THE FATE OF PHENMETRAZINE IN MAN AND ANIMALS WITH OBSERVATIONS ON AMPHETAMINE AND NOREPHEDRINE METABOLISM IN TAMARIN MONKEYS

by

RONALD BRIAN FRANKLIN

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Department of Biochemistry,
St. Mary's Hospital Medical School,
London, W2 1PG.

ABSTRACT

1. The fate of [^{14}C]-Preludin in man, Tamarin monkey, rat and guinea pig has been studied.

2. Synthesis of [^{14}C]-Preludin by two methods, as well as synthesis of the lactam metabolite and attempted syntheses of other possible metabolites was carried out.

3. Three men, dosed orally with 25 mg (about 0.35 mg/kg) excreted about 93% of the ^{14}C in the urine over 4-5 days. About 71% of the ^{14}C was excreted in the first day of which approximately 26% was unchanged drug, 27% a lactam metabolite, 16% as a phenolic metabolite, 11% as a glucuronide conjugate of the phenol, and 7% as an unidentified metabolite.

4. In two Tamarin monkeys dosed intramuscularly (0.28 mg/kg), the metabolites excreted in 24 hours were similar to those in man except that more unchanged drug was excreted.

5. The metabolites in rats receiving 20 mg/kg orally were similar to those found in man except that the major metabolite was the phenolic one and far less of the lactam metabolite was excreted.

6. The guinea pig receiving 20 mg/kg orally, excreted about 55% of the ^{14}C as the lactam metabolite and no phenolic metabolite could be detected.

7. Metabolites were isolated from the urine and in vitro liver preparations in the case of the rat and guinea pig, and their identity was confirmed using a number of physical methods.

8. Pharmacological investigations on the effect of the lactam metabolite on hexobarbitone sleeping-time and spontaneous activity of mice were carried out.

9. The glucuronide of the phenolic metabolite in rat bile after permethylation was identified by means of a combined g.l.c./m.s. system.

10. The possible role of the metabolites in the production of the bizarre effects seen on excessive dosage in man is discussed.

11. The fate of [^{14}C]-(±)-norephedrine administered intramuscularly (0.3 mg/kg) to two Tamarin monkeys was investigated. Over 85% of the ^{14}C was excreted in the first 24 hours and of this approximately 75% was unchanged norephedrine.

12. The metabolism of [^{14}C]-(±)-amphetamine in two Tamarin monkeys dosed intramuscularly (0.3 mg/kg) was studied. Approximately 75% of the administered ^{14}C was accounted for in the first 24 hour urine of which 58% was unchanged amphetamine and 3% was the metabolite, 4-hydroxyamphetamine.

FOREWORD

Species variations in drug metabolism are now widely accepted as an important aspect of the toxicological spectrum of a potential drug. Within this laboratory this dfference in metabolism has been used to investigate some drugs of abuse such as amphetamine, methamphe tamine and the closely related compound, norephedrine. These studies have now been extended to include the amphetamine-like drug, Preludin and furthermore some observations have been made concerning the metabolic fate of [^{14}C]-(±)-amphetamine and [^{14}C]-(±)-norephedrine in the Tamarin monkey, a species of New World monkey which has not been extensively used for drug metabolism studies.

These studies have been carried out in the Biochemistry Department of St. Mary's Hospital Medical School and I would like to thank Professor R. T. Williams, F.R.S., for allowing me the unique opportunity of working under his guidance and encouragement.

I am very much indebted to Dr. Graham Dring for his constant criticisms and appraisals of the work and for imparting to me something of his considerable experience in the study of amphetamines.

As for my colleagues in the laboratory, especially Mr. Z.H. Siddik and Dr. R.D. Barnes, I would like to thank them for the many hours of helpful discussion and advice.

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In writing this thesis, I hope I may have repayed to my parents a little of the encouragement they have shown to me during the various phases of my education.

Finally, I would like to thank my wife, Heulwen, whose love, understanding, patience and support whilst working for and writing this thesis have remained unshaken.

To Heulwen and my parents

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CHAPTER ONE

General Introduction

Contents

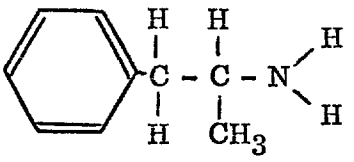
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General Introduction

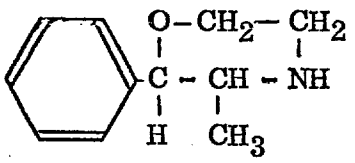
Metabolic studies on amphetamines have been carried out by many workers in order to gain knowledge of different routes of metabolism exhibited by different species, and also to ascertain general patterns of drug metabolism within the particular group of drugs being examined.

The phenomena of tolerance and dependence, as illustrated by the drugs of abuse such as the amphetamines, may prove to have a metabolic origin, thus emphasising the importance of metabolic studies, and identification of metabolites. The discovery that a metabolite of amphetamine, 4-hydroxynorephedrine, might act as a 'false' transmitter within the central nervous system and be responsible for the tolerance exhibited on repeated exposure to the drug was a step forward (Costa and Gropetti, 1970; Lewander, 1971a,b). Although this metabolite has not provided all the answers to the problem of tolerance, it has illustrated that drug metabolism may play a prominant rôle in the pharmacological effects of the amphetamines.

By studying the metabolism of Preludin, see Fig. 1.1, it was hoped to find i) whether the amphetamine-like drug Preludin (phenmetrazine hydrochloride) underwent similar metabolic transformations to amphetamine itself and cogeners such as methamphetamine and norephedrine and ii) to identify the metabolites of Preludin and compare them with that of amphetamine with the idea that the amphetamine-like actions of Preludin may in some way be associated with its metabolism.



Amphetamine



Phenmetrazine

Fig. 1.1

The introduction has included a short general review on the abuse of the amphetamines and their effects on catecholamines in order to demonstrate the similarity of Preludin to other classical amphetamine derivatives such as amphetamine itself and methamphetamine. Further data on the pharmacology of Preludin and amphetamine may be found in Chapters 2 and 3 respectively.

Abuse of Amphetamines

Clinically, amphetamine was found to be of use in the treatment of narcolepsy (Prinzmetal and Bloomberg, 1935), Parkinson's Disease (Solomon et al., 1937), and depression (Myerson, 1936). The anorexigenic effects of amphetamine derivatives were described by Nathanson in 1937 and since then many amphetamine-like drugs have been synthesised which have retained or emphasised the anorexigenic effect but abolished or attenuated the central stimulant effect.

The powerful central stimulant effect of the amphetamines had not stayed unnoticed amongst those people who sought solace with reality-distorting drugs, and as early as 1937, the Journal of the American Medical Association warned of abuse and subsequent dependence.

Unfortunately, human nature has always shown unusual ingenuity and perseverance in discovering new and perverse ways of psychological stimulation

by drugs, in spite of any hazards associated with such practices. Such abuse is by no means restricted to any particular group, society or class of individual indeed such notable personalities as DeQuincy, Baudelaire and Freud have openly indulged in drug abuse (Mason, 1968). Mason, in 1968, attempted to classify drug addiction by social class. Thus, "the lower socio-economic classes, deprived of education, housing and jobs, discriminated against on racial or on an ethnic basis (the Negroes and Puerto Ricans in the U.S.A., the Indians, Pakistanis and West Indians in the United Kingdom) seek solace in opiates and marijuana". He claims that there is a "higher socio-economic group where diet pills (amphetamines) and sleeping pills are used frequently and indiscriminantly, leading to misuse, abuse and eventual addiction". Mason's last and highest "class" consists of "intellectual circles". Amongst these persons, "the use of marijuana or LSD (lysergic acid diethylamide) appears fairly common, not only because of the alleged intellectual and spiritual benefits, but also because it is the "in" thing to do". Phenmetrazine hydrochloride (Preludin) has often been used as a substitute for the classical amphetamine derivatives (Van Praag, 1968) that is, amphetamine, dextro-amphetamine and methamphetamine, especially when these are in short supply. Phenmetrazine abuse was reported by Van Praag in 1968 to be "little less than a national problem in Sweden".

As well as the euphoriant and spiritual uplifting effects of the amphetamines, use has been made of the fact that the amphetamines can delay the onset of fatigue. Thus some students, in preparation for examinations, use the amphetamines. Sportsmen too have tended to abuse the amphetamines, as well as the anabolic steroids, in spite of medical warnings on continued use of either class of drug. Although amphetamine abuse has been noted in all forms of sport, it would appear that the drug is most beneficial in endurance sports such as cycle and cross-

country races (Ostyn, 1972). But, as Ostyn has pointed out, it is precisely these endurance sports which create the hazard for the amphetamine-takers since the feeling of extreme fatigue goes unheeded and normal limits of exertion are exceeded with the possible result of complete exhaustion or death.

