with SKF 525-A (schedule 5, table 11) prior to preparation of the microsomal supernatant for in vitro metabolic studies revealed basically the same pattern of metabolism

of (±)-NPA as discussed above for in vitro studies. Further studies on the role of SKF 525-A in in vitro N-oxidation of secondary amines are needed.

7. In Vitro N-oxidation in the Presence of EDTA or Ascorbic Acid

When liver tissue is homogenized for purposes of in vitro drug metabolism, rupture of the endoplasmic reticulum occurs. The small particles of protein which result, aggregate into spheres referred to as microsomes. These microsomes become encased in a layer of lipid material which is believed to play a role in preserving the metabolic activity of the microsomes (136). Peroxidation of this lipid layer has been shown to be correlated with a decrease in drug metabolizing enzyme activity in vitro. Some other theories (27) also implicate lipids as part of the electron transport system of P-450-mediated C-oxidation reactions.

A recent report (142) examined the role of EDTA in preventing lipid peroxidation and subsequent loss of enzyme activity. Using the N-demethylation of ethylmorphine as a marker, it was found that the presence of EDTA in the incubation mixture prevented the loss of metabolic activity associated with lipid peroxidation.

To examine the role of EDTA in the N-oxidation of (±)-NPA in vitro, standard incubation mixtures were prepared and EDTA (0.1-1.0 mM) was added. Samples were incubated for 1h at 37°, extracted and analyzed as before on g.l.c. When compared to

control samples in which EDTA had been omitted, N-oxidation was virtually unchanged whereas C-oxidation increased in the presence of EDTA (table 20). The qualitative nature of the metabolism was not changed, i.e., no new metabolites were observed. Increasing the amount of EDTA to more than 1 mM did not further improve overall metabolism. This information would seem to support the view (27) that lipids might be involved in P-450 mediated C-oxidation.

Rats treated with 3-MC were also included in this series of experiments. When EDTA (1 mM) was added to *in vitro* incubation mixtures containing microsomes prepared from pretreated animals, no further increase in the metabolism of (±)-NPA beyond that seen in the absence of EDTA was observed. These results indicate that little if any lipid peroxidation has occurred in the *in vitro* system used here and cannot be held responsible for the low metabolic N-oxidation of NPA in rat liver homogenates. In view of this relatively minor effect of EDTA on N-oxidation, routine use of EDTA in *in vitro* studies of N-oxidation is not warranted. Furthermore, EDTA complexes with divalent cations, e.g. Mg^{++}. What affect this has on *in vitro* metabolism is unknown.

Both *in vitro* and *in vivo* metabolism may be affected by ascorbic acid deficiency (143-145). A recent report (146) describes experiments using weanling guinea pigs in which an increase in drug metabolism was produced by large doses (75 mg kg^{-1}) of ascorbic

TABLE 20. EFFECTS OF EDTA AND ASCORBIC ACID ON THE IN VITRO METABOLISM[1] OF (±)-N-PROPYLAMPHETAMINE IN FORTIFIED INCUBATION MIXTURES CONTAINING 12,000 x g SUPERNATANT OF RAT LIVER HOMOGENATE PREPARED FROM CONTROL AND 3-MC TREATED RATS.

Conc. (mM)	μmol N-oxidized[2] products mg^{-1} protein h^{-1}		μmol C-oxidized[2] products mg^{-1} protein h^{-1}	
	control	pretreated	control	pretreated
Control	0.16±0.01	0.22±0.02	0.27±0.02	0.31±0.02
EDTA				
0.1	0.17±0.01	0.22±0.02	0.27±0.02	0.30±0.03
1.0	0.16±0.02	0.20±0.02	0.35±0.04	0.33±0.03
Ascorbic Acid				
0.1	0.16±0.03	0.22±0.03	0.2 0.02	0.32±0.01
1.0	0.14±0.01	0.22±0.02	0.28±0.03	0.33±0.02

[1]Samples incubated for 1h at 37°. Values are (±) S.E.M. (n=2).

[2]see footnote 2 to table 15

acid as compared to non-scorbutic controls. The mechanism of this increased metabolism is not clearly understood but thought to involve P-450.

Other studies (147) indicate that the presence of ascorbic acid in incubation mixtures increases the rate of lipid peroxidation. The result was a decrease in ethylmorphine demethylase activity, and, surprisingly, an increase in aniline hydroxylase. The authors explained this anomaly by pointing out that ethylmorphine exhibits type I binding with P-450 whereas aniline exhibits type II. They suggest that the requirements for lipid in P-450 mediated C-oxidation varies between these two binding sites.

In view of this information and the fact that rats can synthesize ascorbic acid while guinea pigs cannot (146), it seemed possible that ascorbic acid may in fact be involved in metabolic N-oxidation of aliphatic amines in rats.

Standard incubation mixtures were prepared from livers of 3-MC treated and control rats (300-350 g). Ascorbic acid (0.1-1.0 mM) was included and the mixtures incubated for 1h at 37°. G.l.c. analysis of these samples revealed little difference in the metabolism of (±)-NPA between ascorbic acid-treated and non-treated samples (table 20). Including 1 mM EDTA in addition to ascorbic acid did not effect the metabolism.

What role if any ascorbic acid plays in the <u>in vitro</u> N-oxidation of amphetamines in rat liver homogenate is obscure. Resolution

may be found by placing rats on a diet which would promote ascorbic acid deficiency, at which stage, in vitro studies of metabolic N-oxidation could be performed.

B. In Vitro Metabolism of 1-Phenyl-2-Propanone Oxime (X) By Rat Liver Homogenates

Perusal of the literature review reveals that much controversy exists over the mechanism of in vitro and in vivo formation of 1-phenyl-2-propanone oxime from amphetamine and its N-alkylated derivatives. Some believe that the oxime is formed metabolically while others claim that oximes are formed non-enzymatically from metabolically produced hydroxylamines.

The quantities of oxime isolated in reported studies probably do not reflect the amounts of oxime formed since it is known (88) that in the presence of NADPH, the oxime (X) is rapidly destroyed by 9,000 x g supernatant from rat or rabbit liver, but not in the absence of NADPH, thus implicating an enzymatic process. What products were formed was not completely known. Some ketone (IX) was detected but around 50% of the amount of oxime added could not be accounted for (88). It was decided to investigate the metabolic fate of the oxime.

Standard incubation mixtures at pH 7.4 containing oxime (1 μmol) were incubated at 37° for 1 h. The ether extracts of this mixture were pooled, concentrated, and analyzed on g.l.c. column D (155°), using PCP as internal standard.

G. l. c. analysis (fig. 28) showed the extract to contain unmetabolized oxime, X, (50. 5%), a major component, Tr 6. 4 min (36. 1%), and two minor components, Tr 1. 95 min (9. 4%) and Tr 3. 15 (1. 8%). Percentage recoveries were based on the amount of oxime added to the incubation mixture and were obtained by reference to calibration curves. The two minor peaks were identified as 1-phenyl-2-propanone (IX) and benzyl alcohol (XXXV), respectively, by comparison of their g. l. c. and g. [redacted] behavior with authentic samples (fig. 29, 30). Diagnostic [redacted] m. s. of 1-phenyl-2-propanone were of m/e 134, [redacted] ase peak), 77, 65, and 43. These arise as shown in fig. 29. The presence of an ion of m/e 45 and an ion of m/e 92 greater in intensity than expected for an isotope of m/e 91 (see fig. 29), led to the conclusion that there was some 1-phenyl-2-propanol present in the ether extract (cf. fig. 19). Judging by the relative amounts of the ions of m/e 45 and 92, only trace quantities of 1-phenyl-2-propanol were formed, presumably by metabolic reduction of IX. Benzyl alcohol, like 1-phenyl-2-propanone, exhibits a strong molecular ion of m/e 108. Other diagnostic ions in its mass spectrum include m/e 107, 106, 105, 91, 79 (base peak), 77, and 65. The origin of these fragments can be seen in fig. 30 (148).

These conclusions obtained using homogenized rat liver supernatant should be contrasted with the literature report (89) that the

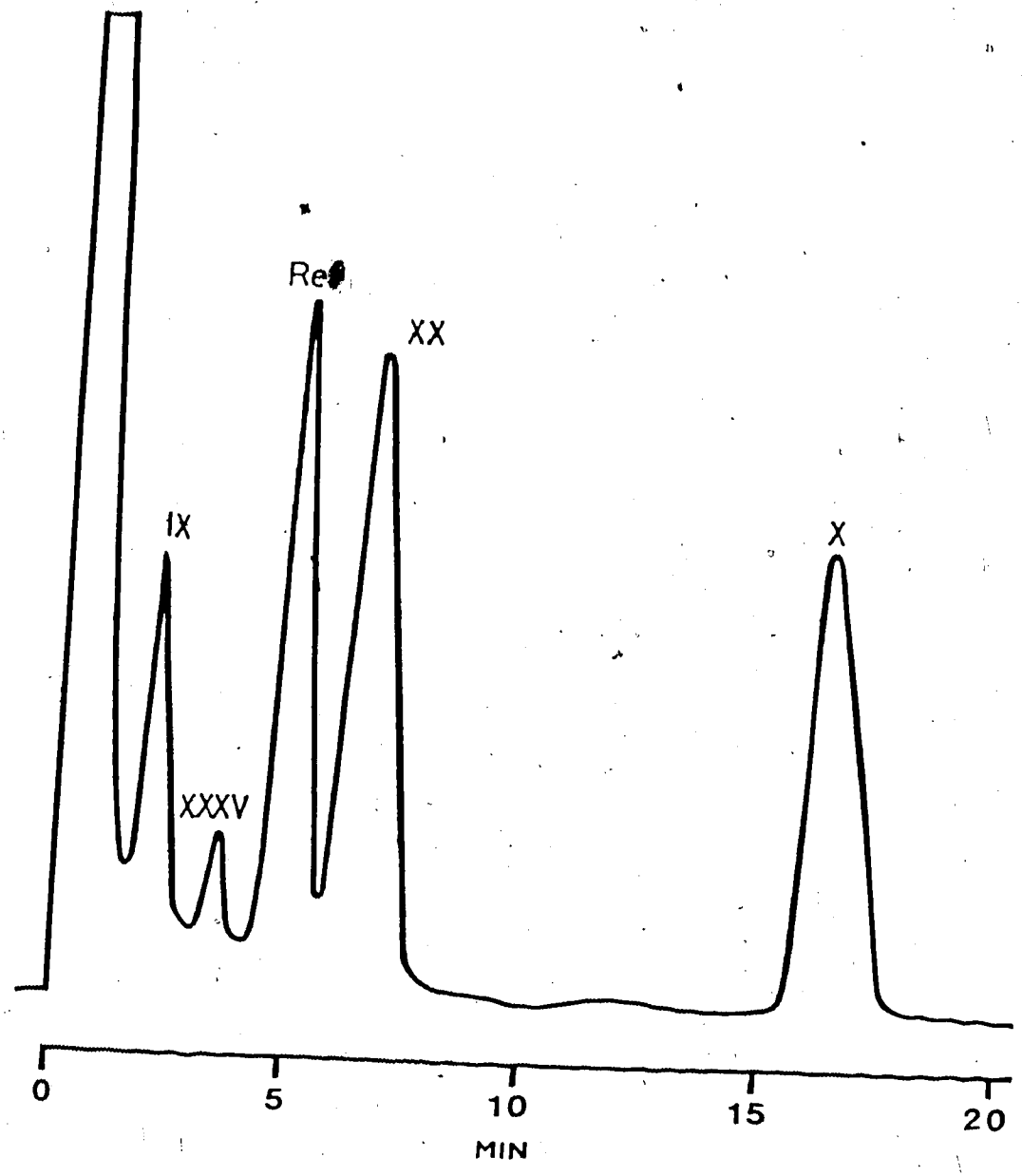


Fig. 28. G. l. c. separation of in vitro metabolites of 1-phenyl-2-propanone oxime (X). (Ref=p-chloropropiophenone)

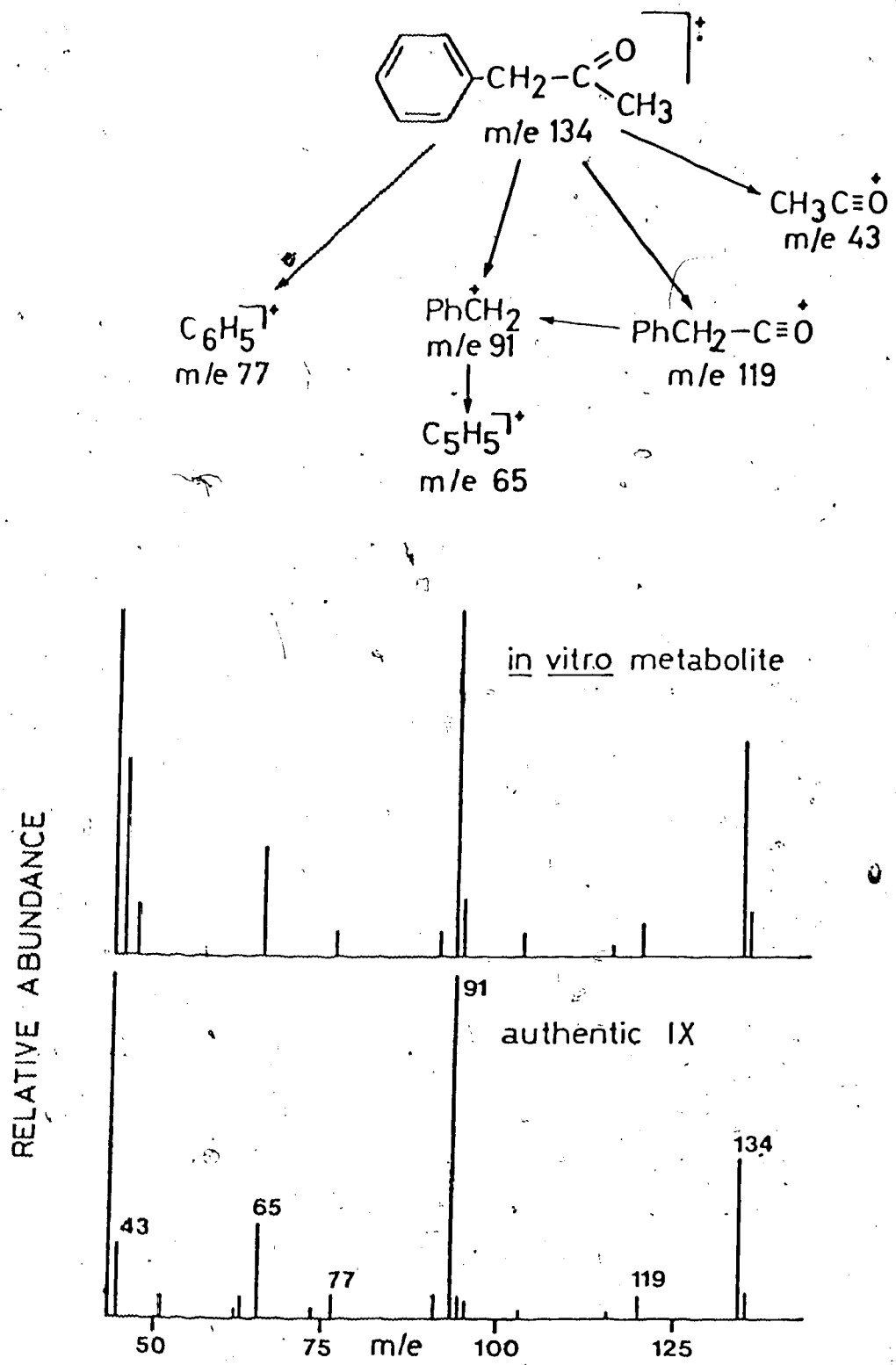
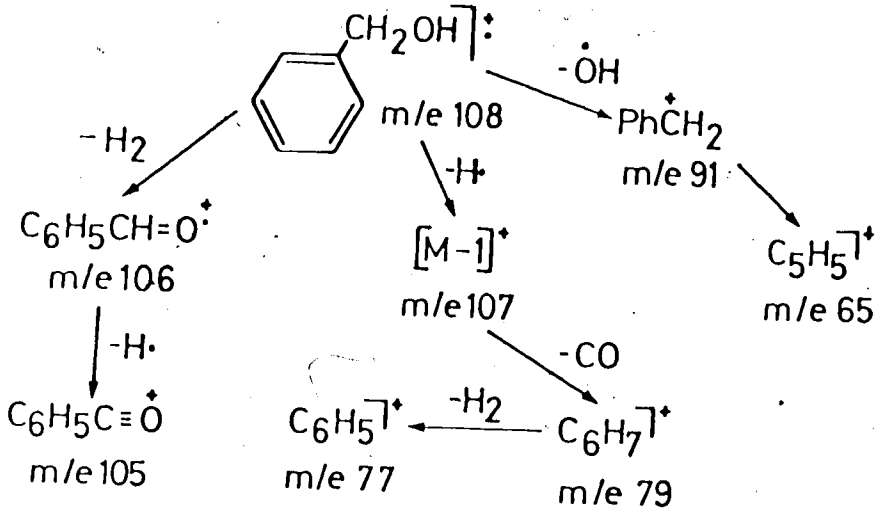


Fig. 29. G.l.c./m.s. identification of 1-phenyl-2-propanone as an in vitro metabolite of 1-phenyl-2-propanone oxime.