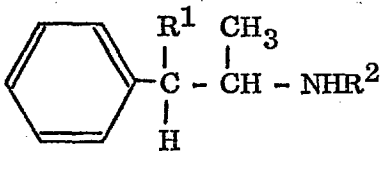
The origin of dependence can be iatrogenic and in 1968, Mason pointed out that drugs that were liable to induce dependence should only be prescribed when necessary, for a limited time, that is, long enough to produce the desired therapeutic effect, and only in sufficient quantity for the length of time needed for therapeutic drug action. In 1972, Van Praag drew attention to the anomaly between the dangers of amphetamines and the readiness with which they were prescribed. In the United Kingdom, the Council of the British Medical Association set up a Working Party in 1967 to investigate the efficacy of amphetamines and amphetamine-like compounds. The Working Party, in 1968, recommended that "amphetamine and amphetamine-like compounds should only be prescribed for those conditions for which no reasonable alternative exists". Also, that "doctors should voluntarily take the same precautions and keep the same records as they already do for those drugs covered by Part 1 of the Schedule of the Dangerous Drugs Act, 1965". Replacement of this Act with the 1973 Misuse of Drugs Act and Misuse of Drugs Regulations has placed further controls on the prescribing of amphetamines, including the proper presentation of prescriptions and legally recording the dispensing of such a compound. Similarly in Canada, amphetamines, phenmetrazine and phendimetrazine may now only be prescribed for six specified conditions (in humans), with special notification regulations to be observed by prescribers. These new regulations allow a doctor to prescribe a thirty-day supply of amphetamines for the conditions of narcolepsy, hyperkinetic disorders in children, mental retardation associated with minimal brain dysfunction, epilepsy, Parkinson's disease and hypotensive states associated

with anaesthesia. On supplying the amphetamines, the doctor must inform the Federal Health Department of the patient's name and details of the therapy. If the amphetamines are required for more than thirty days, then another doctor is required to confirm the original diagnosis (Pharmaceutical Journal, 1973).

Abuse of phenmetrazine leading to its subsequent dependence has been enlarged upon in Chapter 2, Introduction, under the heading of Dependence on and abuse of Preludin. The drug was withdrawn from use in the United Kingdom late in 1973 probably for two reasons, one being the steady rise in the illegal use of the drug and secondly, from the fact that other anorexigenic drugs had a much lower incidence of tolerance and dependence.

Metabolic Pathways and Comparative Metabolism of Some Amphetamines

The structure of amphetamine and some amphetamine-like compounds may be depicted as shown below (Fig. 1.2).



Compound	R^1	R^2
Amphetamine	H	H
Methamphetamine	H	CH_3
Ephedrine	OH	CH_3
Norephedrine	OH	H

Fig. 1.2

Routes of metabolism which have been demonstrated so far are:

i) aromatic hydroxylation
ii) β-hydroxylation
iii) deamination
iv) N-oxidation

Unchanged drug

The quantity of drug excreted unchanged appears to be correlated with lipid solubilities (Williams et al., 1973), since the hydrophilic compounds ephedrine and norephedrine were readily excreted in man (80 and 90% of the dose, respectively; Beckett and Wilkinson, 1965). The more lipophilic drugs, amphetamine and methamphetamine were less readily excreted (20 and 30% of the dose, respectively; Dring et al., 1970; Caldwell et al., 1972a). In the rat less norephedrine, ephedrine, amphetamine and methamphetamine were excreted unchanged (40, 50, 10 and 20% of the dose, respectively). However, the correlation between lipid solubility and excretion of unchanged drug fails to predict that the rabbit extensively metabolised all the aforementioned drugs, leaving less than 5% to be excreted unchanged.

i) Aromatic hydroxylation

Amphetamine is hydroxylated in the 4-position of the aromatic ring to give 4-hydroxyamphetamine. Administration of amphetamine to the rat resulted in extensive 4-hydroxylation (60%; Dring et al., 1970). For methamphetamine the value was 53% (Caldwell et al., 1972a), for norephedrine, 35% (Sinsheimer et al., 1973) and for ephedrine 19% (Bralet et al., 1968) was accounted for as 4-hydroxy metabolites. In man, this ring hydroxylation is much reduced, only between 1 and 5% for amphetamine and 18% for methamphetamine. The guinea pig and rabbit show very little inclination to hydroxylate amphetamine, producing only between 0 - 5% of the dose as the 4-hydroxy metabolite.

ii) Beta-hydroxylation

This reaction is an important step in the biosynthesis of noradrenaline, beta-hydroxylation of dopamine by dopamine-β-hydroxylase occurring to give noradrenaline (see Rôle of Catecholamines in this Chapter). Such a reaction may

occur within the amphetamine group of compounds in vivo and in vitro (Goldstein and Anagnoste, 1965; Goldstein et al., 1964), to give derivatives of norephedrine. Thus with methamphetamine, 4% of the dose was accounted for as the β-hydroxylated compound norephedrine in man (Caldwell et al., 1972a), 16% as norephedrine in the rat and the degree of formation in the guinea pig was found to very between 1 and 19%, depending upon the dose (Caldwell et al., 1972a). Sever and co-workers (1973a)have reported the β-hydroxylation of amphetamine in man, in both normal and dependent subjects and found 2 to 3% was excreted in the urine during the first 24 hours. The aromatic ring hydroxylation product, 4-hydroxyamphetamine (Paredrine), has also been reported to be a substrate for dopamine-β-hydroxylase (Creveling et al., 1962; Sjoerdsma and Von Studnitz, 1963; Kopin et al., 1965) and also in vivo by Sever and co-workers (1973b),the product being 4-hydroxynorephedrine. This is a compound of particular interest since it may be implicated in the induction of tolerance to some of the actions of the amphetamines (see under 'Amphetamine, Phenmetrazine and Norephedrine: Effects on catecholamines and false transmitter theory).

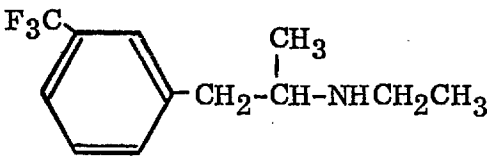
iii) Deamination

The pathway of deamination is favoured by the guinea pig and the rabbit and accounts for between 50 and 90% of the metabolism of amphetamine, methamphetamine and norephedrine (Williams, et al., 1973).

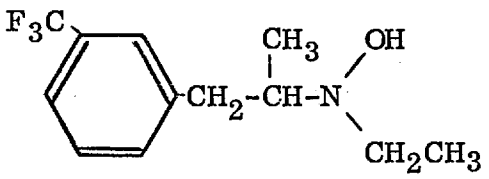
iv) N-Oxidation

This pathway is in no way a new biotransformation route, aromatic N-hydroxylation having been demonstrated by many workers (see review by Weisburger and Weisburger, 1973). The N-oxidation of many secondary and tertiary amines, both in vivo and in vitro has been reported (see Bickel, 1969 and Jenner, 1971, for reviews) and the more recent studies of Beckett (1971) indicated that some primary

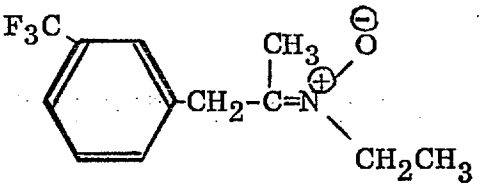
aliphatic amines may also be metabolised by N-oxidation. Beckett and Al-Sarraj (1973) have reported the identification, properties and analysis of N-hydroxy-amphetamine which they reported to be a major metabolite of amphetamine (see also McMahon et al., 1972; Lindeke et al., 1973). N-Hydroxy-4-methoxy-amphetamine has been claimed as a metabolite of 4-methoxyamphetamine (Beckett et al., 1973a). The N-hydroxy derivative of fenfluramine as an in vitro metabolite has been reported by Beckett and co-workers (1973a), although the major isolation product was the nitrone (see Fig. 1.3).



Fenfluramine



N-Hydroxyfenfluramine



Nitrone

Fig. 1.3

The N-hydroxylated compound could not be detected as a metabolite in vivo (Beckett and Brookes, 1967). The N-hydroxy metabolite of phenmetrazine was identified by Beckett and Salami (1972) in liver preparations and also in the whole animal (Beckett and Al-Sarraj, 1972). Using rabbit liver microsomes, Lindeke and co-workers (1973) have shown the hydroxylamines of phenylethylamine, phentermine and 4-chlorphentermine to be the major volatile metabolites using combined gas-liquid chromatography (g.l.c.) and mass spectrometry (m.s.) as well as nuclear magnetic resonance (n.m.r.) spectroscopy.

The Rôle of Catecholamines in Amphetamine Pharmacology

The effects of the amphetamines have been shown by many workers to closely resemble those seen on the stimulation of sympathetic nerves, thus involving the release of the chemical transmitter noradrenaline. The importance of this reaction is further amplified in the section entitled "Amphetamine, Phenmetrazine and Norephedrine: Effects on catecholamines and false transmitter theory". The amphetamines have been called sympathomimetic agents because their effects so closely resemble those of noradrenaline. Therefore it is proposed to present a very brief résumé on the neurohumor noradrenaline and also on its precursor dopamine since they are thought to be vital for the effects of the amphetamines.