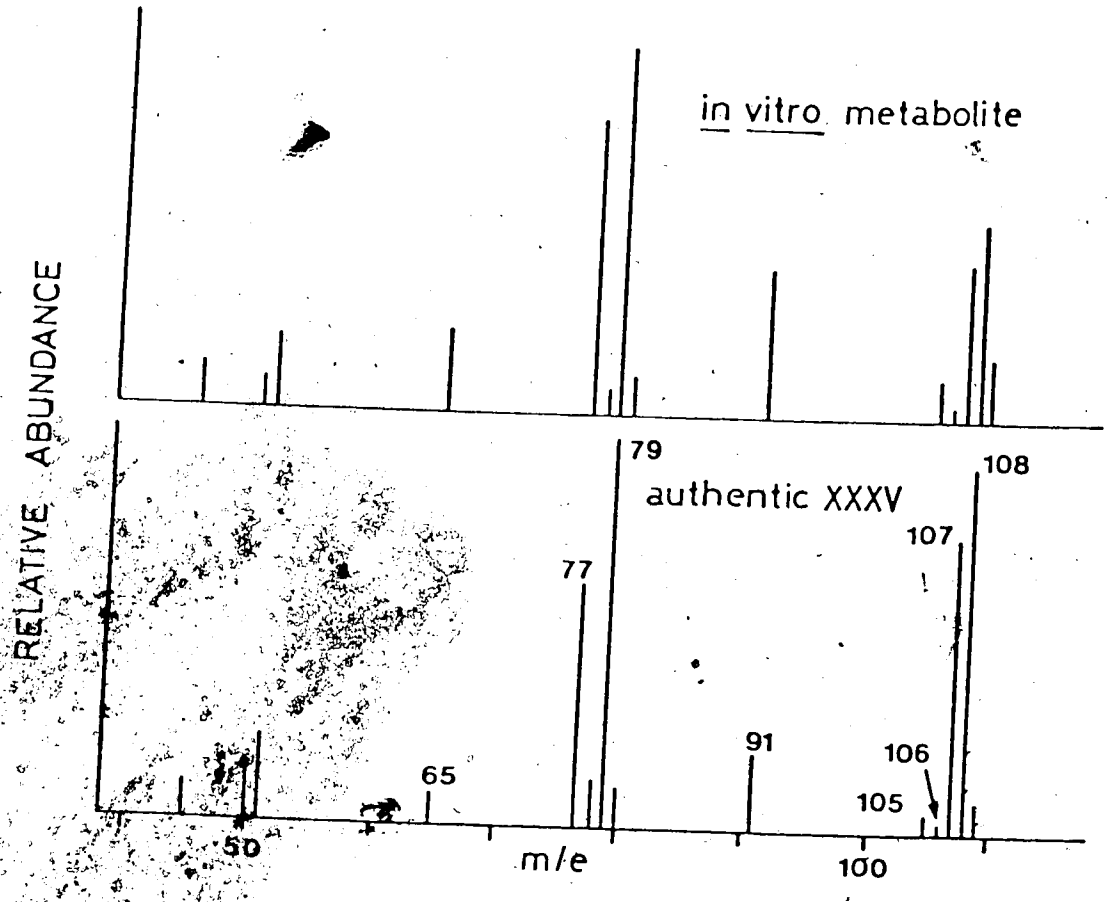


Fig. 30. G.l.c./m[illegible] identification of benzyl alcohol as an in vitro metabolite of 1-phenyl-2-propanone oxime.

oxime was extensively metabolized in vitro by rabbit liver preparations to the ketone (IX) and the related secondary alcohol, 1-phenyl-2-propanol. Apparently a species difference exists in the metabolism of this oxime.

Diagnostic features in the major metabolite's mass spectrum (fig. 31) were the presence of a very weak molecular ion of m/e 165 and a cluster of ions at 117, 118, and 119. Also present were ions diagnostic of the benzyl group at m/e 91 and 65, and the phenyl group at 77 and 51. The intensity of the m/e 119 ion was greater than expected for the isotope peak of ion m/e 118 and was deduced to be an authentic fragment ion of formula XXXVI. The fragment, m/e 118, was obviously ion XXXVII. The same ion is a diagnostic fragment in the mass spectra of N-hydroxylated amphetamine metabolites (98) Interpretation of the mass spectrum in this manner indicated that the probable structure of the major metabolite of the oxime was the nitro-compound (XX) which fragmented in the mass spectrometer as illustrated in fig. 31. An authentic sample of 2-nitro-1-phenylpropane (XX) was then prepared as outlined in scheme 21 (see Methods and Materials, sec. G. 2). Synthetic XX had g. l. c. and g. l. c./m, s. properties identical to those of metabolically produced nitro-compound.

It may be argued that XX was not a true metabolite of the oxime but instead was an artifact formed from the oxime or an initial

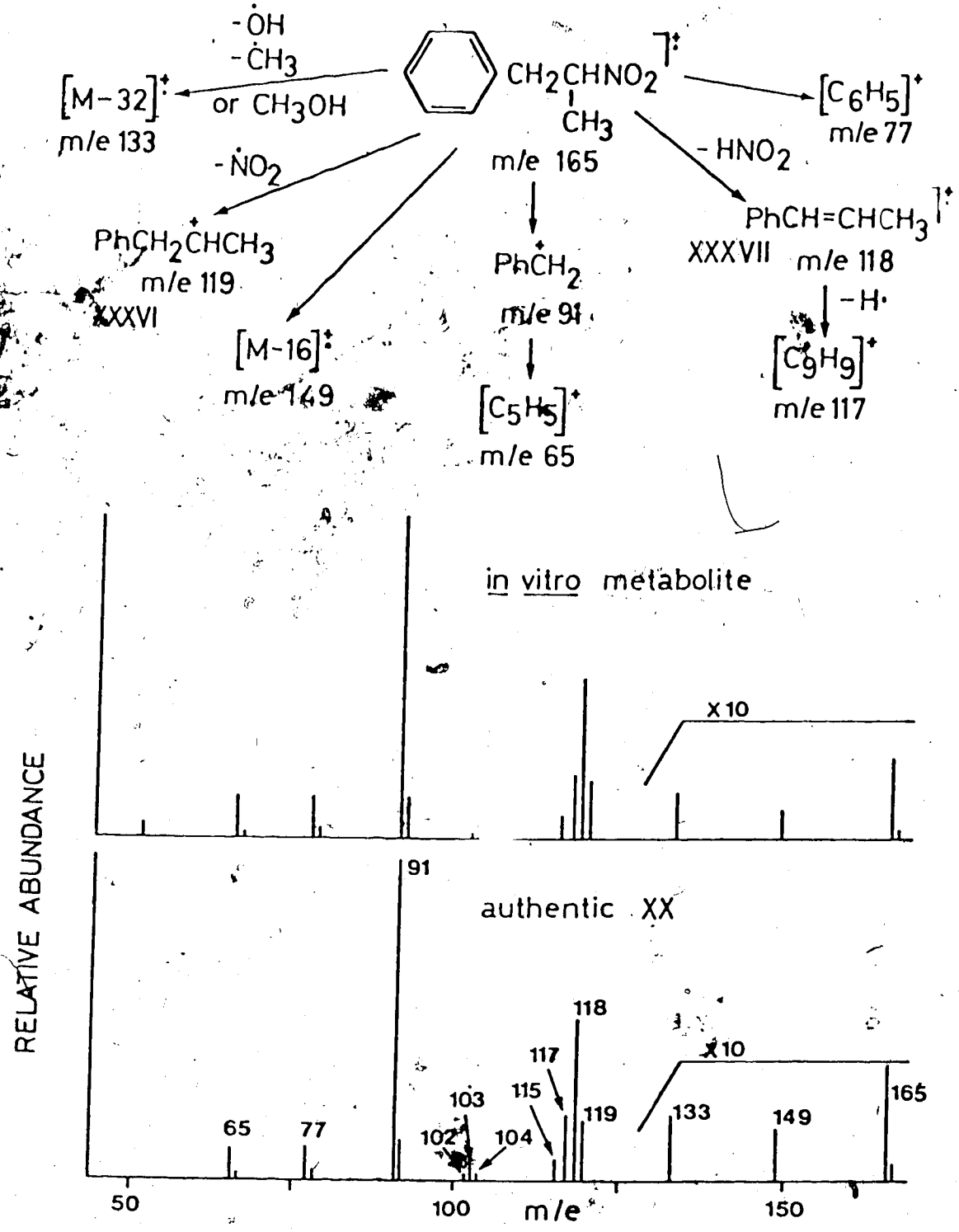
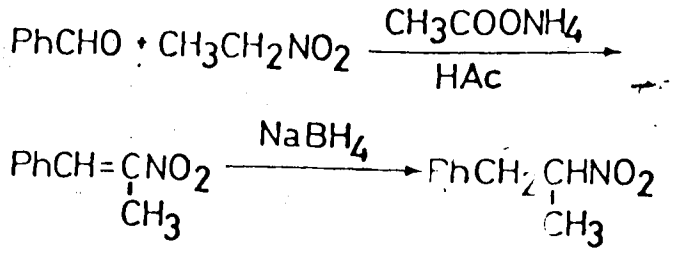


Fig. 31. G.l.c./m.s. identification of 2-nitro-1-phenylpropane as an in vitro metabolite of 1-phenyl-2-propanone oxime.



Scheme 21

metabolite of it, by the action of peroxide present in the ether used in the extraction process. Two experiments were performed to confirm that the nitro-compound (XX) was formed enzymatically, and peroxides, if any were present in the ether, were not involved in the conversion of oxime to XX.

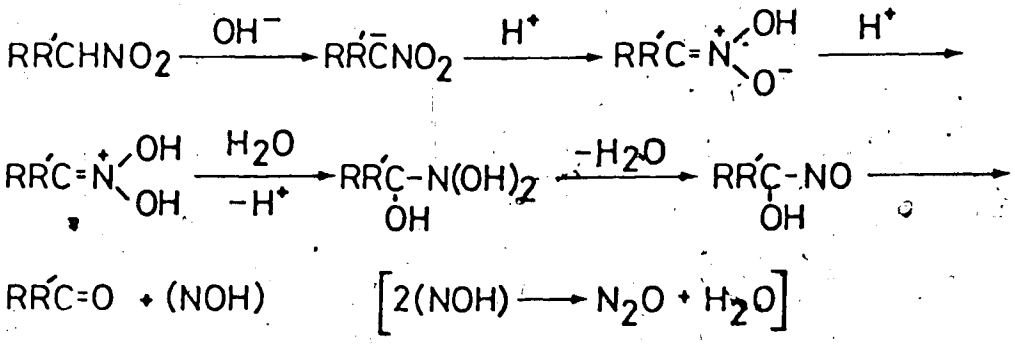
The in vitro metabolism of oxime was repeated exactly as previously described, except that ether was replaced as the extraction solvent. Separate experiments using benzene, methylene chloride, or chloroform for extraction, were performed and when these extracts were treated and examined by g.l.c. exactly as had been done with the ether extracts, the same three metabolites were produced in quantities approximating those shown in fig. 28.

The results shown in fig. 28 were obtained from metabolism mixtures containing (a) oxime, 12,000 x g supernatant of homogenized rat liver, and cofactors. Four related experiments were performed

in which the metabolism mixtures contained: (b) oxime, 12,000 x g supernatant, but no cofactors; (c) oxime and cofactors but no 12,000 x g supernatant; (d) oxime, cofactors and heat denatured 12,000 x g supernatant; and (e) oxime and heat denatured 12,000 x g supernatant.

Maximum metabolic activity was seen in reaction (a); only trace quantities of XX were detected in reaction (b); no metabolism occurred in reactions (c) - (e), substrate only being recovered.

In some experiments, the incubated metabolism mixture of the oxime was basified (pH 12-13) before extraction with ether. This procedure resulted in complete loss of XX and a significant increase (25-40%) in the amount of ketone (IX). The absence of XX in the ether extract was not surprising since nitro-compounds form water-soluble salts in alkaline solution (149); however, the formation of the ketone by this treatment was, at first, difficult to explain. It has been shown that aliphatic nitro-compounds are unstable in acid solution in which they undergo the Nef reaction (scheme 22) which converts them to carbonyl compounds (150),

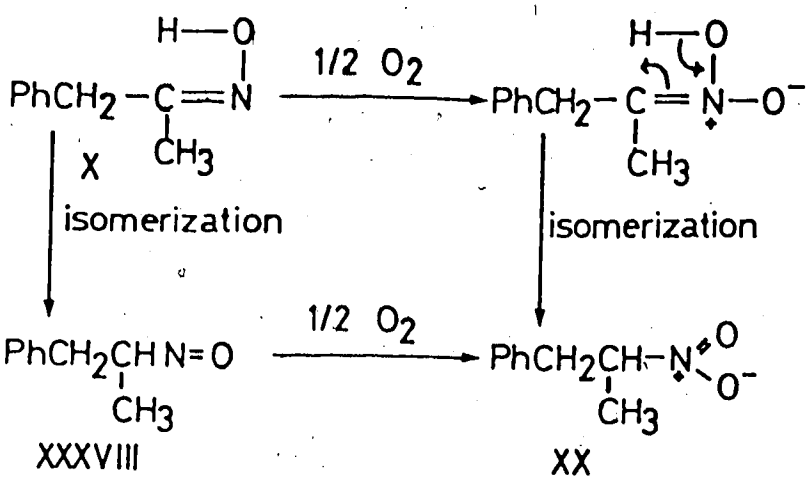


Scheme 22

but this conversion is not reported to occur in basic solution. It was confirmed that dissolution of the nitro-compound (XX) in sodium hydroxide solution alone did not cause any conversion to ketone (IX). When, however, microsomes were added to a solution of XX in aqueous sodium hydroxide, a 40% conversion of XX to IX was observed. It is known (151,152) that nitro-compounds are readily oxidized to ketones in basic solutions of oxidizing agents. It is concluded that the formation of IX from XX in basic solution is mediated by the presence of peroxides or other alkali-stable oxidizing agents in the 12,000 x g supernatant.

It is of interest to speculate on the mechanism involved in the metabolic synthesis of the nitro-compound (XX) from the oxime. Both N-oxidation and isomerization steps are required in the synthesis. Since metabolic N-oxidation of tertiary amines is a common metabolic pathway (72) it is probable that the reaction sequence is initially N-oxidation followed by isomerization, but the alternative pathway in which isomerization to the nitroso-compound (XXXVIII) preceeds oxidation cannot be ruled out (scheme 23).

The results reported in Sec. A. (vide supra) were obtained prior to the discovery of metabolite XX. Experiments have since been performed to determine the extent of the metabolism of oxime (which is formed during the <u>in vitro</u> metabolism of NPA) to XX.



XXXVIII XX

Scheme 23

Although XX could be identified in ether extracts (pH 7.4) of standard in vitro incubation mixtures where NPA was the substrate, the amount of XX was too low for quantitation. Under neutral aqueous conditions the conversion of N-OH-NPA to the oxime is relatively slow, the major product formed being the nitrone (vide supra). As a result, it is not surprising that little XX is formed from the oxime in vitro when NPA was used as the substrate. However, in view of the relative instability of N-OH-amphetamine and its rapid conversion to the oxime in vitro, the metabolic conversion of X to XX may be significant when amphetamine is used as a substrate

in N-oxidation studies. The discovery of this new metabolite (XX) of the oxime necessitates that the in vitro metabolism of medicinal amines, many of which produce oximes as metabolites, will have to be reinvestigated.

C. In Vivo Metabolism of (±)-NPA And Some Related Amphetamines In the Rat

As pointed out in the Literature Survey, the metabolism and excretion of amphetamine and N-methylamphetamine in man and other species have been studied in considerable detail, but similar studies on other N-monoalkylated derivatives of amphetamine have been less extensive (80, 102-104). Investigations have shown that the most important in vivo routes of biotransformation of amphetamines are aromatic hydroxylation, oxidative deamination, aliphatic hydroxylation, and N-dealkylation.

The relative importance of each of these metabolic pathways varies with species. Para-hydroxylation of amphetamine, N-methylamphetamine, ephedrine, norephedrine, N-(3-chloro-n-propyl)amphetamine (Pondinil) and mephentermine occurs extensively in the rat, but in dog, rabbit, guinea pig and man, the extent of metabolic para-hydroxylation is variable and greatly dependent upon the structure of the amphetamine (103). Pondinil is the only amphetamine with an N-alkyl group greater than ethyl which has been studied metabolically in vivo (80). It is para-hydroxylated to greatly differing

extents by all five species listed above (table 9). As mentioned previously, these in vivo results are markedly different than in vitro studies of amphetamine metabolism in rat liver homogenates (105,108) which report very little or no para-hydroxylation. Similar observations (see Results and Discussion, sec. A) have been made when NPA was used as a substrate in vitro.

Further investigation of the in vivo metabolism of N-alkylated amphetamines seemed warranted to determine the extent of aromatic hydroxylation of these compounds.

1. In Vivo metabolism of (±)-NPA

a. Studies with normal rats

Male Wistar rats were administered (±)-NPA by oral intubation according to pretreatment schedule 6 (table 11). Urine samples were collected and extracted with a mixture of ether and methylene chloride. Phenolic metabolites were identified and quantitated on g.l.c. column C (155°).