Discovery, biosynthesis and rate-limiting step of noradrenaline formation

Although Langley (1901) showed that the effects of an extract of adrenal medulla resembled the responses resulting from stimulation of sympathetic nerves, it was Barger and Dale (1910) who first pointed out that this stimulation was mimicked much better by noradrenaline than adrenaline. But it was much later when Cannon and Rosenblueth in 1933 reiterated Bargers and Dale's conclusions but termed their substance "sympathin". It was left to von Euler in 1946 to show that noradrenaline and only a little adrenaline was present in sympathetically innervated tissues and that "sympathin" was identical to noradrenaline. In 1949, Peart conclusively showed that noradrenaline was released from splenic nerves in the cat by post-ganglionic stimulation, and in spite of some objections (Tainter and Luduena, 1950) noradrenaline was accepted as the only transmitter substance in adrenergic nerves in mammals (von Euler, 1959).

Shortly after the discovery of the enzyme, L-dihydroxyphenylalanine (L-Dopa) decarboxylase (Holtz et al., 1938), Blaschko (1939) proposed his biosynthetic

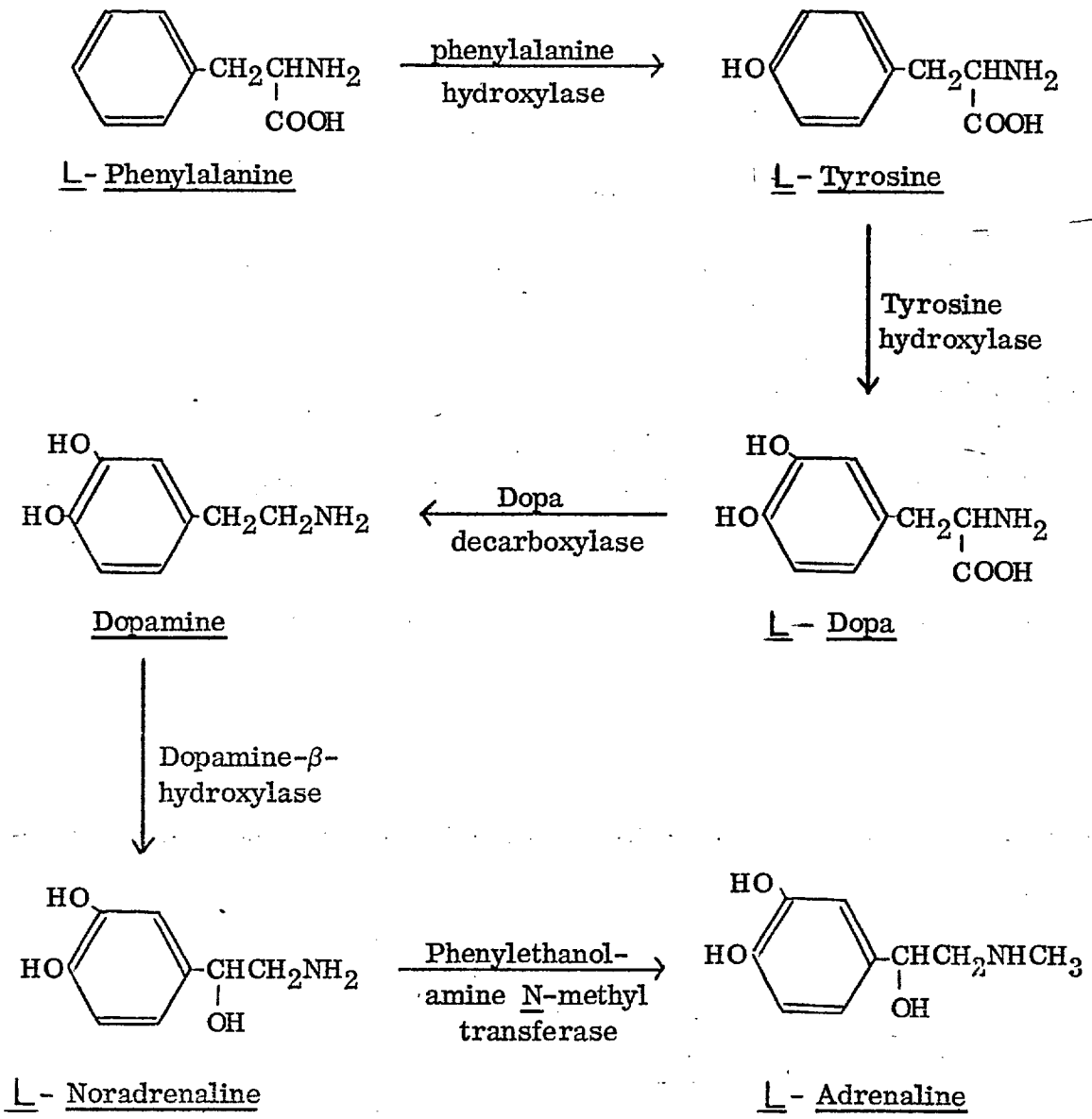


Fig. 1.4. Biosynthesis of Noradrenaline and Adrenaline

(After Blaschko, 1939)

scheme for the formation of adrenaline (Fig. 1.4). Recent research has suggested that this scheme may not provide the complete story. Laduron and Belpaire (1968) reported that tyrosine hydroxylase was not contained within catecholamine granules (see under Storage and uptake of noradrenaline and dopamine) as claimed by Nagatsu and co-workers (1964) and Udenfriend (1966). Thus it was proposed that hydroxylation of tyrosine and decarboxylation of Dopa occurred outside the granules and then the dopamine was taken up again into the granule for conversion to noradrenalin by dopamine-β-hydroxylase. Some noradrenaline, when coming out of the granules, was N-methylated into adrenaline and finally returned to the granules to be stored. Laduron (1972) considered this translocation too complex and reported that in the adrenal medulla, dopamine could be converted to epinine *in vitro*. Considering dopamine to be a neurotransmitter within the brain, Laduron reported that dopamine was converted to epinine in the rat brain but that the methyl donor was 5-methyl-tetrahydrofolic acid (Fig. 1.5) instead of the usual S-adenosylmethionine. Thus Laduron (1973) concluded that both *in vitro* and *in vivo*, epinine rather than noradrenaline was the immediate precursor in the adrenal medulla.

Dopamine + 5-methyltetrahydrofolic acid

$$\xrightarrow{\text{N-methyltransferase}} \text{Epinine + tetrahydrofolic acid}$$

Fig. 1.5

The formation of Dopa is a relatively slow process and Dopa does not accumulate in the tissues but is rapidly converted to dopamine and since dopamine is found in the urine it may indicate that excess is always formed. However, evidence suggests (Levitt *et al.*, 1965) that the rate-limiting step for noradrenaline biosynthesis is the conversion of tyrosine to Dopa, carried out by tyrosine hydroxylase. The

inhibition of tyrosine hydroxylase activity by a high concentration of noradrenaline may represent a mechanism for the positive feedback control of noradrenaline synthesis in vivo (Udenfriend, 1966; Wurtman and Axelrod, 1966).

Release of noradrenaline

Noradrenaline is released from most post-ganglionic sympathetic nerve endings. The primary event that leads to transmitter release is a depolarisation of the presynaptic terminal during the action potential of a nerve impulse. It has been suggested (Katz, 1962) that this depolarisation may be followed by an increase in the number of attachment sites on the inner surface of the presynaptic fibre for the pre-formed storage vesicles, these vesicles then attaching to the sites before extruding their contents into the synaptic cleft, calcium being an essential co-factor in the latter stages. This release of transmitter may take place by several mechanisms (De Potter, 1973), the last of the three being by far the most important.

i) release from vesicles into the cytosol and subsequent diffusion out of the cell,

ii) expulsion of whole vesicles,

and iii) exocytosis.

Two main mechanisms have been put forward to explain local feedback regulation of the release of noradrenaline from nerve terminals. The first is that initiated by Hedqvist in 1970 who proposed that the prostaglandins PGE_1 and PGE_2 mediate this feedback inhibition of noradrenaline release and the second is that noradrenaline itself, as well as sympathomimetic drugs, depress the secretion of noradrenaline by an action on α-receptors (Starke, 1972). Starke and Montel (1973) devised an experiment to determine the relative contribution of each mechanism and concluded that the two mechanisms were independent, but worked in parallel.

Thus there is the "prostaglandin loop" consisting of noradrenaline release - stimulation by noradrenaline of α-receptive sites - liberation of prostaglandins - action of prostaglandins on the nerve endings - inhibition of noradrenaline release. Then there is the "direct loop" consisting of noradrenaline release - stimulation by noradrenaline of (possibly prejunctional) α-receptive sites - inhibition of noradrenaline release.