Acid-hydrolyzed rat urine was first to be analyzed. It was basified to pH 9.5 and extracted with ether-methylene chloride. G.l.c. analysis (fig. 32) of the concentrated extract showed that it contained numerous compounds including two long retention time metabolites (Tr = 8.8 and 13.5 min), designated A and B respectively. Most of the more volatile compounds in this acid-hydrolyzed extract were also present in a similar extract obtained from control rats

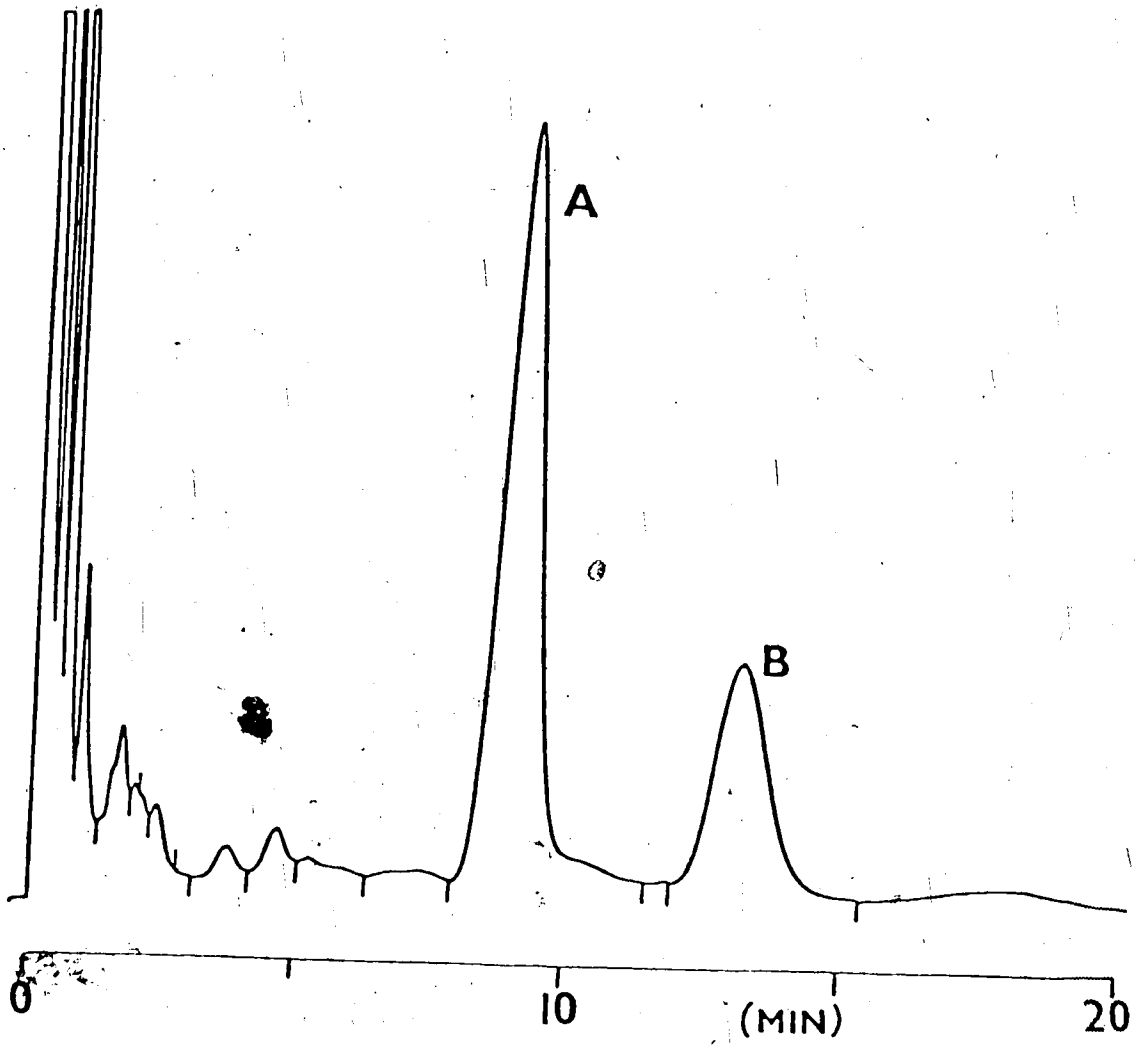


Fig. 32. G. l. c. trace of an ether-methylene chloride extract of hydrolyzed urine obtained from rats after administration of (±)-NPA.

and were not further investigated. The same two metabolites, A and B, were also shown to be present by g.l.c. analysis, in the concentrated ether-methylene chloride extract of enzyme-hydrolyzed rat urine. An unhydrolyzed extract, or urine adjusted to pH 9.5, was found to contain amphetamine, NPA and small amounts of metabolites A and B, indicating A and B were excreted unconjugated to some extent.

The mass spectra of g.l.c. peaks A and B were recorded and are shown in fig. 33. Diagnostic ions in the mass spectrum of compound A were of m/e 192, 178, 164, 107, 86 (base peak), and 44. Corresponding fragments appeared in the m.s. of the substrate NPA (fig. 33) at m/e 176, 162, 148, 91, 86 (base peak), and 44, thus indicating that A was a mono-oxygenated derivative of NPA and that the oxygen atom was located on the ring or on the benzylic carbon but not on the remainder of the side-chain since both metabolite A and NPA give rise to a base peak in their mass spectra of m/e 86. Because N-methylamphetamine is known (99) to be metabolized *in vivo* in the rat to the *para*-hydroxylated derivative, metabolite A was tentatively identified as 1-(4-hydroxyphenyl)-2-(*n*-propylamino)-propane, *i.e.* *p*-OH-NPA (XXIII). Its m.s. fragmentation behavior (fig. 34) was consistent with this conclusion. A subsequent synthesis of XXIII gave a compound with a g.l.c. retention time and a mass spectrum identical to that of metabolite A. In addition, when metabolite

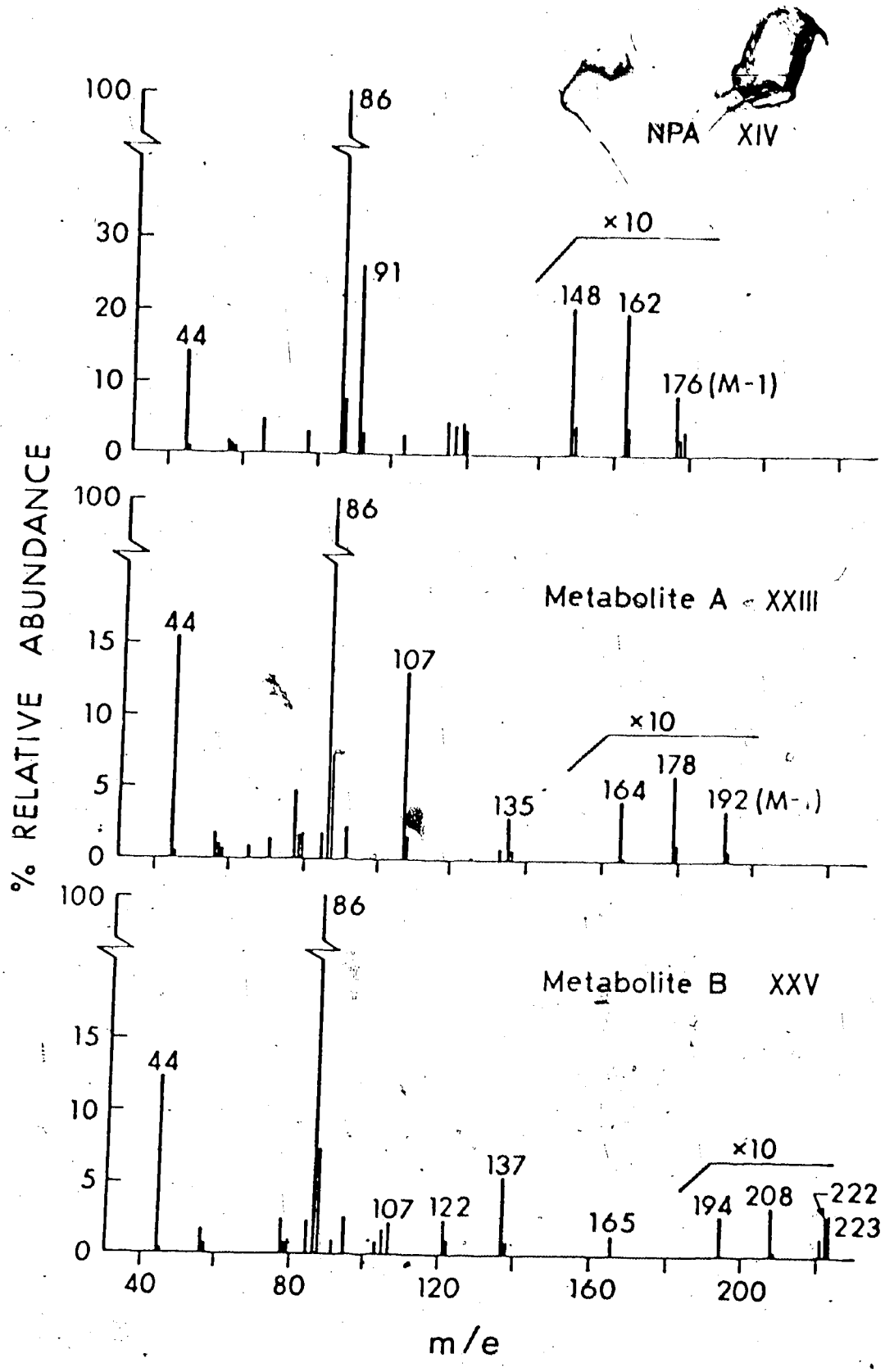


Fig. 33. G.l.c./m.s. identification of para-hydroxy-NPA and 4-hydroxy-3-methoxy-NPA as in vivo metabolites of NPA.

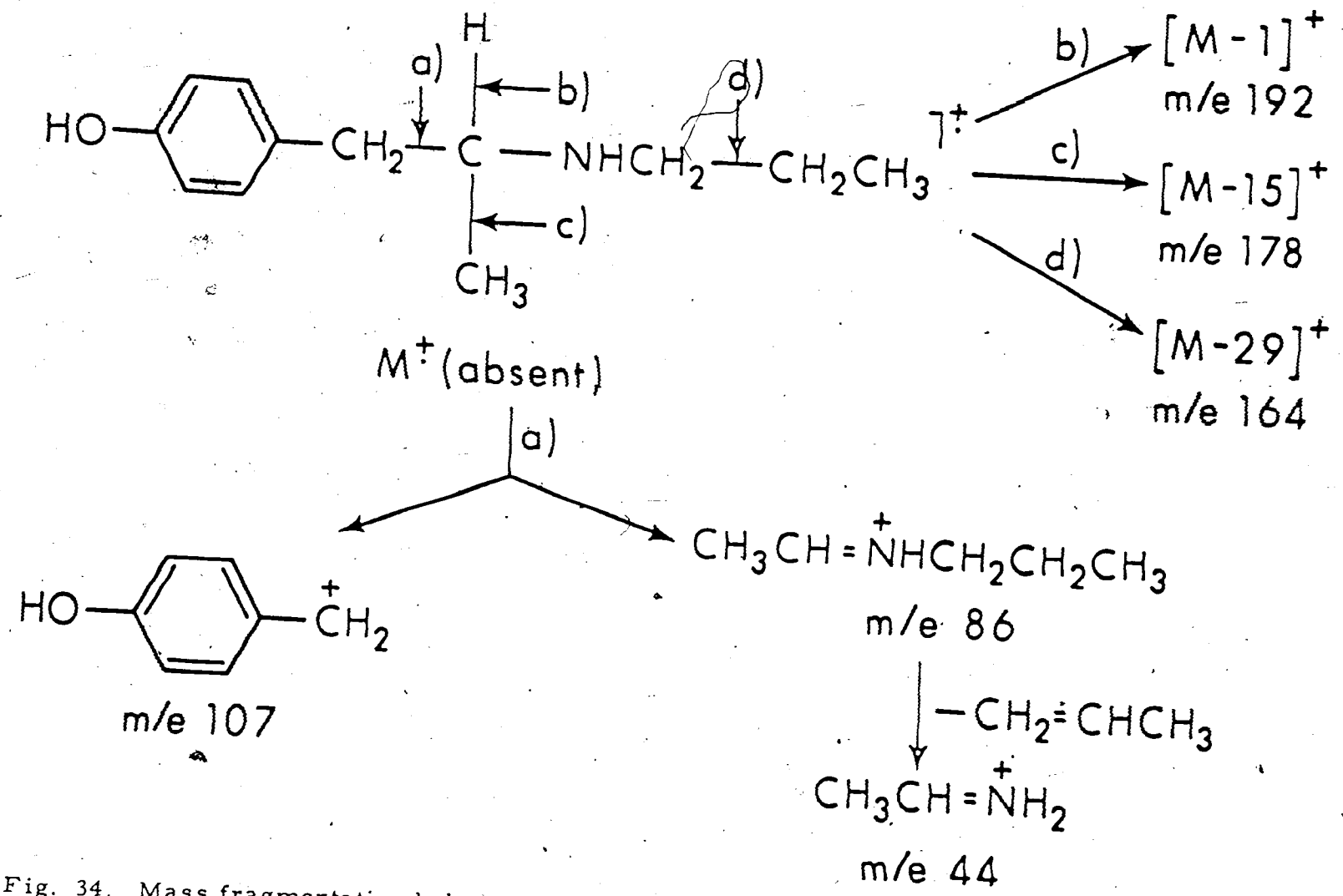


Fig. 34. Mass fragmentation behavior of para-hydroxy-N

A was reacted with trifluoroacetic acid anhydride (TFA), two products with characteristic g.l.c. retention times were obtained and were identified as mono- and di-TFA derivatives XXXIX and XL respectively by their m.s. fragmentation behavior (table 21). Synthetic compound XXIII behaved identically on g.l.c. and g.l.c./m.s. after derivatization with TFA.

Diagnostic ions in the m.s. of metabolite B (fig. 33) were of m/e 223, 222, 208, 194, 137, 122, 107, 86 (base peak), and 44. By employing arguments similar to those used to interpret the m.s. of metabolite A, it was concluded that, since metabolite B retained the $-CH(CH_3)NH\underline{n}Pr$ side-chain of the substrate NPA (base peak of m/e 86), a possible structure for metabolite B was a ring-methoxylated derivative of A (fig. 35). Aromatic methyl ethers expel both a formaldehyde molecule and a methyl radical (153). The production of the odd-electron ion, m/e of 122, from the even-electron ion, m/e of 137, may seem to be an unlikely transition. However, the same ion, m/e of 137, is also a fragment in the m.s. of a metabolite of 3,4-methylenedioxyamphetamine (154) and it decomposes to ions m/e 122 and 107. A metastable ion is present in support of the fragmentation m/e 137 → m/e 122.

The point of attachment of the methoxyl group to the aromatic ring was tentatively deduced to be at C-3 since 4-hydroxy-3-methoxyphenyl derivatives are known metabolites of diphenylhydantoin (155) and

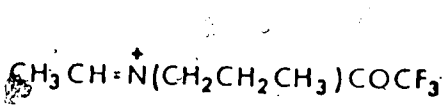
TABLE 21. GAS-LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY PROPERTIES OF TRIFLUOROACETYL DERIVATIVES OF IN VIVO METABOLITES OF NPA AND NBA.

Compound	G.l.c. Tr[1]	m.s.[2,3]
XXXIX	10.1	230 (28), 182 (100), 140 (76).
XLI	18.0	260 (58), 182 (100), 140 (72).
XL	20.4	289 (1), 182 (89), 140 (100), 134 (81), 107 (39).
XLII	25.4	319 (5), 182 (98), 164 (71), 140 (100), 137 (39), 134 (22).

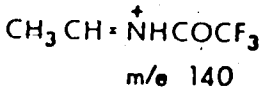
[1] Column C, 155°.

[2] m/e (% relative abundance)

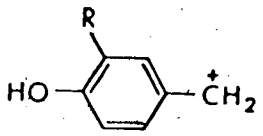
[3] Identity of ions:



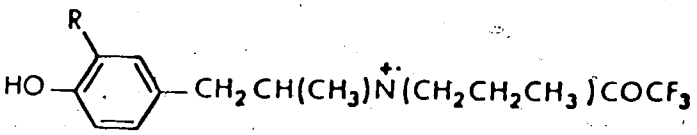
m/e 182



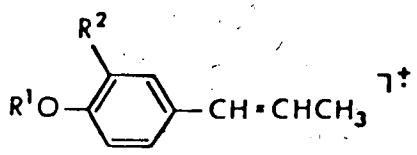
m/e 140



m/e 107 (R=H); 137 (R=OCH3)



M+, m/e 289 (R=H); 319 (R=OCH3)



m/e	R1	R2
134	H	H
164	H	OCH3
230	CF3CO	H
260	CF3CO	OCH3

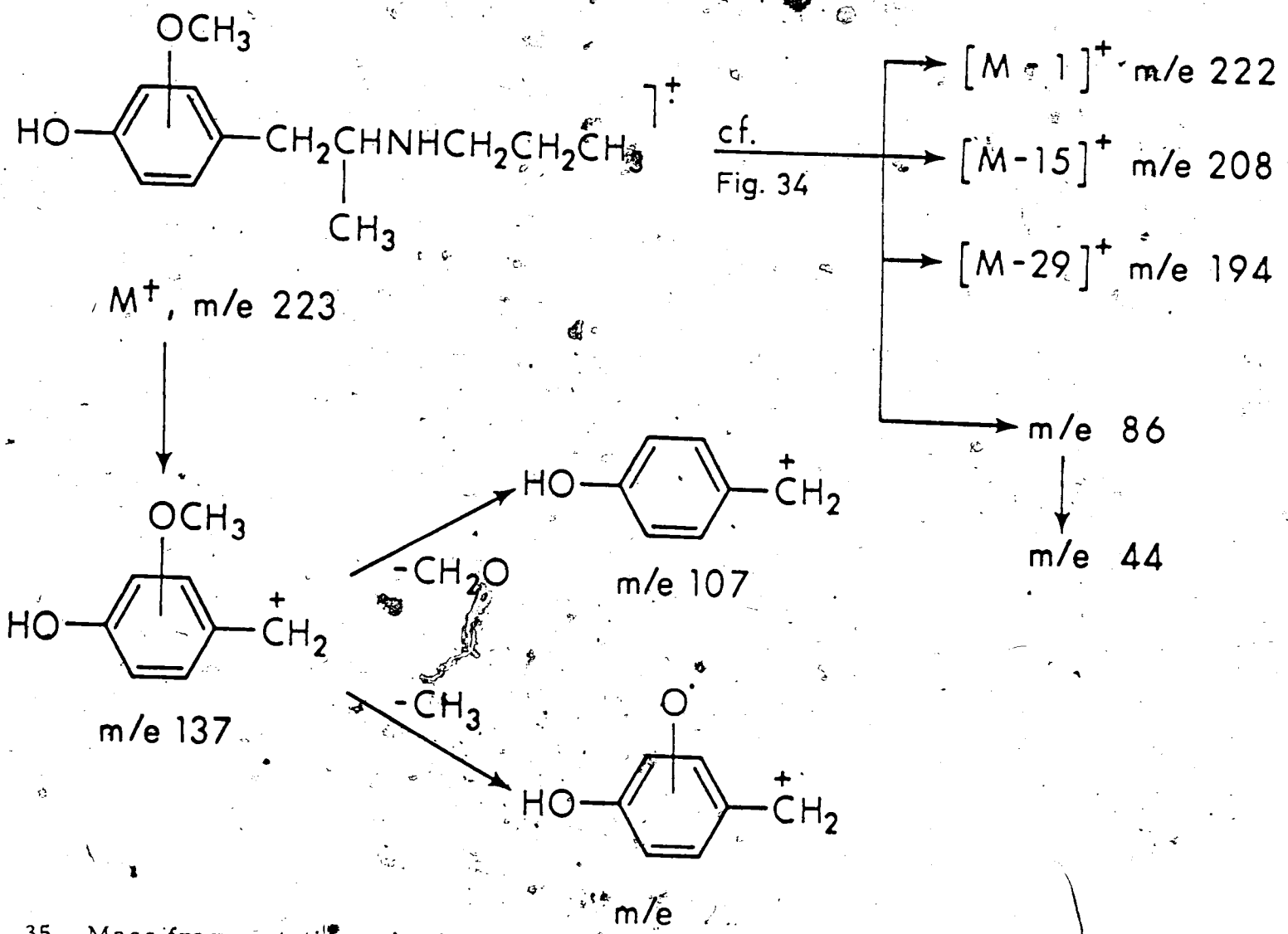


Fig. 35. Mass fragmentation behavior of 4-hydroxy methoxy-NPA.

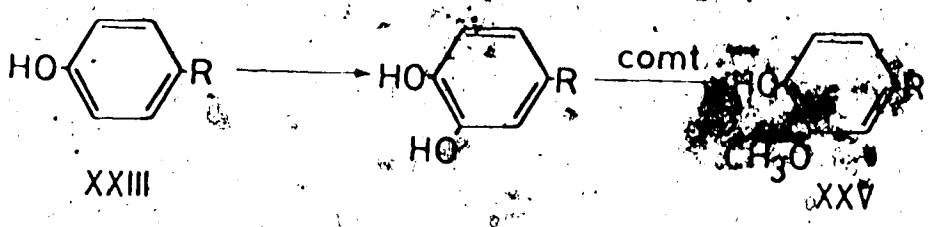
glut[illegible]mide (156) and 4-hydroxy-3-methoxymandelic acid is a major metabolic product of the two catecholamines, norepinephrine and epinephrine, whose enzymatic synthesis begins with the amino acid, phenylalanine (157). As far as could be determined, however, no reports have appeared in the literature which describe the production of ring-hydroxylated and methoxylated metabolites of ring-substituted amphetamine-like substrates.

Confirmation of the identity of metabolite B as structure XXV was obtained by direct comparison of the g.l.c and g.l.c./m.s. properties of the metabolite and its mono- and di-TFA derivatives XLI and XLII (table 21), with those of an authentic sample of XXV and its TFA derivatives.

Quantitative estimation of the amounts of metabolites A and B in extracts of unhydrolyzed and acid-hydrolyzed urine from six individual rats revealed that 18.3% (range 17.3-20.1%) of the dose of NPA was excreted as metabolite XXIII in free (2.3-3.1%), but mainly in conjugated (15.0-17.9%) form, and 3.3% (range 2.3-6.9%) as metabolite XXV in free (0.4-0.6%) and conjugated (1.9-6.3%) form. The average total amounts of XXIII and XXV isolated from enzyme-hydrolyzed urine were calculated to be 18.0% and 2.5% respectively, indicating that glucuronidase hydrolysis under the conditions employed in this study was not as efficient as acid hydrolysis in liberating XXV from its conjugates. Recovery of unchanged NPA averaged

0.88 mg (range 0.48-1.29 mg) or 2.9% of the administered dose. The sum of NPA, metabolite A and B accounted for only about 25% of the administered dose of NPA.

It is assumed that metabolite XXV originated from XXIII via a catechol intermediate (scheme 24) which is O-methylation by



Scheme 24

catechol-O-methyltransferase (COMT) to yield XXV. COMT is known (158) to O-methylate other catechols and catecholamines, and its involvement has been suggested (155) in the in vivo formation of 5-(4-hydroxy-3-methoxy)-5-phenylhydantoin from diphenylhydantoin in the rat. In the present study, the presence of 3,4-dihydroxy-NPA could not be confirmed in the urine of rats which had received NPA.

In attempts to detect the catechol intermediate, p-OH-NPA was administered orally to rats at a dose which was approximately equivalent to the amount of p-OH-NPA seen in the samples from rats

given NPA. The urine was collected, pooled and extracted with ether-methylene chloride as before. G.l.c./m.s. analysis of the concentrated extract before and after TFA treatment did not reveal the presence of a catechol intermediate. It was observed, however, that metabolite XXV was again present (approximately 8% of the dose). In this instance, the ratio of XXV:XXIII (1:3) was greater than that observed when NPA was administered (1:5). This observation would seem to support the claim that XXV arose as a result of metabolism of XXIII.

During the course of the experiments involving quantitation of the in vivo metabolites of NPA, it became readily apparent that only a fraction (about one-third) of the administered dose of NPA was recovered as NPA or metabolites. To determine the excretion pattern of the ring-hydroxylated metabolites of NPA, NPA was administered to rats by oral intubation and the urine and feces collected for 8 days. Extracts of urine and fecal samples for each 24h period were analyzed on g.l.c. for metabolites XXIII and XXV, before and after acid-hydrolysis.

Analysis of these samples revealed that traces of XXIII could be detected in the urine up to 4 days after a single dose of NPA whereas XXV was undetectable after 2 days. Neither of these two metabolites could be detected in the feces. More intensive studies being performed at the time of this writing indicate that p-OH-NPA is only very slowly excreted in vivo in comparison to NPA.

Micro-organisms present in the gastro-intestinal tract (g.i.t.) may play a role in the metabolism of certain drugs (159,160). To what extent the natural flora of the g.i.t. may be involved in drug metabolism is not completely known, but certainly any drug administered orally could conceivably be a candidate for metabolism by bacteria in the gut.

To determine whether or not intestinal microflora were involved in the metabolism of NPA, one group of rats were given NPA orally according to schedule 6 (table 11) and another group received NPA i.p. according to schedule 7 (table 11). Urine samples were collected, pooled, and analyzed as before. G.l.c. analysis revealed no significant difference in the total amounts of metabolism of NPA in either group. This finding and the lack of metabolites in the feces, would seem to indicate that the intestinal micro-flora are not involved in the metabolism, particularly the methoxylation, of NPA. Further studies involving in vitro cultures of intestinal microflora and NPA or p-OH-NPA as substrate would be necessary before eliminating completely the involvement of gut bacteria in the production of XXV.

b. Studies with uremic rats

Chronic uremia in animals and man has been shown to produce a variety of physiological changes including alterations in the metabolism and excretion of many drugs and chemicals (161-164). A model that is particularly suited for in vitro and in vivo metabolic

studies of the effects of uremia because of its biochemical similarities with uremia in humans, is the sub-totally nephrectomized rat (161). Rats used in the present studies, which were prepared by Dr. U. K. Terner, had one entire kidney and approximately two-thirds of the other kidney removed surgically in a two-stage operation. It has been shown (161) that the full effects of this surgical removal are manifest within 30-60 days post-surgically and therefore metabolic studies of chronic uremia can be undertaken within a few weeks after surgery.

Uremic rats that received (±)-NPA (100 mg kg^{-1}) orally exhibited extremely low tolerance to this dosage level and 100% mortality was observed within 24h of dosing. It was not until the dose of NPA was reduced to 15 mg kg^{-1} that the uremic rats survived long enough (i.e. up to 5 days) to collect sufficient urine for g.l.c. analysis.

Urine from the animals treated with the low doses of NPA was extracted with ether in a liquid-liquid extractor, at pH 9.5, before and after acid hydrolysis. G.l.c. analysis of the concentrated ether extracts revealed, as with the normal rats, both p-OH-NPA (XXIII) and the hydroxylated-methoxylated metabolite (XXV) were formed, but in this case, the amounts of each were nearly twice that observed in non-uremic controls (i.e. 41.5-50.1% and 6.25-8.3%, respectively). Greater amounts of unchanged NPA were also recovered (1.7-2.8%) from the urine of the uremic animals than from controls.

The reason for the increased amounts of recovered metabolites and NPA is not clear. Chronic uremia in man and animals has been shown to produce a decreased drug-metabolizing capability (161, 164). However, in uremia, large volumes of dilute urine are excreted. It is conceivable that the increased levels of metabolites observed in the urine of the uremic animals used in this study was not a result of increased metabolism but rather a reflection of an increased rate of excretion of the metabolites once they were formed.

It is difficult to accurately determine the exact cause of the lower LD50 in these animals in response to NPA but it has been shown [redacted] uremic animals are subjected to a great deal of stre[redacted] speculated that these chronically uremic animals cannot adapt physiologically to the CNS stimulation produced by NPA. The result is death, possibly from cardio-vascular collapse.

2. In vivo metabolism of homologs of NPA

Discovery of the ring-hydroxylated and methoxylated metabolites of NPA lead to speculation that amphetamine and its other N-alkyl homologs may also be metabolized in vivo in this manner. Four alternative substrates were readily available for these in vivo studies: amphetamine, N-methylamphetamine, N-ethylamphetamine and N-(n-butyl)amphetamine.

a. (±)-N-(n-butyl)amphetamine (NBA).

(±)-NBA was administered orally to male rats according to schedule 11 (table 11), and the metabolites extracted from pooled

acid-hydrolyzed urine, adjusted to pH 9.5 prior to extraction, with ether-methylene chloride. G.l.c. analysis of the concentrated ether-methylene chloride extract revealed the presence of two major metabolites (Tr=14.8 and 20.6 min) designated C and D respectively, together with amphetamine and unchanged NBA. The mass spectra of C and D were recorded and compared with the mass spectrum of NBA (fig. 36) and the mass spectra of metabolites A and B (fig. 33). It was obvious that metabolites C and D (fig. 36) behaved similarly to A and B in the mass spectrometer. Using the same arguments as applied to metabolites A and B, metabolite C was deduced to be of structure XXXIII and metabolite D to be of structure XXXIV. Subsequent synthesis and g.l.c./m.s. characterization of each compound confirmed these structures.

The results of quantitation of the amounts of XXXIII and XXXIV present in the urine in free and conjugated forms are shown in table 22. N-dealkylation of NBA to amphetamine occurred only to a very small extent (2%) and no p-OH-amphetamine could be detected.

b. (±)-Amphetamine

Para-hydroxyamphetamine is the major *in vivo* metabolite of amphetamine in the rat (103). It was speculated, as outlined previously, that para-hydroxylation was the first step in the metabolic synthesis of the hydroxylated, methoxylated metabolites, i.e. XXIII → XXV (vide supra), and this prompted the thought that amphetamine

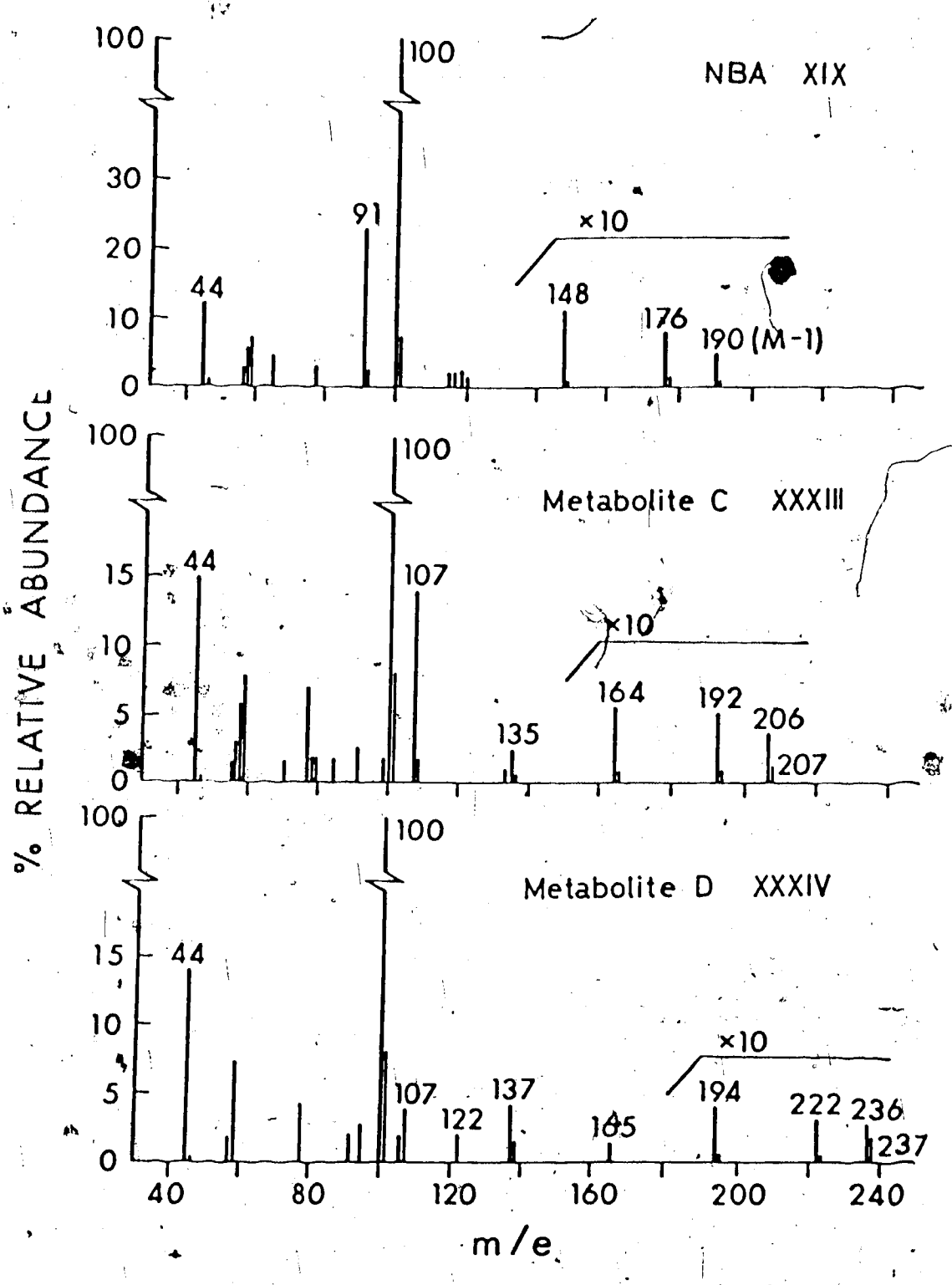


Fig. 36. G.l.c./m.s. identification of para-hydroxy-NBA and 4-hydroxy-3-methoxy-NBA as in vivo metabolites of NBA.

TABLE 22. SUMMARY OF THE QUANTITIES OF RING-HYDROXYLATED AND HYDROXYLATED-METHOXYLATED IN VIVO METABOLITES OF SOME AMPHETAMINES IN THE RAT.

	Drug or Metabolite	% of Dose Recovered		Total
		Free	Conjugated	
1.	(±)-NPA	2.9(2.5-3.3)	--	2.9(2.5-3.3)
	p-OH-NPA	2.7(2.3-3.1)	17(15.0-17.9)	18.3(17.3-21)
	4-OH-3-MeO-NPA	0.5(0.4-0.6)	2.9(1.9-6.3)	3.3(2.3-6.9)
2.	(±)-NBA	5.1(2.1-8.1)	--	5.1(2.1-8.1)
	p-OH-NBA	ND[2]	ND	22.3(19.3-25.3)
	4-OH-3-MeO-NBA	ND	ND	9.5(8.3-10.8)
3.	(±)-Amphetamine	< 1		< 1
	p-OH-Amphetamine	ND	21.6(16.1-28.5)	21.6(16.1-28.5)
	4-OH-3-MeO-Amphetamine	--		--
4.	(+)-NMA	13.9(9.4-18.5)	--	13.9(9.4-18.5)
	p-OH-NMA	--	23(15.1-33.2)	23(15.1-33.2)
	4-OH-3-MeO-NMA	--	--	--
5.	(±)-NEA	4(2.5-5.5)	--	4(2.5-5.5)
	p-OH-NEA	1.3(1.1-1.5)	--	49.8(43-56.8)
	4-OH-3-MeO-NEA	--	--	6.9(5.3-8.4)

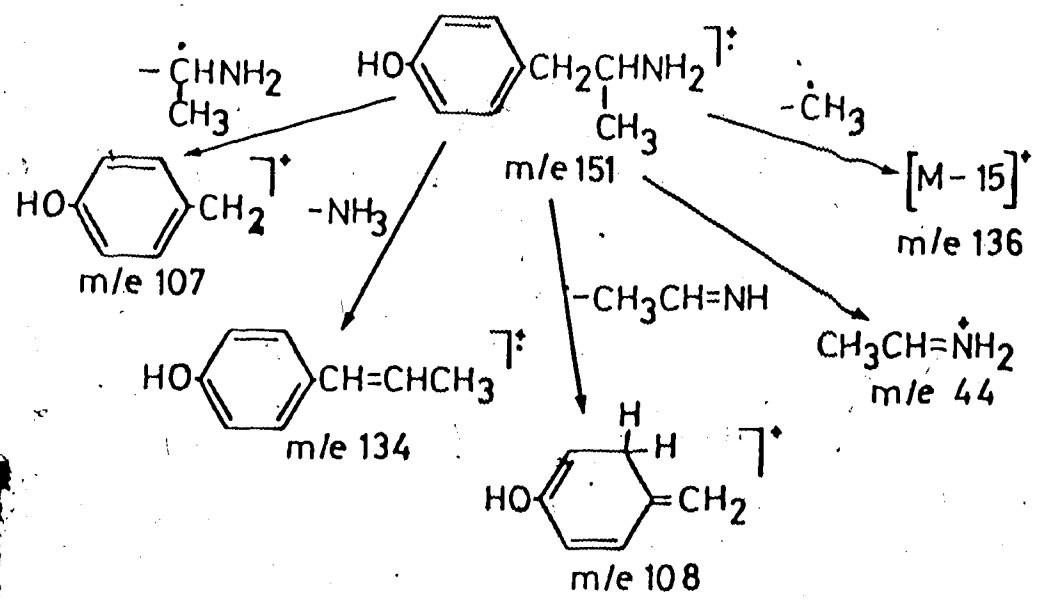
[1] range is given in parenthesis

[2] not determined

might also be metabolized to a hydroxylated, methoxylated derivative in vivo.