Inactivation of noradrenaline and dopamine

The action of noradrenaline on effector cells is terminated when the concentration is reduced below a critical level. This level may be reached by (a) enzymatic action or (b) re-uptake mechanisms.

(a) The enzyme system principally concerned with deactivation is monoamine oxidase (MAO), discovered by Hare in 1928 and named MAO by Zeller in 1951 to distinguish it from the enzyme responsible for the oxidation of histamine and other diamines. It is possible that MAO is made up of several iso-enzymes (Sandler and Youdim, 1972). The enzymatic degradation of noradrenaline and dopamine may be found in Fig. 1.6. They are both oxidatively deaminated in the brain by MAO to their corresponding aldehyde metabolites.

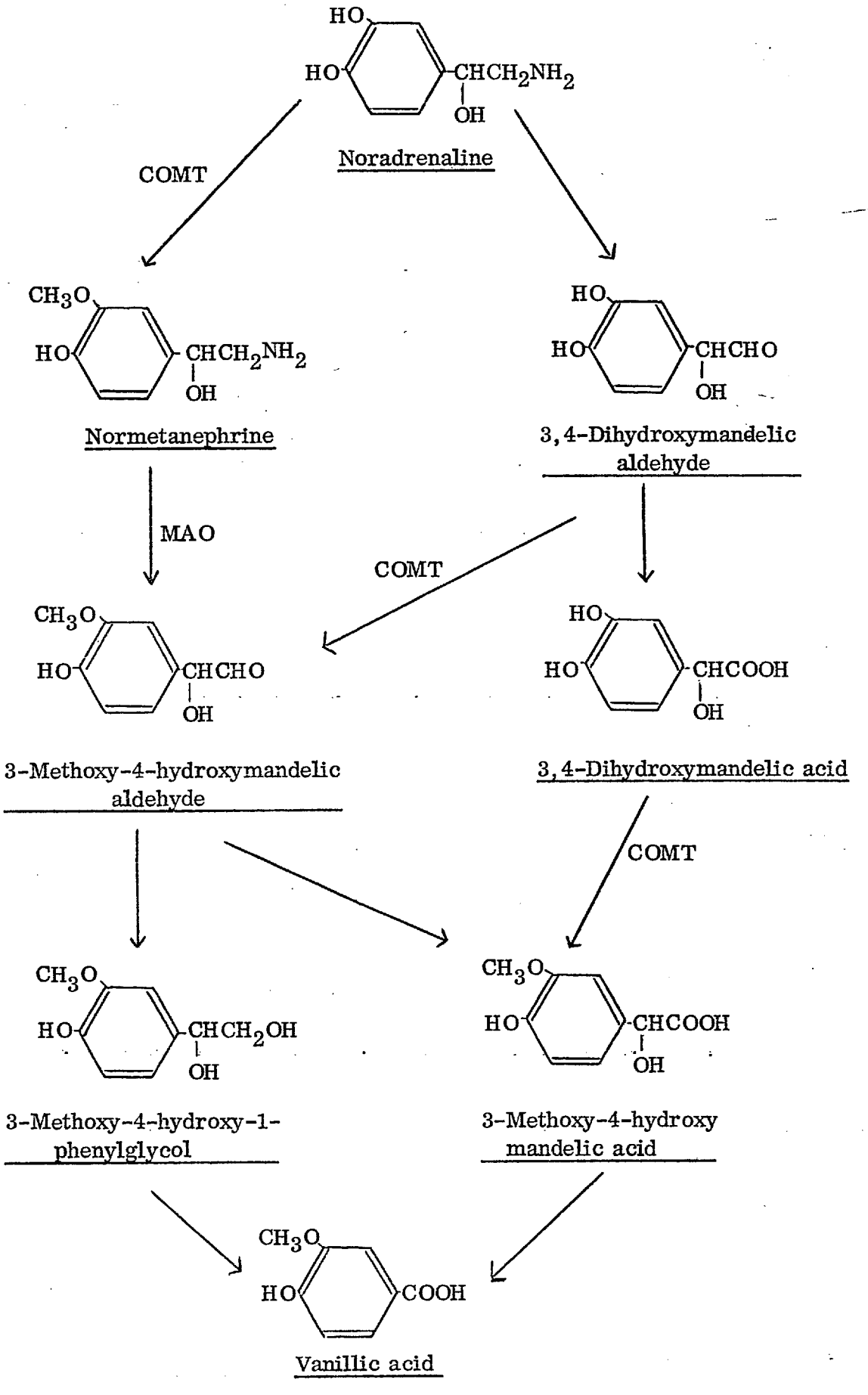
$$RCH_2\text{-}NH_2 + O_2 + H_2O \longrightarrow RCHO + H_2O_2 + NH_3.$$

The noradrenaline in the brain is metabolised primarily to the corresponding alcohol by an NADPH-dependent aldehyde reductase (Erwin, 1973) and dopamine is converted by brain tissue to an acid metabolite by an NAD-dependent aldehyde dehydrogenase.

(b) Strömblad and Nickerson (1961) suggested that re-uptake of catecholamines into neurones might represent an important mechanism for their inactivation. This uptake has been shown to proceed against a considerable concentration gradient (Axelrod et al., 1959; Whitby et al., 1961) and some authors (for example, Iversen, 1963) suggested that this uptake was mediated by a saturable membrane-transport process. This neuronal uptake showing structural and stereo-chemical specificity

Fig. 1.6. Main Pathways of Noradrenaline Breakdown

(After Axelrod, 1966)



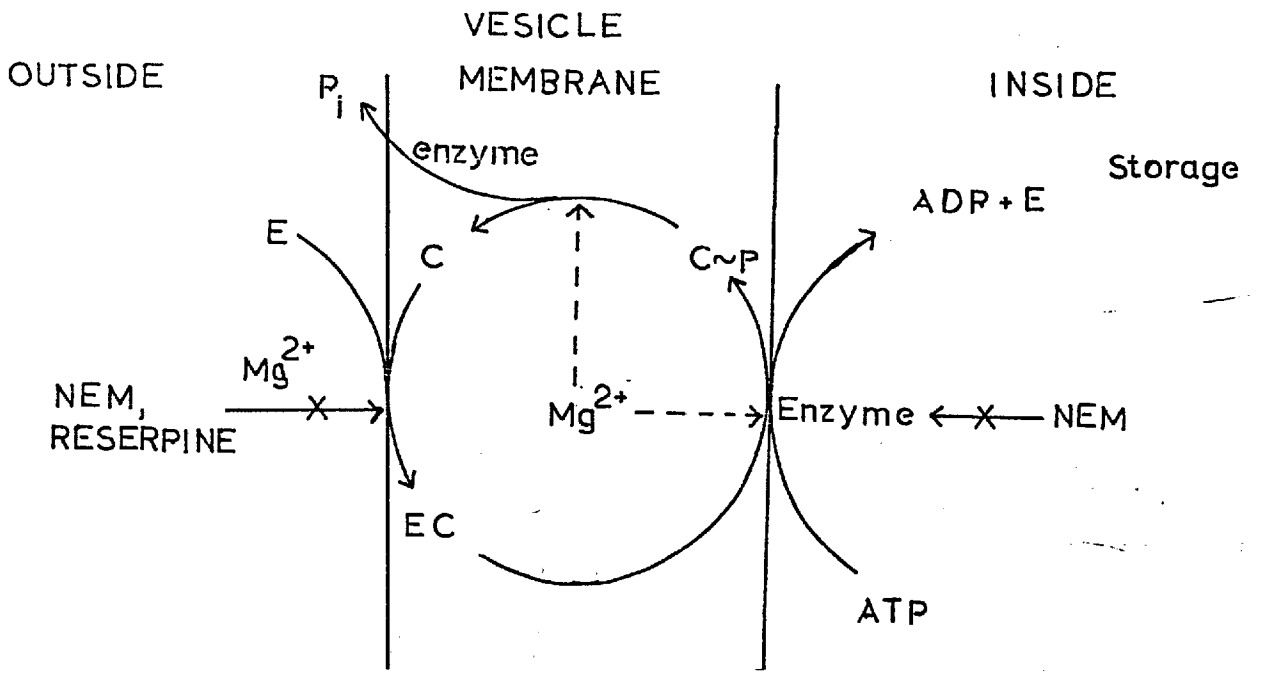
COMT = Catechol-O-methyltransferase. MAO = Monoamine oxidase.

has been termed "Uptake $_1$" (see Iversen, 1967). Some post-synaptic tissues, for example, cardiac muscle, may take up catecholamines by a different mechanism, termed "Uptake $_2$" by Iversen (1967) which is not so stereo-specific but may be important for inactivating circulating catecholamines (Iversen, 1973).

Storage and uptake of noradrenaline and dopamine

Catecholamine-containing granules were separated from the adrenal medulla by Blaschko and Welch (1953) and Hillarp and co-workers (1953) by density gradient centrifugation. Noradrenaline storage particles from the terminals of sympathetic nerves in the central nervous system have been isolated (Michaelson et al., 1964; Snyder et al., 1964) and found to resemble those of the adrenal medulla apart from size and density (Stjärne, 1964). Since the concentration of catecholamines is so high in the granules, it has been suggested that the amines form a storage complex with adenosine triphosphate (ATP) within the granule (Hillarp et al., 1958, 1959, 1960).

Administration of reserpine has been shown by many workers to deplete stores of noradrenaline, but on the administration of an indirectly-acting sympathomimetic amine, the action which the drug may cause is not grossly attenuated until the total noradrenaline content is very low indeed. This led Crout and his fellow-workers, in 1962, to postulate two pools of noradrenaline in sympathetic nerves. One store of "bound" noradrenaline which contained most of the noradrenaline and was capable of being depleted by reserpine and a second "available" pool of noradrenaline which may not be depleted by reserpine but which eventually emptied in the presence of reserpine when the reservoir of bound noradrenaline (from which the "available" pool was replenished) had been completely depleted. Recently pools containing newly synthesised catecholamines and "older"



Hypothetical model of catecholamine transport in storage vesicles, showing probable sites of action of reserpine and N-ethylmaleimide (NEM). E is epinephrine (adrenaline), C is carrier, EC is epinephrine carrier complex, C~P is phosphorylated carrier and P_i is inorganic phosphate

(After Slotkin, 1973).

Fig. 1.7

catecholamines have been postulated to explain the actions of some drugs, for example, the amphetamines (Fuxe and Ungerstedt, 1970), the newly synthesised catecholamines being stored in the reserpine-resistant pool. Besson and co-workers (1969) and Glowinski (1970) have demonstrated that amphetamine released newly synthesised dopamine from the corpus striatum.

The processes of storage and uptake are very different (Slotkin et al., 1971; Slotkin and Kirshner, 1971) and using data from a variety of sources, Slotkin (1973) has been able to construct a hypothetical model of catecholamine transport in adrenal medullary storage vesicles. This may well be extended to cover the processes in neurones. The model is illustrated in Fig. 1.7.

Amphetamine, Phenmetrazine and Norephedrine: Effects on Catecholamines and False Transmitter Theory

In 1954, Vogt suggested that the pharmacological effects of amphetamine were mediated by noradrenaline, a conclusion also reached by Moore and Lariviere in 1963 and also by Quinton and Halliwell in the same year. Stein in 1964 claimed that amphetamine exerted an indirect effect, that is, by causing the release of noradrenaline, as against a direct action with noradrenaline receptors as claimed by Van Rossum and co-workers (1962).

On studying the effect of amphetamine on noradrenaline, Axelrod et al., (1961) found that the drug initially increased plasma levels of the catecholamine but decreased the uptake of noradrenaline in the cat heart, spleen, adrenal gland and liver, but not skeletal muscle. Using ^{3}H-tyrosine and ^{3}-H-Dopa, Lewander (1970) found that phenmetrazine and, to a minor extent, amphetamine reduced the disappearance of brain dopamine, but not brain noradrenaline. Lewander claimed that it might have been caused by neuronal feed-back inhibition of the impulse flow in the dopamine neurone (see also Bunney and Aghajanian (1973). Lewander (1970) also found phenmetraz

to decrease the incorporation of ^{14}C-noradrenaline (produced in vivo from ^{14}C-tyrosine) into the brain which he thought due to either (i) interference with dopamine-β-hydroxylase, or (ii) the uptake of newly synthesised ^{14}C-noradrenaline into the storage granules. Since there is a small increase in the incorporation of ^{14}C-dopamine after phenmetrazine treatment, the former alternative (i) was favoured.

Injection of α-methyl-para-tyrosine (to inhibit catecholamine biosynthesis) in reserpinised rats, followed by an injection of phenmetrazine has been shown (Fuxe and Ungerstedt, 1970) to deplete amines from the noradrenaline nerve terminals in the septal area of the brain and, to a lesser extent, to cause a depletion at the dopamine nerve terminals. Cerebral dopamine has been cited by Randrup and Munkvad (1968) to be mainly responsible for the stereotyped behaviour (sniffing, licking, biting) seen after the administration of amphetamine. Extensive experimentation by Scheel-Krüger (1971) using both reserpine and α-methyl-para-tyrosine has shown that the locomotor stimulation and stereotyped behaviour produced by amphetamine, phenmetrazine and methamphetamine in rats pretreated with a high dose of reserpine, was correlated with the influence of these drugs on dopamine and its metabolism.

Many studies have indicated (Goldman et al., 1971; Berger et al., 1971; Holtzman and Jewett, 1971) that catecholamines, especially noradrenaline, are mediators for the anorexigenic effect of amphetamine. Sletten and co-workers in 1967, found that administration of chlorpromazine (which is a blocker of noradrenaline and dopamine receptors; Carlsson and Lindqvist, 1963; Corrodi et al., 1967; Andén et al., 1967) prevented the anorexigenic effects of phenmetrazine (Preludin) and chlorphentermine. Fenfluramine and 4-chloroamphetamine are also

anorexigenic compounds which have a strong effect on the metabolism of 5-hydroxytryptamine (Fuller et al., 1965; Opitz, 1967) and recently Frey and Schulz (1973) have suggested that noradrenaline, dopamine and 5-hydroxytryptamine are all involved in the anorexigenic effect of 4-chloroamphetamine.

The central actions of norephedrine and its effect on catecholamines have been less well documented than those of amphetamine or phenmetrazine. The general pharmacology of norephedrine may be found in Chapter 4. Burgen and Iversen (1965) reported that the (±)-form of norephedrine had a relatively low uptake affinity for the site of noradrenaline uptake, about five times less effective than (±)-amphetamine. The 4-hydroxylated derivative, 4-hydroxynorephedrine, has recently become important because of its possible involvement in the induction of tolerance to some of the effects of amphetamine which occurs in animals (Tormey and Lasagna, 1960) and in man (Rosenberg et al., 1963). (+)-Amphetamine has been shown to form 4-hydroxynorephedrine in the rat heart (Goldstein and Anagnoste, 1965; Brodie et al., 1970) and spleen (Thoenen et al., 1966) and also in central adrenergic neurones (Groppetti and Costa, 1969, 1970; Brodie et al., 1970; Lewander, 1970). It has also been found in the urine of human subjects (Caldwell et al., 1972b; Sever et al., 1973a)dosed with amphetamine. Tolerance develops to the anorexigenic and hyperthermic effects of amphetamine on chronic administration (Harrison et al., 1952; Kosman and Unna, 1967) together with a decline in the stores of noradrenaline (Brodie et al., 1969). But the prolonged depletion of noradrenaline far exceeded the half-life of amphetamine in the body and was related to the amount of 4-hydroxynorephedrine formed as a metabolite (Costa and Groppetti, 1970). The metabolite, 4-hydroxynorephedrine was taken up by sympathetic nerve endings (Carlsson and Waldeck, 1964) and liberated by subsequent nerve stimulation (Thoenen et al., 1966). Further, it was released

by (+)-amphetamine preferentially with respect to noradrenaline (Brodie et al., 1970). However, this apparent 'false' transmitter, 4-hydroxynorephedrine only produced tolerance to the peripheral effects of amphetamine such as the increased urinary excretion of noradrenaline, lipolysis and cardiovascular effects (Lewander, 1971b) and hyperthermia (Gessa et al., 1969). Central effects such as anorexia, central excitation or increased urinary excretion of adrenaline (Lewander, 1971b) to which tolerance develops, were not affected by 4-hydroxynorephedrine. Other central effects such as increased motor activity and stereotyped behaviour to which tolerance does not develop was also unaffected by the 'false' transmitter, implying that different mechanisms were involved in the induction of tolerance to the central effects.

Further evidence that 4-hydroxynorephedrine cannot totally explain tolerance to some of the actions of amphetamine may be found in the fact that the (-)-antipode of amphetamine fails to form the 'false' transmitter (Goldstein and Anagnoste, 1965) and yet noradrenaline is released by (-)-amphetamine from various tissues and elicits peripheral and central effects similar to the (+)-isomer. The depletion of noradrenaline after administration of (+)-amphetamine to rats has been shown to be greater than in guinea pigs (Costa and Gropetti, 1970). This was supposedly due to the fact that whereas the rat was capable of both β-hydroxylation and extensive ring hydroxylation, the guinea pig extensively deaminated the amphetamine (Williams, et al., 1973) thus implying that hardly any 4-hydroxynorephedrine would be formed in the guinea pig and therefore the guinea pig should not become tolerant to the peripheral effects of amphetamine. However, it has been demonstrated in our laboratory (Sever, 1973 - unpublished results) that not only does the guinea pig produce 4-hydroxynorephedrine from (+)-amphetamine, but it does so in the same amount as in the rat similarly dosed (approx. 3%). Further,

tolerance has been demonstrated to the hyperthermic effect of amphetamine, a peripheral effect supposedly mediated by noradrenaline.