Male rats were given (±)-amphetamine orally according to schedule 8 (table 11). The urine was collected, pooled and extracted with ether-methylene chloride at pH 9.5 after acid-hydrolysis. G.l.c. and g.l.c./m.s. analysis of the concentrated ether-methylene chloride extract revealed only a very small amount of p-OH-amphetamine (fig. 37) and no ring-hydroxylated and methoxylated metabolite. It should be pointed out that it was known that amphetamine is metabolized to a variety of products in vivo (103) but in the experiments just described, the primary interest was to determine whether a hydroxylated and methoxylated metabolite of amphetamine was formed.

Early in the quantitative studies of the in vivo para-hydroxylation of amphetamine, very poor extraction (20-25%) of p-OH-amphetamine with ether from aqueous solutions at pH 9.5 was observed. When an ether-methylene chloride (14:11) mixture was used for extraction, recovery of p-OH-amphetamine from doped solutions was still very poor (30%) in comparison to the same extraction procedure for p-OH-NPA (recovery 60-70%). Varying the pH of the doped solutions within the range 8.5-10.5 did not improve the efficiency of p-OH-amphetamine extraction over that observed at pH 9.5. Furthermore, when calibration curves for p-OH-amphetamine were constructed from these ether extracts, they were found to be variable and poorly reproducible.



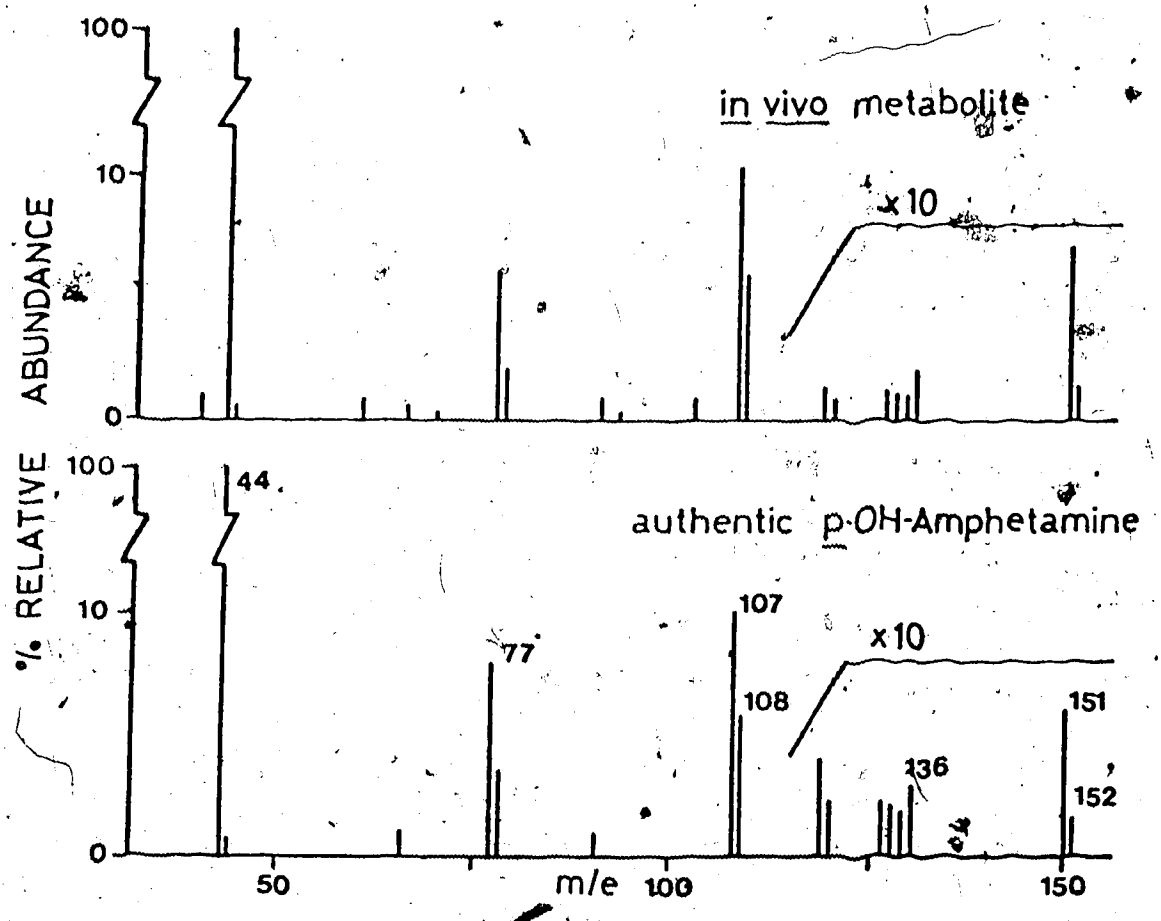


Fig. 37. G.l.c./m.s. identification of para-hydroxy-amphetamine as an in vivo metabolite of amphetamine.

In an attempt to improve on the quantitation of ring-hydroxylated metabolites, a continuous liquid-liquid extractor was employed. When ether alone was percolated for 24h through a control urine sample, which had been doped with p-OH-amphetamine and adjusted to pH 9.5, efficiency of the extraction improved, but only to a total of 30-35%. However, subsequent construction of calibration curves from data obtained in this system were found to be reproducible and linear in the concentration ranges encountered in the in vivo experiments. A major problem of the liquid-liquid extractor was the tendency of the ether-urine mixtures to form emulsions. When emulsions formed, extraction efficiency of all ring-hydroxylated metabolites was markedly reduced. Formation of emulsions by shaking extraction vessels containing solvent and urine was also an ever present problem which contributed to the very low recoveries of p-OH-amphetamine observed in early studies.

The in vivo metabolism of amphetamine was reinvestigated taking special care to avoid formation of emulsions and using the carefully [redacted] calibration curves. Despite correction for the low recovery of [redacted]etamine, only 20-25% of the dose of amphetamine was observed in the urine as the para-hydroxy metabolite. This finding contrasts markedly with a published report (103) that claims 60% of the dose of amphetamine is excreted as p-OH-amphetamine.

A possible explanation of the apparent differences in the in vivo amphetamine metabolism observed may be in the fact that the dose of amphetamine employed in this study produced marked CNS stimulation. The net effect on the rats as a result of this stimulated state was a marked decrease in food and water intake. Subsequently, very little urine was excreted. Lowering the dose of amphetamine to 10 mg kg^{-1} eliminated much of the overt CNS stimulation but also decreased the amount of p-OH-amphetamine in the urine to the point that quantitation was made more difficult.

In the absence of authentic 4-hydroxy-3-methoxy-amphetamine, its g.l.c. and g.l.c./m.s. characteristics could not be determined. However, repeated g.l.c./m.s. scanning of that region of the g.l.c. chromatogram where this metabolite might elute, did not reveal any indication that even trace amounts were present. TFA treatment of a portion of the concentrated ether-methylene chloride extract and subsequent g.l.c./m.s. analysis also failed to reveal any ring-hydroxylated and methoxylated metabolite of amphetamine.

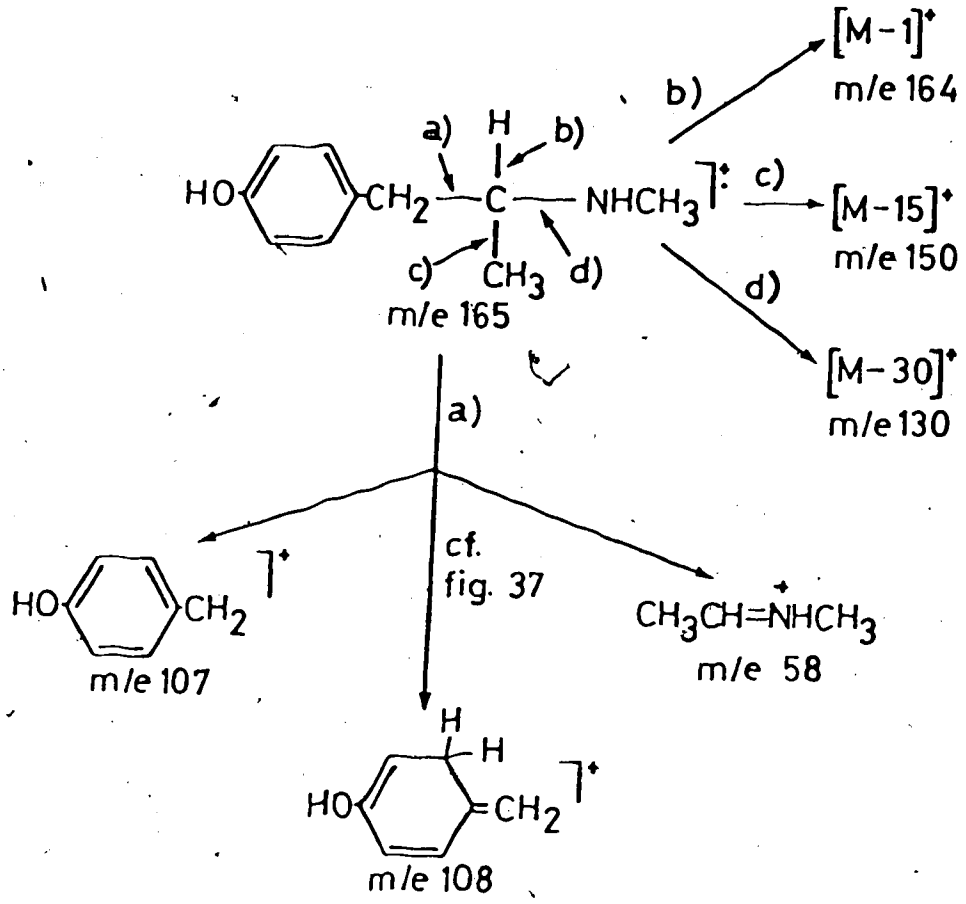
In a final attempt to detect a ring-hydroxylated and methoxylated metabolite of amphetamine, rats were administered p-OH-amphetamine (10 mg kg^{-1}) orally. Subsequent g.l.c. and g.l.c./m.s. analysis of the ether-methylene chloride extract of the acid-hydrolyzed urine, did not produce the metabolite in question. The conclusion from these studies was that neither amphetamine nor p-OH-amphetamine was converted to a ring-hydroxylated and methoxylated metabolite in vivo.

c. (+)-N-methylamphetamine (NMA)

The in vivo excretion and metabolic reactions of NMA have been reported elsewhere (99,103). It was not the intent to repeat those previously performed studies but rather an attempt was made to isolate and identify 4-hydroxy-3-methoxy-NMA (XXVIII) as an in vivo metabolite of NMA.

Oral administration of (+)-NMA according to schedule 9 (table 11) and subsequent g.l.c. and g.l.c./m.s. analysis of the concentrated ether-methylene chloride extracts of acid-hydrolyzed urine which had been basified to pH 9.5, revealed that approximately 30% of the dose of NMA was excreted as p-hydroxy-N-methylamphetamine (p-OH-NMA), XXVII (table 22), and about 14% as unchanged NMA (fig. 38). These findings correlate well with those of Caldwell et al. (99) who used (±)-^{14}C-NMA, and recovered 30 and 11%, respectively, of these two compounds. They also found a number of other metabolites but could only account for 73% of the administered dose of radioactivity. Confirmation of the presence of metabolically produced 4-hydroxy-3-methoxy NMA could not be obtained in ether-methylene chloride extracts from the urine of animals treated with (+)-NMA, either by direct g.l.c./m.s. or g.l.c./m.s. after TFA treatment of urine extracts.

In an attempt to see if the ring-hydroxylated and methoxylated metabolite (XXVIII) was formed in vivo, rats were administered


[M-1]+
m/e 164
b)
a) H b)
HO CH2 C NHCH3]+·
c)
[M-15]+
m/e 150
c) CH3 d)
m/e 165
d)
[M-30]+
m/e 130
a)
cf.
fig. 37
HO CH2]+
m/e 107
CH3CH=NHCH3
m/e 58
H
H
HO =CH2]+
m/e 108

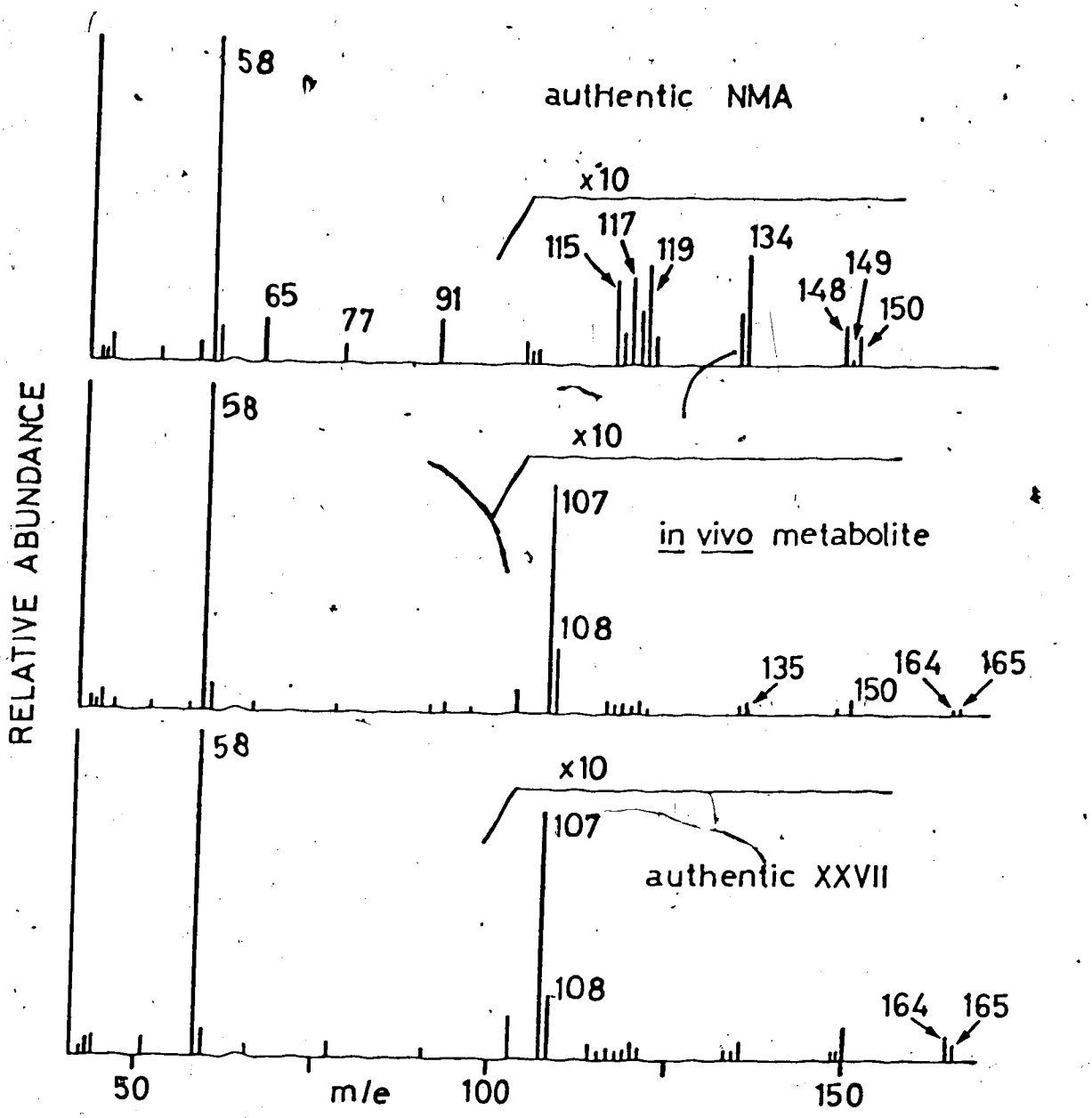


Fig. 38. G.l.c./m.s. identification of para-hydroxy-NMA as an in vivo metabolite of NMA.

p-OH-NMA (30 mg kg^{-1}) orally and the urine was collected for three days. G.l.c. and g.l.c./m.s. analysis of the acid-hydrolyzed extract, adjusted to pH 9.5, revealed the presence of p-OH-NMA (about 50% of the dose), two unidentified metabolites in small quantities (Tr 1.2 and 2.57 min) and another peak (Tr 6.2 min) whose mass spectrum was reminiscent of the ring-hydroxylated and methoxylated compounds seen previously (cf. fig. 33, 36). However, background in the m.s. from interfering peaks in this area of the chromatogram was so great, that small amounts of 4-hydroxy-3-methoxy-NMA (XXVIII) that might have been present, were masked, making positive identification impossible.

It should be pointed out that because of low extraction efficiency of p-OH-NMA (30-40%) and the relative toxicity of (+)-NMA necessitating small doses (cf. NPA and NBA), the ring-hydroxylated and methoxylated metabolite (XXVIII) may be formed, but in such small quantities as to make positive identification impossible.

It is important to note that, because of its ready availability, (+)-NMA was used in these studies whereas (±)-NMA was used in the studies of Caldwell et al. (99). Beckett and Brookes (104) have reported that the (+)-isomer of NMA is excreted in the urine at a faster rate than (-)-NMA. It may be that the rate of excretion of (+)-NMA was too rapid to permit metabolism to a ring-hydroxylated and methoxylated metabolite.

During the studies just described, it was observed that in all animals which had received (+)-NMA, the pH of the urine was consistantly lower (i.e. pH 5-6) than that of controls or animals which had received NPA or NBA (i.e. pH 7.5-8.5). A lower urinary pH has been reported (104) to increase the rate of urinary excretion of amphetamines.