CHAPTER TWO

Synthesis of [^{14}C]-Preludin and the Fate of the Drug in the Rat, Guinea Pig, Tamarin Monkey and Man

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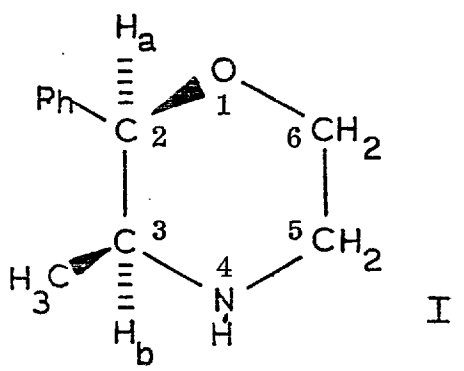
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INTRODUCTION

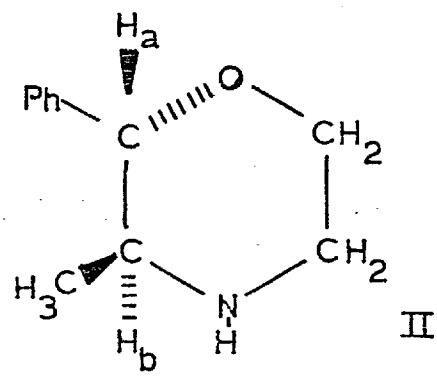
Phenmetrazine hydrochloride (Preludin), (±)-threo-2-phenyl-3-methyl-morpholine was first marketed in 1954 as an anorexigenic compound, and like nearly all of the successful anorexigenic drugs, it was based on the amphetamine nucleus and hence liable for abuse as a central nervous stimulant. Van Praag (1968) has, in a review, quoted many relevant German reports concerning the demonstration of the central effects of Preludin, both in animals and man, although its action was seven to ten times weaker than amphetamine. More detailed information concerning the pharmacology and abuse of the drug will be discussed later in the relevant sections, but primarily it is necessary to clarify the chemistry of the drug.

Chemical aspects of Preludin

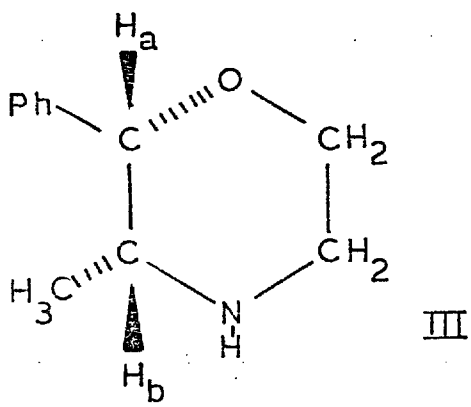
There are two asymmetric carbons in the phenmetrazine molecule, namely those at positions 2 and 3 in the ring structure. There are therefore four isomers and these are shown below.



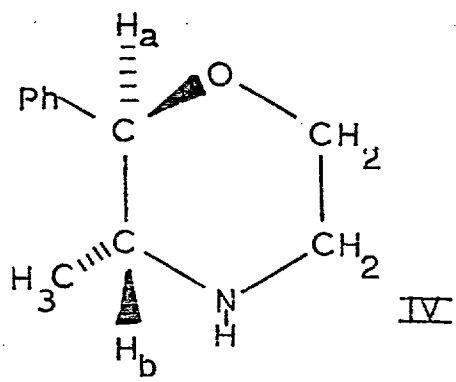
I



II

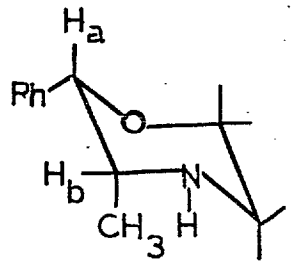


III

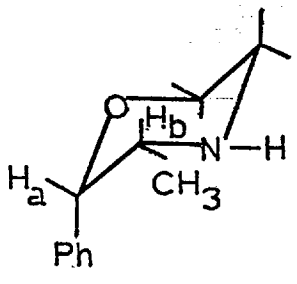


IV

In structures I and III, the protons H_a and H_b are cis- to one another, that is on the same side of the ring structure, and thus represent the cis-configuration. In structure II and IV, the protons H_a and H_b are on opposite sides of the ring structure, that is in the trans-configuration. Furthermore, structures I and III have the erythro-configuration and structures II and IV in the threo-configuration. Each of these configurations may exist in a different conformation, for example:-

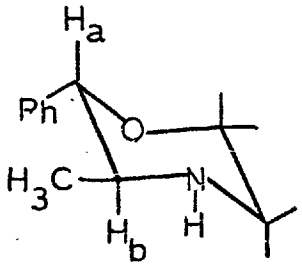




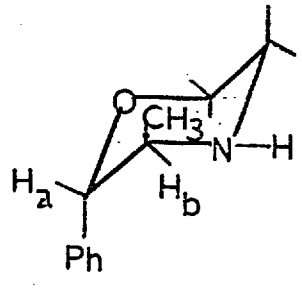


Conformer A cis-

Conformer B cis-



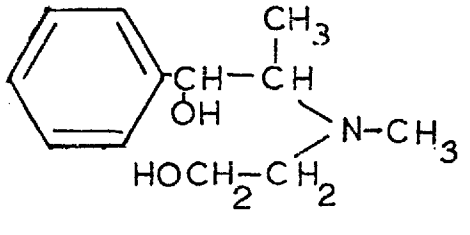


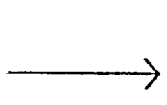


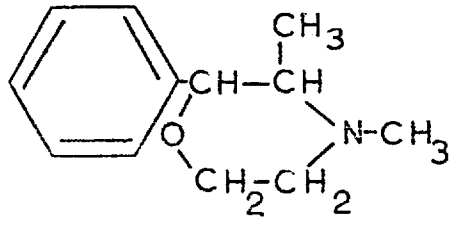
Conformer C trans-

Conformer D trans-

Synthesis of the N-methyl analogue of phenmetrazine, phendimetrazine (for structure see below) was carried out in 1962, utilizing the acid-catalysed cyclization of N-(2-hydroxyethyl)-(-)-ephedrine (Dvornik and Schilling, 1965).







N-(2-Hydroxyethyl)-(-)-ephedrine

(+)-3,4-Dimethyl-2-phenylmorpholine (phendimetrazine)

Dvornik and Schilling (1965) established a threo-configuration for the cyclized product and also assigned the absolute configurations around carbons 2 and 3, namely 2S:3S (Cahn et al., 1956) for the threo-configuration and 2R:3S for the erythro-configuration. Clarke in 1962 also showed a threo-configuration for phenmetrazine but to obtain this form he used the N-(2-hydroxyethyl) derivatives of racemic norephedrine and pseudo-norephedrine (for the configurations of norephedrine and pseudo-norephedrine see Zimmerman and English, 1954).

A point worthy of comment, is that concerning a change in configuration when cyclization takes place in an acid medium. Thus, in the synthesis of phendimetrazine, a change is seen in the optical rotation of the N-(2-hydroxyethyl) derivative from -30.5° (erythro-configuration) to +132.0° for phendimetrazine in the threo-configuration. This is due to a Walden inversion at position 2 in the ring structure (Foltz and Wilkop, 1957).

Preludin is the racemic threo-form of phenmetrazine and Clarke (1962) has stated that optically active cyclized products would be obtained if the norephedrines were first resolved. Clarke also synthesised the erythro-and threo-forms of the lactam derivatives of phenmetrazine. More will be said concerning the complex stereochemistry of phenmetrazine in the Discussion, in relation to the structural possibilities of the metabolites.

Pharmacology

The pharmacological effects of Preludin may be subdivided into cardiovascular, central and anorexigenic efficacy of Preludin.

a) Cardiovascular effects

Like amphetamine, Thomä and Wick (1954) found that Preludin was a pressor

agent, although its action was approximately one thousand times less than that of adrenaline. During a study of the effects of Preludin in human cardiovascular disease, Fletcher (1961) administered the drug intravenously to normotensive and hypertensive patients. In both cases, he noted a rise in systolic and diastolic arterial pressures which fell to their previous levels approximately an hour later. Administration of the drug every thirty minutes resulted in a stepwise increase in both systolic and diastolic pressures. The increase in blood pressure was proportionally higher in the hypertensive patients. He observed no effect on the pulse rate.