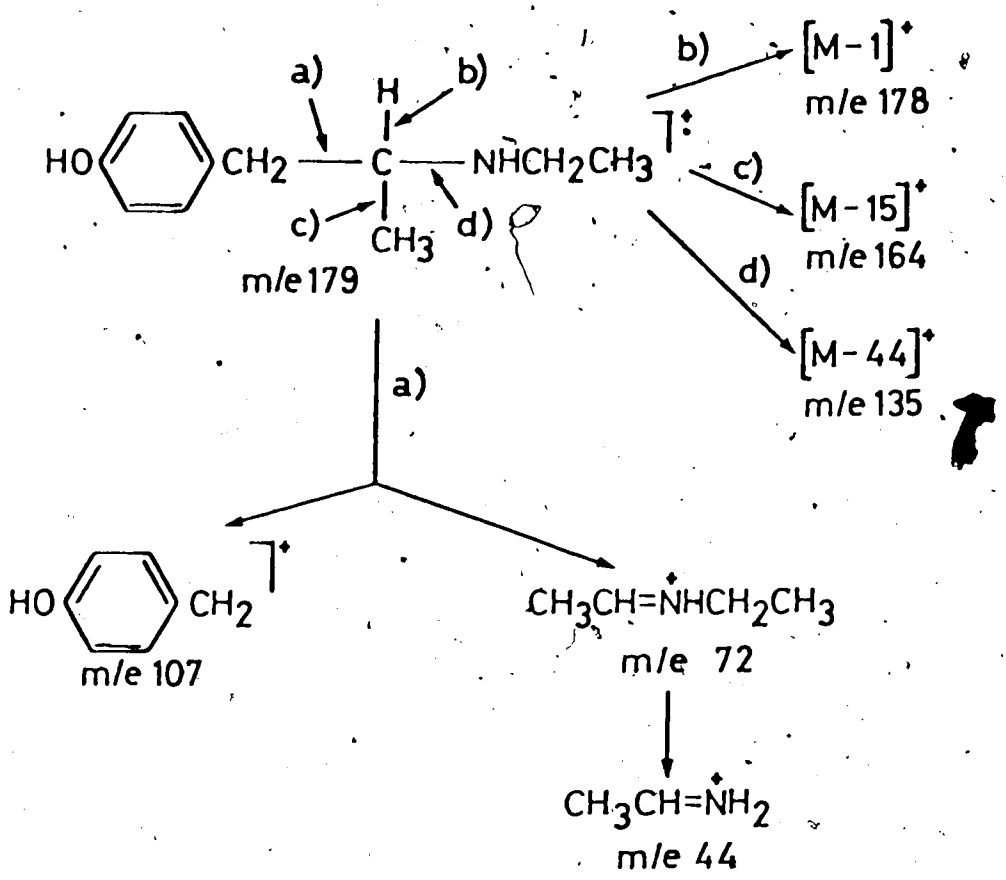
d. (±)-N-ethylamphetamine (NEA)

Oral administration of (±)-NEA to rats according to schedule 10 (table 11) and subsequent g.l.c. and g.l.c./m.s. analysis of the ether-methylene chloride extract of acid-hydrolyzed urine, after adjusting to pH 9.5, revealed that nearly half of the administered dose of NEA was excreted as p-hydroxy-N-ethylamphetamine (p-OH-NEA; XXX) and 5.3-8.4% as the ring-hydroxylated and methoxylated metabolite, XXXI (table 22; fig. 39). The same arguments were applied to the g.l.c./m.s. identification of XXXI as was used to identify XXIII and XXV (fig. 33).

It is worthy to note that with p-OH-NEA, as with p-OH-NPA and p-OH-NBA, the extraction efficiency from aqueous solutions was quite good (50-60%) as compared to the extraction efficiency of p-OH-amphetamine and p-OH-NMA (vide supra).

e. In vivo metabolism of (±)-NPA in man

In view of the abuse potential and psychological effects of the amphetamines and the psychological effects of certain methoxylated


a)
b)
H
HO
CH2
C
NHCH2CH3
c)
d)
CH3
m/e 179
b)
[M-1]+
m/e 178
c)
[M-15]+
m/e 164
d)
[M-44]+
m/e 135
a)
HO
CH2
m/e 107
CH3CH=NHCH2CH3
m/e 72
CH3CH=NH2
m/e 44

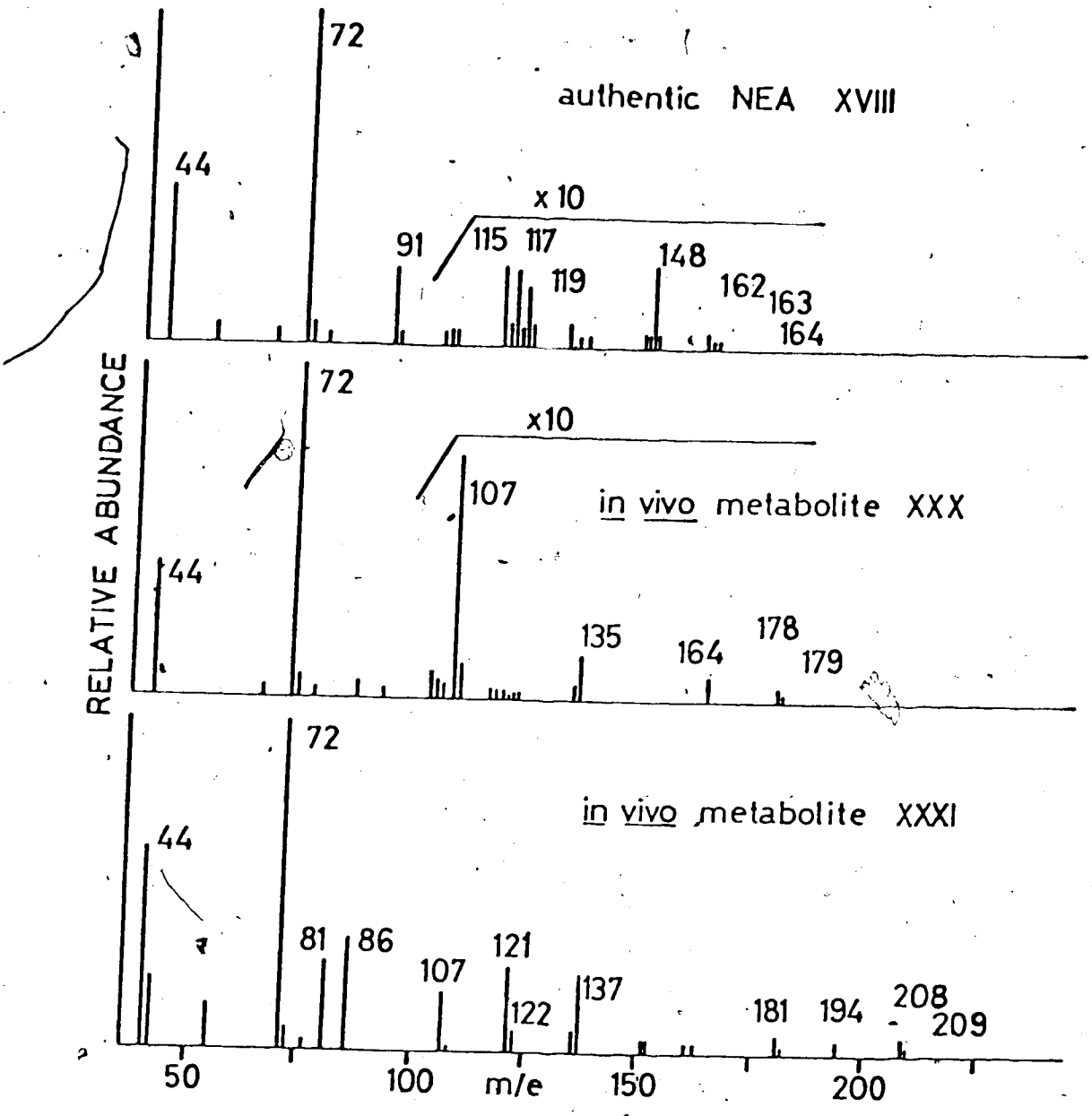


Fig. 39. G.l.c./m.s. identification of para-hydroxy-NEA and 4-hydroxy-3-methoxy-NEA as in vivo metabolites of NEA.

derivatives of amphetamine, it seemed appropriate to determine whether or not amphetamines formed hydroxylated and methoxylated metabolites in man. Because of the toxicity of (+)-NMA, (±)-NPA was chosed as the substrate for in vivo study in man.

Two healthy male volunteers were given 25 mg (±)-NPA orally and urine samples were collected for 24h. No attempts were made to control urinary pH. The urine samples from each volunteer were pooled and divided into three equal portions which were treated as follows: (1) one portion was adjusted to pH 9.5 and extracted with ether for 24h on a liquid-liquid extractor; (2) a second portion was acid-hydrolyzed, adjusted to pH 9.5 and extracted as in (1); and (3) a third portion was enzyme-hydrolyzed over-night and adjusted to pH 9.5 and extracted with ether as in (1).

G.l.c. and g.l.c./m.s. analysis of the concentrated ether extracts of enzyme- and acid-hydrolyzed urine revealed that 14-15% (3.6-3.7 mg) of the dose of NPA was excreted as p-OH-NPA (XXIII) and that 2-7% (0.5-1.6 mg) was excreted as 4-OH- 3-MeO-NPA (XXV). There have been no previous reports in the literature of the formation of hydroxylated and methoxylated metabolites of any amphetamine in vivo in man. Compounds similar to these hydroxylated and methoxylated metabolites, such as methylenedioxyamphetamine (MDA), are reported to be potent hallucinogens (165). The discovery of these hydroxylated and methoxylated metabolites necessitates a

reinvestigation of the in vivo metabolism of N-alkylated amphetamines and amphetamine-like compounds in man.

During the course of the in vivo studies on amphetamine and its N-alkylated derivatives, attempts were made to identify N-oxygenated metabolites by means of g. l. c. and combined g. l. c. / m. s. Although several g. l. c. peaks were observed when extracts from non-hydrolyzed and enzyme-hydrolyzed urine were chromatographed, scanning of these peaks in the mass spectrometer revealed that none of these peaks were N-oxygenated compounds which would imply that in vivo N-oxidation had not occurred.

Beckett et al. (62, 63, 97) have claimed that in vivo N-oxidation does occur with N-methyl- and N-ethylamphetamine. Dring et al. (99), on the other hand, could not identify any in vivo N-oxygenated metabolites of N-methylamphetamine. The possibility exists (65), however, that N-hydroxy metabolites may be formed in vivo and subsequently metabolically reduced back to the parent amine, or to, as yet, unidentified metabolites.

VI. SUMMARY AND CONCLUSIONS

1. N-(n-Propyl)amphetamine (NPA) was found to be a suitable substrate for both in vitro and in vivo metabolic C- and N-oxidation. Numerous metabolites were isolated in both in vitro and in vivo studies, and identified by means of gas-liquid chromatography (g.l.c.) and combined gas-liquid chromatography/mass spectrometry (g.l.c /m.s.). Proposed structures of metabolites were confirmed by direct comparisons with g.l.c. and g.l.c./m.s. behavior of authentic synthetic reference compounds.

2. In agreement with literature claims, standard rat liver homogenate preparations were found to be low in their C- and N-oxidative capabilities. However, several in vitro metabolites of NPA were formed and identified through the use of g.l.c. and combined g.l.c./m.s. The products identified included two N-oxygenated metabolites, N-hydroxy-1-phenyl-2-(n-propylamino)propane (N-OH-NPA), and N-[(1-methyl-2-phenyl)ethyl]-propanimine N-oxide (NPA-nitrone) as well as amphetamine, 1-phenyl-2-propanone, 1-phenyl-2-propanone oxime, and a previously unreported metabolite 2-(2-hydroxypropylamino)-1-phenylpropane.

3. Previously accepted procedures for the quantitation of in vitro metabolism were found to require modification to insure an acceptable level of accuracy and specificity. The standard method of g.l.c. column preparation was ultimately found to be unacceptable and an improved method of preparation was developed. As a result of this increased

g.l.c. column efficiency, calibration curves were found to be more accurate and reproducible. In addition, refinements in the extraction procedure were introduced to further increase the reliability of the quantitative analytical procedure.

4. Rat liver homogenate could be induced to more rapidly N-oxidize NPA when the rat was given 3-methylcholanthrene (3-MC) 24h prior to preparation of the homogenate for in vitro assay. Phenobarbital pretreatment of rats for 5 days prior to preparation of the liver homogenate did not increase in vitro metabolic N-oxidation. Both pretreatments, however, increased the rate of in vitro metabolic C-oxidation of NPA. Pretreatment of rats with NPA decreased the absolute amount of in vitro metabolism of NPA but was shown to have little effect when the results were expressed as μmol of product formed mg^{-1} protein h^{-1}. This difference is explained in the fact that the amount of microsomal protein is reduced as a result of the NPA treatment but the specific activity of the enzymes present has not changed.

5. The pH of the buffer used in the in vitro incubation mixture had a marked effect on the rates of both N- and C-oxidation of NPA by rat liver microsomes. Adjusting the pH from 7.4 to 8.4 resulted in a 2-3 fold increase in in vitro metabolism of NPA. This increased metabolism may be a reflection of the increased amount of the unionized, lipophilic form of NPA (pKa = 9.9) at the higher pH. As a result, more drug was able to interact with the enzymes responsible for its

metabolism. As far as could be determined, no reports have appeared in the literature regarding the effect of pH on the in vitro metabolic N-oxidation of amphetamine substrates.

6. SKF 525-A, a known inhibitor of cytochrome P-450, markedly reduced in vitro metabolic C-oxidation, and to some extent, the N-oxidation, of NPA. Cytochrome P-450 has been implicated in the metabolism of many amines to their corresponding N-oxides, but it is not believed to be responsible for the N-oxidation of amphetamines. Further, FAD-amine oxidase, the enzyme thought to be responsible for the N-oxidation of amphetamines, has not been shown to be sensitive to SKF 525-A inhibition as is cytochrome P-450. The conclusion was that SKF 525-A, a tertiary amine, might be a substrate for the amine oxidase enzyme and, therefore, might compete with NPA for the active site on this enzyme. The result was a decrease in the N-oxidation of NPA. Further studies of the kinetics of this interaction are needed.

7. Nicotinamide was clearly shown to be an inhibitor of in vitro N-oxidation of NPA. In view of the reports that nicotinamide is required for efficient N-oxidation of certain amphetamine-like substrates related to NPA, e.g. phentermine, the requirement of nicotinamide for in vitro N-oxidation must be determined for each substrate under investigation. Much of the published data on in vitro N-oxidation of amphetamines has been obtained from in vitro incubation mixtures in which nicotinamide had been included but usually no attempts were made

to determine the effects of nicotinamide on the N-oxidation of the particular substrate under study.

8. The inclusion of EDTA or ascorbic acid in the in vitro incubation mixtures had little effect on the N-oxidation of NPA. From these results, it was concluded that lipid peroxidation (inhibited by EDTA) or ascorbic acid (synthesized by the rat) were not probable causes of the comparatively low levels of N-oxidation observed in rat liver homogenate.

9. In vitro metabolism of NPA by rat liver homogenate was, to some extent, stereoselective. The rate of metabolic C-oxidation of (+)-NPA was greater than that for (-)-NPA whereas the opposite was true for the rate of N-oxidation. This implies that there is a stereochemical requirement for optimum fit of substrate to cytochrome P-450 and FAD-amine oxidase.

10. N-hydroxy-NPA, the initial N-oxidized metabolite of NPA, was oxidized to the related nitrone in aqueous solutions. This nitrone in turn hydrolyzed to N-hydroxyamphetamine which was highly unstable and oxidized further to 1-phenyl-2-propanone oxime. Although this sequence of events is generally thought to be a direct result of physical or chemical action, i.e. non-enzymatic, it was shown that microsomal enzymes might play a role in this conversion.

11. The in vitro metabolite of many amphetamines, i.e. 1-phenyl-2-propanone oxime, is metabolized further in vitro. The major metabolite,

2-nitro-1-phenylpropane, and two minor metabolites, 1-phenyl-2-propanone and benzyl alcohol, were identified by g.l.c. and combined g.l.c./m.s. and by comparison with authentic synthetic reference compounds. It is possible that if 1-phenyl-2-propanone oxime is further metabolized *in vitro*, it might also be metabolized *via* this route *in vivo*, and this might explain why conflicting reports exist in the literature regarding the significance of *in vivo* N-oxidation. In view of this discovery, the *in vivo* metabolic N-oxidation of "amphetamines" capable of forming oximes should now be re-investigated.

12. *In vivo* metabolism in rats of NPA, or its analogs, amphetamine, N-methylamphetamine (NMA), N-ethylamphetamine (NEA), and N-(n-butyl)amphetamine (NBA), resulted in the formation of numerous metabolites including the *para*-hydroxylated derivatives of the respective substrates but no N-oxidized metabolites were isolated. Metabolism of NEA, NPA, and NBA, also resulted in the isolation of previously unreported ring-hydroxylated and methoxylated metabolites. These hydroxylated and methoxylated metabolites apparently arise as a result of O-methylation of catechol intermediates which have not yet been isolated.

The hydroxylated and methoxylated metabolite of NPA was also identified in the urine of human volunteers given NPA. Since ring-hydroxylated and methoxylated derivatives of amphetamine are known to be hallucinogenic, it would be of interest to re-investigate the *in vivo* metabolism of other ring-unsubstituted amphetamines and analogs to

determine whether combined ring-hydroxylation and -methoxylation is a common metabolic reaction, and, if so, to establish what pharmacological properites these metabolites possess.

13. Quantitation of the amount of in vivo ring-hydroxylation of the amphetamine analogs in rats demonstrated that an increase in the extent of ring-hydroxylation generally occurred as the N-alkyl side-chain increased in length. The one exception was the metabolism of NEA, which resulted in nearly twice as much para-hydroxylated metabolite as any other substrate studied. The reason for this anomaly awaits elucidation.

VII. BIBLIOGRAPHY

1. Mueller, G. C. and Miller, J. A. 1949. The reductive cleavage of 4-dimethylaminoazobenzene by rat liver: the intracellular distribution of the enzyme system and its requirement for triphosphopyridine nucleotide. J. Biol. Chem. 180, 1125-1136.

2. Mueller, G. C. and Miller, J. A. 1953. The metabolism of methylated aminoazo dyes. II. Oxidative demethylation by rat liver homogenates. J. Biol. Chem. 202, 579-587.

3. Brodie, B. B., Axelrod, J., Cooper, J. R., Gaudette, L., LaDu, B. N., Mitoma, C. and Udenfriend, S. 1955. Detoxication of drugs and other foreign compounds by liver microsomes. Science 121, 603-604.

4. Posner, H. S., Mitoma, C., Rothberg, S. and Udenfriend, S. 1961. Enzymatic hydroxylation of aromatic compounds. III. Studies on the mechanism of microsomal hydroxylation. Arch. Biochem. Biophys. 94, 280-290.

5. Lake, B. G., Hopkins, R., Chakraborty, J., Bridges, J. W. and Parke, D. V. W. 1973. The influence of some hepatic enzyme inducers on extrahepatic drug metabolism. Drug Metab. Disp. 1, 342-349.

6. Orrenius, S., Ellin, A., Jakobsson, S. V., Thor, H., Cinti, D. L., Schenkman, J. B. and Estabrook, R. W. 1973. The cytochrome P-450-containing mono-oxygenase system of rat kidney cortex microsomes. Drug Metab. Disp. 1, 350-357.

7. Bend, J. R., Hook, G. E. R. and Gram, T. E. 1973. Characterization of lung microsomes as related to drug metabolism. Drug Metab. Disp. 1, 358-367.

8. Omura, T. and Sato, R. 1964. The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. J. Biol. Chem. 239, 2379-2385.

9. Schenkman, J. B., Remmer, H. and Estabrook, R. W. 1967. Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3, 113-123.

10. Sato, R., Satake, H. and Imai, Y. 1973. Partial purification and some spectral properties of hepatic microsomal cytochrome P-450. Drug Metab. Disp. 1, 6-13.

11. Fouts, J.R. 1971. In, Methods in Pharmacology, vol. 1. Arnold Schwartz (ed.). Appleton-Century-Crofts, New York, New York. p. 287.

12. Anders, M.W. and Mannering, G.J. 1966. Inhibition of drug metabolism. I. Kinetics of the inhibition of the N-demethylation of ethylmorphine by 2-diethylaminoethyl-2,2-diphenylvalerate HCl (SKF 525-A) and related compounds. Mol. Pharmacol. 2, 319-327.

13. Kratz, F. 1973. In, Progress In Drug Research, vol. 17, Ernst Jucker (ed.). Birkhäuser Verlag, Basel. p. 488.

14. Gillette, J.R., Conney, A.H., Cosmides, G.J., Estabrook, R.W., Fouts, J.R. and Mannering, G.J. (eds.). 1969. Microsomes and Drug Oxidations, Academic Press, New York. 547 p.

15. Estabrook, R.W., Gillette, J.R. and Leibman, K.C. (eds.). 1972. The Second International Symposium On Microsomes and Drug Oxidation. Williams and Wilkins, Baltimore. 486 p.

16. Schreiber, E.C. 1974. Metabolically oxygenated compounds: Formation, conjugation, and possible biological implications. J. Pharm. Sci. 63, 1177-1190.

17. Williams, R.T. 1971. In, Handbook of Experimental Pharmacology, vol. 28, part 2. B.B. Brodie and J.R. Gillette (eds.). Springer-Verlag, New York. p. 226.

18. Mason, H.S., North, J.C. and Vanneste, M. 1965. Microsomal mixed-function oxidations: the metabolism of xenobiotics. Fed. Proc. 24, 1172-1180.

19. Gillette, J.R. 1963. In, Progress In Drug Research, vol. 6. Ernst Jucker (ed.). Birkhäuser Verlag, Basel. p. 13.

20. Estabrook, R.W., Cooper, D.Y. and Rosenthal, O. 1963. The light reversible carbon monoxide inhibition of the steroid C21-hydroxylase system of the adrenal cortex. Biochem. Z. 338, 741-755.

21. Cooper, D.Y., Schleyer, H. and Rosenthal, O. 1973. Chemistry of cytochrome P-450 purified from endocrine systems. Drug Metab. Disp. 1, 21-28.

22. Yohro, T. and Horie, S. 1967. Subcellular distribution of P-450 in bovine corpus luteum. J. Biochem. 61, 515-517.

23. Omura, T., Sato, R., Cooper, D.Y., Rosenthal, O. and Estabrook, R.W. 1965. Function of cytochrome P-450 of microsomes. Fed. Proc. 24, 1181-1189.

24. Omura, T., Sanders, E., Estabrook, R.W., Cooper, D.Y. and Rosenthal, O. 1966. Isolation from adrenal cortex of a nonheme iron protein and a flavoprotein functioning as a reduced triphosphopyridine nucleotide-cytochrome P-450 reductase. Arch. Biochem. Biophys. 117, 660-673.

25. Maclennan, D.H., Tzagoloff, A. and McConnell, D.G. 1967. The preparation of microsomal electron-transfer complexes. Biochim. Biophys. Acta. 131, 59-80.

26. Coutts, R.T. Private Communication.

27. Coon, M.J., Strobel, H.W. and Boyer, R.F. 1973. On the mechanism of hydroxylation reactions catalyzed by cytochrome P-450. Drug Metab. Disp. 1, 92-97.

28. Kaufman, S. 1957. The enzymatic conversion of phenylalanine to tyrosine. J. Biol. Chem. 226, 511-524.

29. Cooper, D.Y., Estabrook, R.W. and Rosenthal, O. 1963. The stoichiometry of C21 hydroxylation of steroids by adrenal microsomes. J. Biol. Chem. 238, 1320-1323.

30. Ernster, L. and Orrenius, S. 1965. Substrate-induced synthesis of the hydroxylating enzyme systems of liver microsomes. Fed. Proc. 24, 1190-1199.

31. Estabrook, R.W. and Cohen, B. 1969. In, Microsomes and Drug Oxidations. J.R. Gillette, A.H. Conney, G.J. Cosmides, R.W. Estabrook, J.R. Fouts and G.J. Mannering (eds.). Academic Press, New York. p. 95.

32. Gillette, J.R. 1966. Biochemistry of drug oxidation and reduction by enzymes in hepatic endoplasmic reticulum. Advan. Pharmacol. 4, 219-261.

33. Sasame, H.A., Mitchell, J.R., Thorgeirsson, S. and Gillette, J.R. 1973. Relationship between NADH and NADPH oxidation during drug metabolism. Drug Metab. Disp. 1, 150-155.

34. Haugen, D.A., Van der Hoeven, T.A. and Coon, M.J. 1975. Purified liver microsomal cytochrome P-450. Separation and characterization of multiple forms. J. Biol. Chem. 250, 3567-3570.

35. Longshaw, R. N. 1973. Inhibition of hepatic drug metabolism. Drug Intel. Clin. Pharm. 7, 263-270.

36. Brown, B. R. 1973. Hepatic microsomal enzyme induction. Anesthesiology 39, 178-187.

37. Sher, S. P. 1971. Drug enzyme induction and drug interactions: literature tabulation. Toxicol. Appl. Pharmacol. 18, 780-834.

38. Conney, A. H. 1967. Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19, 317-377.

39. Kuntzman, R. 1969. Drugs and enzyme induction. Ann. Rev. Pharmacol. 9, 21-36.

40. Gelehrter, T. 1976. Enzyme induction: New Eng. J. Med. 294, 523-526, 589-595, 646-651.

41. Brock, N. 1967. Pharmacologic characterization of cyclophosphamide (NSC-26271) and cyclophosphamide metabolites. Cancer Chemother. Rep. 51, 315-325.

42. Mannering, G. J. 1968. In, Selected Pharmacological Testing Methods, vol. 3. Alfred Burger (ed.). Marcel Dekker, New York. p. 51.

43. Schulte-Hermann, R. 1974. In, CRC Critical Reviews in Toxicology, vol. 3. Leon Goldberg (ed.). CRC Press, Cleveland, Ohio. p. 97.

44. Anders, M. W., Alvares, A. P. and Mannering, G. J. 1966. Inhibition of drug metabolism. II. Metabolism of 2-diethylaminoethyl-2,2-diphenylvalerate HCl (SKF 525-A). Mol. Pharmacol. 2, 328-334.

45. Anders, M. W. and Alvares, A. P. 1966. Inhibition of drug metabolism. IV. Induction of drug metabolism by 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) and 2,4-dichloro-6-phenylphenoxyethyldiethylamine HBr (Lilly 18947) and the effect of induction on the inhibitory properties of SKF 525-A type compounds. Mol. Pharmacol. 2, 341-346.

46. Williams, R. T. 1959. In, Detoxication Mechanisms. The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds. John Wiley and Sons, New York. 796 p.

47. Heubner, W. 1913. Studien über methämoglobinbildung. Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 72, 241-281.

48. Kiese, M. 1959. Die bedeutung der oxydation von anilin zu nitrosobenzol für die hämiglobinbildung nach aufnahme von anilin in dem organismus. Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 235, 360-364.

49. Cramer, J.W., Miller, J.A. and Miller, E.C. 1960. N-hydroxylation: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J. Biol. Chem. 235, 885-888.

50. Lotlikar, P.D., Scribner, J.D., Miller, J.A. and Miller, E.C. 1966. Reaction of esters of aromatic N-hydroxy amines and amides with methionine in vitro: A model for in vivo binding of amine carcinogens to protein. Life Sci. 5, 1263-1269.

51. Miller, J.A. and Miller, E.C. 1969. The metabolic activation of carcinogenic aromatic amines and amides. Progr. Exp. Tumor Res. 11, 273-301.

52. Miller, J.A. 1970. Carcinogenesis by chemicals: An overview. Cancer Res. 30, 559-576.

53. Boyland, E. and Mason, D. 1966. The biochemistry of aromatic amines. The metabolism of 2-naphthylamine and 2-naphthylhydroxylamine derivatives. Biochem. J. 101, 84-102.

54. Miller, E.C. and Miller, J.A. 1966. Mechanisms of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. Pharmac. Rev. 18, 805-838.

55. Clayson, D.B., Dawson, K.M. and Dean, H. 1971. Aromatic amine carcinogenesis: The importance of N-hydroxylation. Xenobiotica 1, 539-542.

56. Lotlikar, P.D. 1971. Acylation of carcinogenic aromatic hydroxamic acids by acetyl-CoA and carbamoyl phosphate to form reactive esters. Xenobiotica 1, 543-544.

57. Williams, K. and Nery, R. 1971. Aspects of the mechanism of urethane carcinogenesis. Xenobiotica 1, 545-549.

58. Irving, C.C. 1971. Metabolic activation of N-hydroxy compounds by conjugation. Xenobiotica 1, 387-398.

59. Weisburger, J.A. and Weisburger, E.K. 1973. Biochemical formation and pharmacological, toxicological and pathological properties of hydroxylamines and hydroxamic acids. Pharmacol. Rev. 25, 1-66.

60. Bickel, M.H. 1969. The pharmacology and biochemistry of N-oxides. Pharmacol. Rev. 21, 325-355.

61. Uehleke, H. 1973. The role of cytochrome P-450 in the N-oxidation of individual amines. Drug Metab. Disp. 1, 299-321.

62. Beckett, A.H., Van Dyke, J.M., Chissick, H.H. and Gorrod, J.W. 1971. Metabolic oxidation on aliphatic basic nitrogen atoms and their α-carbon atoms-some unifying principles. J. Pharm. Pharmacol. 23, 809-812.

63. Beckett, A.H. 1971. Metabolic oxidation of aliphatic basic nitrogen atoms and their α-carbon atoms. Xenobiotica 1, 365-384.

64. Lindeke, B., Cho, A.K., Thomas, T.L. and Michelson, L. 1973. Microsomal N-hydroxylation of phenylalkylamines. Identification of N-hydroxylated phenylalkylamines as their trimethylsilyl derivatives by GC/MS. Acta Pharm. Suecica 10, 493-506.

65. Cho, A.K., Lindeke, B. and Hodshon, B.J. 1972. The N-hydroxylation of phentermine (2-methyl-1-phenylisopropylamine) by rabbit liver microsomes. Res. Commun. Chem. Pathol. Pharmacol. 4, 519-528.

66. Beckett, A.H. and Bélanger, P.M. 1974. Metabolism of chlorphentermine and phentermine in man to yield hydroxylamino, C-nitroso, and nitro-compounds. J. Pharm. Pharmacol. 26, 205-206.

67. Beckett, A.H. and Bélanger, P.M. 1974. The mechanism of metabolic N-oxidation of phentermine and chlorphentermine to their hydroxylamino- and nitroso-compounds. J. Pharm. Pharmacol. 26, 558-560.

68. Beckett, A.H., Coutts, R.T. and Ogunbona, F.A. 1973. N-ethyl- α -methyl- α (m-trifluoromethylbenzyl)nitrone-the major in vitro metabolic product of fenfluramine in guinea-pig liver microsomal fractions. J. Pharm. Pharmacol. 25, 190-192.

69. Beckett, A.H. and Essien, E.E. 1973. Chlorpromazine 'hydroxylamines' in red blood cells as major metabolites of chlorpromazine in man. J. Pharm. Pharmacol. 25, 188-189.

70. Beckett, A.H. and Midha, K.K. 1974. The identification of four metabolic products after incubation of p-methoxyamphetamine with liver preparations of various species. Xenobiotica 4, 297-311.

71. Ziegler, D.M., Mitchell, C.H. and Jallow, D. 1969. In, Microsomes and Drug Oxidations. J.R. Gillette, A.H. Conney, G.J. Cosmides, R.W. Estabrook, J.R. Fouts, G.J. Mannering (eds.). Academic Press, New York. p. 173.

72. Gorrod, J.W. 1973. Differentiation of various types of biological oxidation of nitrogen in organic compounds. Chem.-Biol. Interactions 7, 289-303.

73. Beckett, A.H. and Bélanger, P.M. 1975. Metabolic incorporation of oxygen into primary and secondary aliphatic amines and the consequences in carbon-nitrogen bond cleavage. J. Pharm. Pharmacol. 27, 547-552.

74. Beckett, A.H. and Skeikh, A.H. 1973. In vitro metabolic N-oxidation of the minor tobacco alkaloids, (-)-methylanabasine and (-)-anabasine to yield a hydroxylamine and a nitrone in lung and liver homogenates. J. Pharm. Pharmacol. 25, 171P.

75. Beckett, A.H. and Ogunbona, F.A. 1973. The importance of hydrolysis and N-oxidation in the in vitro metabolism of N-2-benzoyloxyethylnorfenfluramine. J. Pharm. Pharmacol. 25, 170P.

76. Beckett, A.H. and Salami, M.A. 1972. A note on the identification of N-hydroxyphenmetrazine as a metabolic product of phendimetrazine and phenmetrazine. J. Pharm. Pharmacol. 24, 900-902.

77. Midha, K.K., Beckett, A.H. and Saunders, A. 1974. Identification of the major metabolites of propylhexedrine in vivo (in man) and in vitro (in guinea pig and rabbit). Xenobiotica, 4, 627-635.

78. Caldwell, J., Köster, U., Smith, R. L. and Williams, R. T. 1975. Species variations in the N-oxidation of chlorphentermine. Biochem. Pharmacol. 24, 2225-2232.

79. Beckett, A. H. and Al-Sarraj, S. 1972. The mechanism of oxidation of amphetamine enantiomorphs by liver microsomal preparations from different species. J. Pharm. Pharmacol. 24, 174-176.

80. Smith, R. L. and Dring, L. G. 1970. In, Amphetamines and Related Compounds, E. Costa and S. Garattini (eds.). Raven Press, Washington, D. C. p. 121.

81. Axelrod, J. 1954. Studies on sympathomimetic amines. II. The biotransformation and physiological disposition of D-amphetamine, D-p-hydroxyamphetamine and D-methamphetamine. J. Pharmacol. Exp. Therap. 110, 315-326.

82. Axelrod, J. 1955. The enzymatic deamination of amphetamine (Benzedrine). J. Biol. Chem. 214, 753-763.

83. Dring, L. G., Smith, R. L. and Williams, R. T. 1966. The fate of amphetamine in man and other mammals. J. Pharm. Pharmacol. 18, 402-405.

84. Dring, L. G., Smith, R. L. and Williams, R. T. 1968. A precursor of benzyl methyl ketone in amphetamine urine. Biochem. J. 109, 10P.

85. Brodie, B. B., Gillette, J. R. and LaDu, B. N. 1958. Enzymatic metabolism of drugs and other foreign compounds. Ann. Rev. Biochem. 27, 427-454.

86. Hucker, H. B., Michniewicz, B. M. and Rhodes, R. E. 1971. Phenylacetone oxime-An intermediate in the oxidative deamination of amphetamine. Biochem. Pharmacol. 20, 2123-2128.

87. Parli, C. J., Wang, N. and McMahon, R. E. 1971. The mechanism of the oxidation of d-amphetamine by rabbit liver oxygenase. Oxygen-18 studies. Biochem. Biophys. Res. Commun. 43, 1204-1209.

88. Parli, C. J. and McMahon, R. E. 1973. The mechanism of microsomal deamination: Heavy isotope studies. Drug. Metab. Disp. 1, 337-341.

89. Hucker, H.B. 1973. Phenylacetone oxime - An intermediate in amphetamine deamination. Drug. Metab. Disp. 1, 332-336.

90. Beckett, A.H., Coutts, R.T. and Ogunbona, F.A. 1973. Synthesis of N-alkyl-N-hydroxyamphetamines and related nitrones. Tetrahedron 29, 4189-4193.

91. Das, M.L. and Ziegler, D.M. 1970. Rat liver oxidative N-dealkylase and N-oxidase activities as a function of animal age. Arch. Biochem. Biophys. 140, 300-306.

92. Ziegler, D.M. and Mitchell, C.H. 1972. Microsomal oxidase IV: Properties of a mixed-function amine oxidase isolated from pig liver microsomes. Ibid. 150, 116-125.

93. Cho, A.K., Lindeke, B. and Sum, C.Y. 1974. The N-hydroxylation of phentermine (2-methyl-2-amino-1-phenylpropane): Properties of the enzyme system. Drug Metab. Disp. 2, 1-8.