However, Ehrlich (1962) experimenting with dogs dosed with approximately clinical doses of Preludin exhibited a slowing of the pulse and an increased blood pressure at rest. Martin and co-workers (1971) found a dose-related increase in blood pressure of human subjects dosed subcutaneously with Preludin and the blood pressure changes were more characteristic of those produced by noradrenaline than by adrenaline. Martin also reported that a tachycardia was produced but there proved to be an overall negative correlation between pulse rate (which increased) and blood pressure. In their studies, Thomä and Wick (1954) indicated that the pressor action of Preludin was due to a direct action since not only its pressor action but also its effect on cat nictitating membrane were enhanced rather than antagonised by cocaine. This fact tends to link with the idea that noradrenaline does not mediate all the central actions of the drug (see later under (b) Central Actions).

Recent cardiovascular studies have been concerned with the possible link between anorexigenic drugs and progressive pulmonary hypertension. Blumberger and Lorenz (1971), using the guinea pig isolated heart, defined the cardiotoxicity of some anorexigenic drugs by the occurrence of a negative inotropic effect, as

shown by the decrease in the maximum speed of increase in pressure measured in the left ventricle. They produced a list of anorexigenic compounds in increasing order of cardiotoxicity:- amphetamine, aminorex, phenmetrazine, diethylpropion, phentermine, chlorphentermine and fenfluramine. They also measured the increase in coronary blood flow and heart rate and found lower values with the above anorexigenic drugs than they did with noradrenaline, adrenaline, ephedrine and tyramine. They concluded that a connection between possible pulmonary hypertension and cardiotoxicity was unlikely with the anorexigenic drugs tested.

A similar conclusion was reached by Engelhardt and co-workers (1971) during acute and chronic toxicity testing in dogs. From the results of the acute tests they were able to divide the anorexigenic compounds tested into three subdivisions.

i) Those drugs which had a parallel effect on aortic and pulmonary arterial pressure, for example amphetamine, methamphetamine, phenmetrazine.

ii) Those drugs showing a relatively stronger pressor effect on pulmonary arterial pressure than on aortic pressure, for example fenfluramine, aminorex, chlorphentermine. Aminorex gave the largest rise in pulmonary arterial pressure. (Gurtner et al., 1968, have been reported by Seiler and Wasserman, 1973, as having demonstrated that aminorex gives rise to severe pulmonary hypertension in man).

iii) Those drugs which showed no effect on either pulmonary, arterial or aortic circulation, for example, prolintane.

On examining the mechanism involved in drug-induced pulmonary hypertension, Seiler and Wasserman (1973) found a correlation between the increase of 5-hydroxytryptamine (5-HT) concentration in the lungs and the degree of pulmonary hypertension. Besides affecting the liberation and accumulation of 5-HT, some

anorexigenic drugs seemed to influence the metabolic breakdown of the amine. Therefore, using aminorex, chlorphentermine, phentermine and phenmetrazine (Preludin) they determined the monoamine oxidase activity of these compounds in vitro. They concluded that since Preludin did not inhibit the monoamine oxidase activity of rat liver mitochondria, but did increase the excretion of 5-hydroxyindol-3-acetic acid, there must be another mechanism by which 5-HT biosynthesis was stimulated (Seiler and Wasserman, 1973).

b) Central actions

The central actions of phenmetrazine are very similar to those of amphetamine, that is, motor stimulation, hyperactivity and stereotyped behaviour. It is generally accepted that the catecholamines dopamine and noradrenaline act as neurotransmitters in the central nervous system and it has also become increasingly clear that the central stimulant effects of amphetamine and its closely related congeners such as Preludin rely on an interaction with the brain catecholamines for some of their actions. A more detailed discussion of the interaction of amphetamine and phenmetrazine (Preludin) with the catecholamines is to be found elsewhere in this thesis (see under "Amphetamine, Phenmetrazine and Norephedrine: Effects on catecholamines and false transmitter theory"). Pharmacological evidence for such an indirect action has accumulated using α-methyl-para-tyrosine to inhibit the biosynthesis of the catecholamines (Hanson, 1967; Randrup and Munkvad, 1966; Weissman and Koe, 1965; Weissman et al., 1966), as well as using dopamine-β-hydroxylase inhibitors such as diethyldithiocarbamate (Jonsson and Lewander, 1973). Van Rossum and co-workers, in 1962, proposed that the central action of minimal effective doses of (+)-amphetamine did not involve a release of brain noradrenaline, a result also reported by Smith

(1963, 1965). This result was extended to incorporate Preludin (as well as aminorex and 4-chloroamphetamine) by Costa and co-workers (1971). Whilst many researchers still attempt to resolve which of the catecholamines is responsible for psychomotor stimulation, the production of the syndrome known as stereotyped behaviour appears to be brought about by an action of the drugs on dopamine in the corpus striatum of the brain (Fog et al., 1970, 1971; Jonas and Scheel-Krüger, 1969; Scheel-Krüger, 1971, 1972). In its extreme form stereotyped activity consists of continuous repetition of several types of behaviour. In rats for example, dosed with amphetamine, locomotion, rearing and sniffing are increased. Grooming may decrease, and after approximately one hour, locomotion and rearing disappear. Sniffing at the bottom of the cage, together with movement of the head and forelegs remain. The sniffing may then become associated with licking and biting cage wires (Randrup and Munkvad, 1972). This stereotyped activity has been shown to occur in many species when dosed with amphetamine and closely related compounds such as Preludin. This drug has been shown to be capable of inducing stereotyped behaviour in rats, cats and dogs by Wallach et al., (1973), corroborating the data of Fog (1969) and Scheel-Krüger (1971) who found stereotyped behaviour in rats. This behaviour has also been observed in humans and has been termed "punding" in Sweden (Rylander, 1971).

c) Anorexigenic efficacy of Preludin

Western Society has recognised that the disease of obesity can affect general health as well as life insurance policies. Obesity has been shown to induce cardiovascular complaints, diabetes mellitus and ischaemic heart disease (Court, 1972). Eastern culture has reached no such frightening conclusion since

in India, for example, the girth of one's wife (or wives) is directly proportional to one's social status. However, in the West, whether for reasons of health or vanity, much research has gone on in the field of anorexigenic compounds. The aetiology of obesity is not relevant here but the site of action of Preludin is probably within the hypothalamus. Stark and Totty, in 1967, carried out experiments using rats with chronically implanted electrodes in the lateral hypothalamus and they measured the current intensity necessary to elicit eating after the rats had been fed. They then administered Preludin by the intra-peritoneal route at several dose levels and found that the current intensity had to be increased to elicit feeding. Kabak and Nikitina (1966) used rats whose ventromedial nucleus (important in the termination of feeding) had been destroyed. Over a period of 1.5 to 2.0 months, obesity in the rats could be controlled by giving daily doses of Preludin ranging from 2 to 15 mg. Kabak and Nikitina also reported on the induction of tolerance to the anorexigenic effect of Preludin. Preludin has been shown to decrease food consumption in rats (Spengler and Waser, 1959; Varela and Moreiras-Varela, 1964; Carpi and Giaroli, 1966; Hoffman, 1966) and in mice (Friedman, 1962; Cullen and Swartz, 1964; Arney and Swartz, 1965) and Mercier and Dessaigne (1968) have shown a depressed voracity in the Praying Mantis given a dose of 100-1000 μg/g of Preludin. Farner, (1961) using rats, found that the appetite depressant effect of Preludin was greater in older rats than in younger ones.

Investigations in humans have shown phenmetrazine to be an effective appetite supressant (Laurian, 1968; Müting, 1957; Scheffler, 1956). Weill and Bernfeld (1957) claimed a cure for infantile obesity with Preludin on obese two to fifteen year old children. In diabetic patients, an increase in body weight not only affects the heart and circulation, but the whole metabolism (Müting, 1957). Thus, an overweight diabetic tends to exercise less and

therefore reduce carbohydrate metabolism. Müting successfully reduced the weight of a group of obese diabetics using Preludin. Furthermore, in four out of seven diabetics having insulin, after treatment with Preludin it was found that the dose of insulin could be reduced.

Biochemistry of Preludin

The biochemical effects of Preludin have been studied mainly in experiments concerning obese patients. Data has been accumulated from both normal and obese subjects, given a single dose, or series of doses of Preludin and parameters measured have included blood glucose, free fatty acid (FFA) levels and insulin (bound and free).

The relationship of the sympathetic nervous system to fat mobilization (reviewed by Havel in 1965) suggested that the metabolic effect of anorexigenic compounds in raising FFA levels was due to the sympathomimetic activity of the compounds. In experiments carried out by Laurian, (1968) using Preludin on obese and normal patients, he found that the levels of serum FFA in obese patients were increased to those levels found in normal patients. But this effect of correcting the FFA levels was only maintained during drug therapy and the levels returned to their original state once the drug was withdrawn. Contrary to these results, both Penick and Hinkle (1963) and Gaut and co-workers (1969) failed to detect any significant difference in serum FFA levels in obese patients treated with Preludin. Amphetamine and amphetamine-like compounds can stimulate fat mobilization but appear to have no direct lipolytic action (Finger et al., 1966; Finger and Feller, 1966; Mühlbachova et al., 1964). They therefore appear to act via some indirect mechanism (Opitz, 1970) which is not fully understood but may involve endogenous catecholamine release (Pinter and Pattee, 1970).