94. Beckett, A.H. and Al-Sarraj, S. 1973. Metabolism of ephedrine on or near its basic center. J. Pharm. Pharmacol. 25, 169-170P.

95. Beckett, A.H. and Al-Sarraj, S. 1973. Metabolism of amitriptyline, nortriptyline, imipramine and desipramine to yield hydroxylamines. J. Pharm. Pharmacol. 25, 335-336.

96. Beckett, A.H. and Al-Sarraj, S. 1972. N-oxidation of primary and secondary amines to give hydroxylamines - a general metabolic route. J. Pharm. Pharmacol. 24, 916-917.

97. Beckett, A.H. and Al-Sarraj, S.M. 1972. The metabolism of (+)- and (-)-amphetamine and (+)- and (-)-dimethylamphetamine in rabbits in vivo. Biochem. J. 130, 14P.

98. Beckett, A.H., Coutts, R.T. and Ogunbona, F.A. 1973. Metabolism of amphetamines. Identification of N-oxygenated products by gas chromatography and mass spectrometry. J. Pharm. Pharmacol. 25, 708-717.

99. Caldwell, J., Dring, L.G. and Williams, R.T. 1972. Metabolism of (14C)methamphetamine in man, the guinea pig and the rat. Biochem. J. 129, 11-22.

100. Fuller, R.W., Parli, C.J. and Molloy, G.G. 1973. Metabolism of amphetamine and β, β-difluoroamphetamine in phenobarbital-treated rats. Biochem. Pharmacol. 22, 2059-2061.

101. Vree, T. B. and van Rossum, J. M. 1970. In, Amphetamines and Related Compounds, E. Costa and S. Garattini (eds.). Raven Press, Washington, D. C. p. 165.

102. Gorrod, J. W. 1973. In, Frontiers In Catecholamine Research, E. Usdin and S. H. Snyder (eds.). Permagon Press, New York, p. 945.

103. Williams, R. T., Caldwell, J. and Dring, L. G. 1970. Ibid. p. 927.

104. Beckett, A. H. and Brookes, L. G. 1970. In, Amphetamines and Related Compounds, E. Costa and S. Garattini (eds.). Raven Press, Washington, D. C. p. 109.

105. Dingell, J. V. and Bass, A. D. 1969. Inhibition of the hepatic metabolism of amphetamine by desipramine. Biochem. Pharmacol. 18, 1535-1538.

106. Jonsson, J. A. 1974. Hydroxylation of amphetamine to para-hydroxyamphetamine by rat liver microsomes. Biochem. Pharmacol. 23, 3191-3197.

107. Rommelspacher, H., Horrecker, H., Schulze, G. and Strauss, S. M. 1974. The hydroxylation of D-amphetamine by liver microsomes of the male rat. Biochem. Pharmacol. 23, 1065-1071.

108. Cho, A. K., Hodshon, B. J., Lindeke, B. and Jonsson, J. 1975. The p-hydroxylation of amphetamine and phentermine by rat liver microsomes. Xenobiotica 5, 531-538.

109. Beckett, A. H., Coutts, R. T. and Ogunbona, F. A. 1974. The structure of nitrones derived from amphetamines. J. Pharm. Pharmacol. 26, 312-316.

110. Coutts, R. T., Dawe, R. and Beckett, A. H. 1975. A re-examination of the synthesis and some properties of the in vitro metabolite, N-hydroxyphenmetrazine. Biomed. Mass Spectrom. 2, 137-141.

111. Coutts, R. T. and Kovach, S. H. Private Communication.

112. Beckett, A. H. and Al-Sarraj, S. 1973. The identification, properties and analysis of N-hydroxyamphetamine - a metabolite of amphetamine. J. Pharm. Pharmacol. 25, 328-334.

113. Bremmer, J. M. 1954. Identification of hydroxylamine and hydrazine by paper chromatography. Analyst 79, 198-201.

114. Feigl, F. 1937. Microchemical detection of organic compounds by means of spot plate reactions. Mikrochim. Acta 1, 127-141.

115. Barton, G. M., Evans, R. S. and Gardner, J. A. F. 1952. Paper chromatography of phenolic substances. Nature 170, 249-250.

116. Leonard, N. J., Adamcik, J. A., Djerassi, C. and Halpern, O. 1958. Transanhular nitrogen-carbonyl interaction in cyclic aminoketones and optical rotatory dispersion. J. Am. Chem. Soc. 80, 4858-4862.

117. Temmler, T. H. 1952. German patent 767,263 through Chem. Abstr. 47, 2772c (1953).

118. Shiho, D. 1944. A new process of alkylation of amines. J. Chem. Soc. Japan. 65, 237-239.

119. Priebs, B. 1884. Ueber die einwirkung des benzaldehyds auf nitromethan und nitroäthan. Ann. Chem. 225, 319-364.

120. Hoover, F. W. and Hass, H. B. 1947. Synthesis of paredrine and related compounds. J. Org. Chem. 12, 501-505.

121. Pearl, I. A. and Beyer, D. I. 1951. Reactions of vanillin and its derived compounds. XII. Benzyl methyl ketones derived from vanillin and its related compounds. J. Org. Chem. 16, 221-224.

122. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Miller, G. L. 1959. Protein determination for large numbers of samples. Anal. Chem. 31, 964.

124. Omura, T. and Sato, R. 1964. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370-2378.

125. Leibrand, R. J. and Dunham, L. L. 1973. Preparing high efficiency packed GC columns. Res. Development 24, 32-36.

126. Schenkman, J. B., Ball, J. A. and Estabrook, R. W. 1967. On the use of nicotinamide in assays for microsomal mixed-function oxidase activity. Biochem. Pharmacol. 16, 1071-1081.

127. Parli, C. J. and Mannering, G. J. 1971. Inhibition of microsomal mixed-function oxidase activity with nicotinamide. Biochem. Pharmacol. 20, 2118-2121.

128. Chaykin, S. and Bloch, K. 1959. The metabolism of nicotinamide-N-oxide. Biochim. Biophys. Acta. 31, 213-216.

129. Sparthan, M. and Chaykin, S. 1969. Determination of nicotinamide N-oxide - a human excretory product. Anal. Biochem. 31, 286-295.

130. Booth, J. and Boyland, E. 1970. The metabolism of nicotine into two optically-active stereoisomers of nicotine-1'-oxide by animal tissues in vitro and by cigarette smokers. Biochem. Pharmacol. 19, 733-742.

131. Booth, J. and Boyland, E. 1971. Enzymic oxidation of (-)-nicotine by guinea-pig tissues in vitro. Biochem. Pharmacol. 20, 407-415.

132. Gueffroy, D. E. 1975. In, A Guide For the Preparation and Use of Buffers in Biological Systems, Calbiochem, LaJolla, California. 24 p.

133. Fouts, J. R. 1970. Some in vitro assay conditions that affect detection and quantitation of phenobarbital-induced increases in hepatic microsomal drug-metabolizing enzyme activity. Toxicol. Appl. Pharmacol. 16, 48-65.

134. Henderson, P. Th., Vree, T. B., van Ginneken, C. A. M. and van Rossum, J. M. 1974. Activation energies of α-C-oxidation and N-oxidation of N-alkyl-substituted amphetamines by rat liver microsomes. Stereochemistry and deuterium isotope effects. Xenobiotica 4, 121-130

135. Beckett, A. H. and Shenoy, E. V. B. 1973. The effect of N-alkyl chain length and stereochemistry on the absorption, metabolism and urinary excretion of N-alkylamphetamines in man. J. Pharm. Pharmacol. 25, 793-799.

136. Mazel, P. 1969. In, Fundamentals of Drug Metabolism and Drug Disposition, B. N. LaDu, H. G. Mandel and E. L. Way (eds.). Williams and Wilkins Co., Baltimore. P. 527.

137. Beckett, A. H. and Gibson, G. G. 1975. Microsomal N-hydroxylation of dibenzylamine. Xenobiotica 5, 677-686.

138. Magour, S., Coper, H. and Fähndrich, C. H. 1974. Effect of chronic intoxication with (+)-amphetamine on its concentration in liver and brain and on [^{14}C] leucine incorporation into microsomal and cytoplasmic proteins of rat liver. J. Pharm. Pharmacol. 26, 105-108.

139. Kosman, M. E. and Unna, K. R. 1968. Effects of chronic administration of the amphetamines and other stimulants on behavior. Clin. Pharm. Therap. 9, 240-254.

140. Lewander, T. 1971. A mechanism for the development of tolerance to amphetamine in rats. Psychopharmacologica 21, 17-31.

141. Beckett, A. H., Gorrod, J. W., and Lazarus, C. R. 1971. The in vitro metabolism of (35S) chlorpromazine. Xenobiotica 1, 535-536.

142. Kamataki, T. and Kitagawa, H. 1974. Effects of EDTA on stability of drug metabolizing enzymes in liver microsomes of rats. Chem. Pharm. Bull. 22, 1041-1045.

143. Zannoni, V. G., Flynn, E. J. and Lynch, M. 1972. Ascorbic acid and drug metabolism. Biochem. Pharmacol. 21, 1377-1392.

144. Zannoni, V. G. and Lynch, M. M. 1973. The role of ascorbic acid in drug metabolism. Drug Metab. Rev. 2, 57-69.

145. Ginter, E. 1973. Cholesterol: vitamin C controls its transformation to bile acids. Science 179, 702-704.

146. Sato, P. H. and Zannoni, V. G. 1974. Stimulation of drug metabolism by ascorbic acid in weanling guinea pigs. Biochem. Pharmacol. 23, 3121-3128.

147. Kitada, M., Kamataki, T. and Kitagawa, H. 1974. Effects of lipid peroxidation on the microsomal electron transport system and the rate of drug metabolism in rat liver. Chem. Pharm. Bull. 22, 752-756.

148. Budzikiewicz, H., Djerassi, C. and Williams, D.H. 1967. In, Mass Spectrometry of Organic Compounds, Holden-Day San Francisco. p. 119.

149. Nielson, A.T. 1969. In, The Chemistry of the Nitro and Nitroso Groups, part I, H. Feuer (ed.). Interscience Publishers, New York. p. 364.

150. Noland, W.E. 1955. The Nef reaction. Chem. Revs. 55, 137-155.

151. Shechter, H. and Williams, F.T. 1962. An effective general method for oxidizing salts of mononitro compounds with neutral permanganate to aldehydes and ketones. J. Org. Chem. 27, 3699-3701.

152. Shechter, H. and Kaplan, R.B. 1953. The action of oxidizing agents on salts of nitroalkanes. I. Oxidative dimerization. J. Amer. Chem. Soc. 75, 3980-3983.

153. Budzikiewicz, H., Djerassi, C. and Williams, D.H. 1967. In, Mass Spectrometry of Organic Compounds, Holden-Day, San Francisco. p. 237.

154. Midha, K.K. Private Communication.

155. Chang, T., Okerholm, R.A. and Glazko, A.J. 1972. A 3-0-methylated catechol metabolite of diphenylhydantoin (Dilantin) in rat urine. Res. Commun. Chem. Pathol. Pharmacol. 4, 13-23.

156. Andresen, B.D., Hammer, R.H., Templeton, J.L., Moldowan, M.J. and Panzik, H.L. 1975. Phenolic metabolite, 2-ethyl-2-(4-hydroxyphenyl)-glutarimide in human urine following chronic ingestion of glutethimide (Doriden). Res. Commun. Chem. Pathol. Pharmacol. 10, 443-453.

157. Axelrod, J. 1963. In, The Clinical Chemistry of the Monoamines, H. Varley and A.H. Gowenlock (eds.). Elsevier, Amsterdam. p. 5.

158. Axelrod, J. and Tomchick, R. 1958. Enzymatic O-methylation of epinephrine and other catechols. J. Biol. Chem. 233, 702-705.

159. Scheline, R. R. 1968. Drug metabolism by intestinal micro-organisms. J. Pharm. Sci. 57, 2021-2037.

160. Hartiala, K. 1973. Metabolism of hormones, drugs and other substances by the gut. Physiol. Rev. 53, 496-534.

161. Terner, U. K. 1976. Ph. D. Thesis, University of Alberta.

162. Letteri, J. M., Mellk, H., Louis, S., Kutt, H., Durante, P., and Glazko, A. 1971. Diphenylhydantoin metabolism in uremia. New Eng. J. Med. 285, 648-652.

163. Leber, H. W. and Schütterle, G. 1972. Oxidative drug metabolism in liver microsomes from uremic rats. Kidney Int. 2, 152-158.

164. Reidenberg, M. M. 1971. In, Renal Function and Drug Action, W. B. Saunders, Philadelphia. 113 p.

165. Aldous, F. A. B., Barrass, B. C., Brewster, K., Buxton, D. A., Green, D. M., Prinder, R. M., Rich, P., Skeels, M. and Tutt, K. J. 1974. Structure-activity relationships in psychotomimetic phenylakylamines. J. Med. Chem. 17, 1100-1111.

APPENDIX

Computer program for linear regression and correlation calculations

```
*
*01.05 E
*01.09 A ! !"   X",X; I (X) 1.20
*01.10 S  C=C+X;S D=D+X↑2;S N=N+1;GOTO 4.15
*01.20 S R=(H-C*E/N)/FSQT((D-C↑2/N)*(G-E↑2/N))
*01.30 S A=(H-C*E/N)/(D-C↑2/N)
*01.35 S B=(E-A*C)/N
*
*02.10 T !!"   R",%8.04,R
*02.15 T !"   D.F.",N-2
*02.20 T !!"   Y   ",A," X +(",B,")"!!
*02.30 T !!!"DO YOU WISH TO PREDICT X FROM Y?  IF YES TYPE -1;
*02.32 T !"IF NO TYPE 0."
*02.35 A Z
*02.40 I (Z)5.10,5.20
*
*04.15 A "   Y",Y;S E=E+Y;S G =G+Y↑2;S H=H+Y*X;GOTO 1.09
*
*05.10 A !"   Y",W;I  (W)5.20;S T=(W-B)/A;T "   X",T
*05.12 GOTO 5.10
*05.20 Q
**
*
*
*G

   X:1   Y:1
   X:2   Y:2
   X:3   Y:3
   X:4   Y:4
   X:-1

   R=     1.0000
   D.F.=     2.0000

   Y   =     1.0000  X  +(=     0.0000)


DO YOU WISH TO PREDICT X FROM Y?  IF YES TYPE -1;
IF NO TYPE 0.:-1
   Y:1     X=     1.0000
   Y:2     X=     2.0000
   Y:3     X=     3.0000
   Y:4     X=     4.0000
   Y:-1 *
*
```

```
01.01 E
01.10 T !!"CORRECTION AND NORMALIZATION OF GLC INTEGRATOR DATA"!
01.20 D 2;
01.30 D 3;D 4;T !
01.40 G 1.3

02.10 A !!"HOW MANY STANDARD CURVES ? ",NS,! ,"DEFINE CURVES "!
02.15 F I=1,NS;D 6
02.20 A !!"HOW MANY SAMPLE PEAKS ? "NP,!
02.25 F I=1,NP;D 7
02.30 A !!"AMOUNT OF PROTEIN PER INCUBATION IN GRAMS ",MP,!;R

03.01 T !"******"
03.10 A !!"SAMPLE INPUT DATA  I.S. "IS," ";I (IS)3.4,3.4
03.12 I (3-NP)3.2
03.15 F I=1,NP; D 8;
03.16 R
03.20 F I=1,3;D 8
03.24 T !
03.25 F I=4,NP;D 8
03.30 R
03.40 T !!!!!; Q

04.10 F I=1,NP;S P(I)=(P(I)/IS-BI(CS(I)))/BS(CS(I))
04.20 T !!"MICROMOLES";D 9
04.30 F I=1,NP;S P(I)=P(I)/MP
04.40 T !!"MICRO-MOLES/GM PROTEIN";D 9;R

06.10 X;T !"CURVE "%1,I," :-  SLOPE (1/UM) ";X;
06.20 A BS(I),"   INTERCEPT "BI(I);R

07.10 X;T !"FOR PEAK ",I," USE CURVE ";X;A CS(I),"  M.W. ",MW(I);R

08.10 X;T "P"%1,I;X;A P(I)," " ;R

09.01 S J=0
09.10 I (2-NP)9.2
09.15 F I=1,NP;D 10
09.16 R
09.20 F I=1,2;D 10
09.30 S J=J+1;T !
09.40 I (2+4*J-NP)9.5
09.45 F I=4*J-1,NP;D 10
09.46 R
09.50 F I=4*J-1,4*J+2;D 10
09.60 G 9.30

10.10 X;T " P"%1,I;X;T %,P(I)
*
*
*G
```

CORRECTION AND NORMALIZATION OF GLC INTEGRATOR DATA

cont'd

Computer program continued

```
HOW MANY STANDARD CURVES ? :4
DEFINE CURVES

CURVE 1 :-  SLOPE (1/UM) :.5    INTERCEPT :.002
CURVE 2 :-  SLOPE (1/UM) :.4    INTERCEPT :0
CURVE 3 :-  SLOPE (1/UM) :.5    INTERCEPT :-.001
CURVE 4 :-  SLOPE (1/UM) :.4    INTERCEPT :0

HOW MANY SAMPLE PEAKS ? :4

FOR PEAK 1 USE CURVE :1   M.W. :177
FOR PEAK 2 USE CURVE :2   M.W. :149
FOR PEAK 3 USE CURVE :3   M.W. :193
FOR PEAK 4 USE CURVE :4   M.W. :191

AMOUNT OF PROTEIN PER INCUBATION IN GRAMS :.0018

******

SAMPLE INPUT DATA  I.S. :1000000  P1:2000000  P2:20000  P3:20000
P4:20000

MICROMOLES P1= 0.399600E+01 P2= 0.500000E-01
 P3= 0.420000E-01 P4= 0.500000E-01

MICRO-MOLES/GM PROTEIN P1= 0.222000E+04 P2= 0.277778E+02
 P3= 0.233333E+02 P4= 0.277778E+02

******
```