In normal persons, the most important carbohydrate metabolism pathway is the glycolytic pathway, but in obese persons there occurs an increase in the activity of the pentose phosphate pathway. The level of this particular pathway was found to be high in the erythrocytes of overweight women. Sonka and co-workers (1963) proceeded to show that treatment with the dextrorotatory isomer of phenmetrazine inhibited changes in the reduction of the pentose phosphate pathway due to diet and muscular exercise, thus implying that (+)-phenmetrazine did not exert an anorexigenic effect via the pentose phosphate pathway.

Balaz (1963) has been reported as concluding that Preludin has a normalising effect on some biochemical parameters since he demonstrated a higher blood-glucose level for sixty minutes after a dose of Preludin in obese subjects. No appreciable changes in blood glucose levels have been found in normal subjects receiving Preludin (Thomä and Wick, 1954; Fineberg, 1962; Howard, 1964). On treating mice with Preludin, Ohnesorgeu and Khan (1961) showed a high level of oxygen consumption in the mice, possibly demonstrating that the glucose being taken up by the tissues was metabolised and not synthesised into lipids or stored.

Investigations have been carried out on free and bound insulin levels in human plasma. Dascalu and co-workers (1965) have been reported as having treated obese patients with Preludin and found that it appeared to normalise the plasma level of free insulin, whilst that value in untreated obese patients was approximately 50% greater. The plasma-bound insulin activity appeared not to be affected by obesity or Preludin. A similar conclusion has been reported by Pora in 1965, using rats. He is also reported to have found that intravenous administration of glucose to untreated rats liberated almost all the plasma-bound insulin, but the effect was less marked when given to rats treated with Preludin.

Preludin has been reported to decrease high blood-cholesterol levels and

increase the level of blood phospholipids. D'Agata and co-worker (1958) were reported as having found improved anaesthesia with frequent decrease of appetite in patients. chronically dosed with Preludin. A similar situation has been reported recently by Johnston and co-workers (1972) whereby chronic ingestion of (+)-amphetamine led to a lower halothane requirement in dogs for anaesthesia, although this was not so for an acute dose of the drug. The suggested explanation for this was that whilst an acute dose of the drug released catecholamines from adrenergic nerve terminals in the central nervous system as well as peripherally, chronic administration caused a depletion of central nervous system catecholamines.

Dependence on and abuse of Preludin

The amphetamine-like anorexigenic drugs have been prescribed for long periods and due to the rapidly developing tolerance to their anorexigenic effect, it was tempting for both the patient and the doctor to increase the dose for the required effect, thus increasing the risk of dependence. Reports on dependence on Preludin began to appear from 1956 and Van Praag (1968), in a review, has collected together nearly all the early reports of dependence to the drug. Initially there was some uncertainty as to whether Preludin was really a drug of dependence, or whether its abuse relied upon habituation or tolerance. Although it was demonstrated that a patient could have an overwhelming craving for the drug and become mentally dependent on it, (Oswald and Thacore, 1963), the occurrence of a withdrawal syndrome was refuted. However, it was later reported (Van Praag, 1968) that the effects of withdrawal were largely psychological, for example, lethargy. The physical symptoms were far less prominant than with other major drugs, for example, the opiates. However, in 1963, Oswald and Thacore demonstrated that physical dependence can occur, by showing that rapid eye movement (REM) sleep showed a marked increase when Preludin was

discontinued, and vanished abruptly when Preludin was resumed.

Prior to 1960, major abuse of the amphetamines as well as Preludin was by the oral route, but by 1963-1964, Preludin was being injected intravenously. The addicts would inject 1000-2000 mg of Preludin in twenty-four hours, this being approximately four times the therapeutic dosage of 50-75 mg per day taken orally (Rylander, 1971). After such a massive dose, self-confidence is increased and associations of ideas are hastened. Perceptions of all senses are sharpened and partially changed, for example, the addicts cannot stand spiced food or alcohol for it stings the tongue and burns the throat. Changes in hearing and sight may be of importance in the development of ideas of reference, of illusions, and of hallucinations leading to psychotic reactions (Rylander, 1972). Strong sexual stimulation can occur with such large doses of Preludin. Rylander (1971, 1972), has classified stereotyped behaviour in Preludin addicts as consisting of two types of behaviour:-

i) "Punding" (a term originally coined by addicts). This type of movement denotes a sense of purpose organised and directed, but totally meaningless. Such activities may be considered as analagous to the stereotyped behaviour seen in animals.

ii) Athetotic-like movements (the jerking syndrome). These movements consist of meaningless, automatic, unwilled movements.

"Punding" movements may manifest themselves in activities such as polishing nails (even until they bleed, which does not prevent the addict continuing), dismantling clocks and motors, wandering the streets aimlessly, combing the hair, tidying up things, or driving a car until it runs out of petrol. Some addicts indulge in artistic "punding", for example, drawing, painting, writing or playing an instrument. Generally, the addict is doing something he is used to, or likes to do when he "punds". The majority of addicts have reported the onset of their "punding"

one to two hours after the Preludin injection, and this state may go on for hours or days. It appears that "punding" is not broken off voluntarily and addicts may become severely agitated if forced to return to their everyday life. There is very little anxiety associated with "punding" and whilst in this state addicts do not eat or drink and may postpone defaecation and micturition.

Some addicts described screwing or twisting movements of the body and some facial grimaces, chewing and/or grinding of teeth and constant biting of the tongue (Rylander, 1972). Odd finger and arm movements may become apparent and the addicts may stare at their finger tips and claim to have animals on them. Whilst walking, the addict may tend to lift his legs very high.

Chronic abuse of Preludin can lead to psychoses (Quadbeck and Schmitt, 1966), although the smallest single dose known to be followed by a psychosis was 50 mg (see Van Praag, 1968). Preludin psychoses have wide and varying forms. These may be conveniently sub-divided thus:

i) Deleriant syndromes in which motor unrest occurs. Mainly visual hallucinations and delusions of an anorexigenic effect occur. Thinking may become muddled.

ii) Schizophrenic syndromes in which paranoid thoughts are developed. The addict becomes restless and agitated, has a sense of being threatened and hears voices which confirm his suspicions.

iii) Organic brain syndromes. The Japanese have reported (see Van Praag, 1968) that in chronic abuse of amphetamine derivatives approximately 10% of cases have histo-pathologically demonstrable brain lesions leading to a form of dementia.

iv) Neurological effects. Bartholomew and Marley, (1959) studied twelve cases of Preludin intoxication. They found that physical signs included mydriasis,

impaired pupillary response to light, impaired reaction to accommodation/ convergence, tremor of the tongue and upper limbs together with augmented tendon reflexes, facial twitchings and excessive small movements of the hands, as well as mood changes including depression. Paranoid ideas were present in many addicts as well as auditory hallucinations, impaired attention, concentration and disorientation. Psychoses to central stimulant drugs are also discussed by Rylander (1972).

Van Praag (1968) discussed Sweden's dilemma, the number of Preludin addicts in 1966 being approximately 4000. The black market price for one tablet then was approximately 20p and according to Van Praag (1968), some 15 million Preludin tablets were being annually smuggled into the country. He also reported that the Swedish Medical Association had requested that the manufacturer cease production of Preludin. In fact what happened was the introduction of "Preludin compositum" a preparation containing 15 mg Preludin and 5 mg of a laxative, 3,3-bis-(4-acetoxyphenyl)-2-indolinone (Bienert and Uberla, 1967). This proved no solution to the problem and late in 1966 the drug was banned in Sweden. This was no deterrent to the drug smugglers as a recent report in a daily newspaper indicated. About £5 million worth of Preludin was seized in Germany prior to its illegal shipment to Scandinavia by lorry (The Times, 1972).

Materials and Methods

Compounds

(±)-Norephedrine hydrochloride, m.p. 190-192°C, saccharo-1,4-lactone, NAD^+, $NADP^+$ (sodium salt), D-glucose-6-phosphate (disodium salt), glucose-6-phosphate dehydrogenase (ex. Baker's Yeast) were all purchased from the Sigma Chemical Company Ltd., London, U.K. 4-Aminoantipyrine, m.p. 107.5-109°C (Ralph N. Emanuel Ltd., Wembley, Middx., U.K.), (±)-4-hydroxy-norephedrine, m.p. 198°C (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.), m-chloroperbenzoic acid, m.p. 92-94°C (decomp.), ethyl chloroacetate, $n_D^{20} = 1.4226$ (B.D.H. Chemicals Ltd., Poole, Dorset, U.K.), ethylene chlorhydrin, $n_D^{20} = 1.4419$, N-benzylethanolamine, $n_D^{20} = 1.5434$ (Koch-Light Ltd., Colnbrook, Bucks., U.K.), (±)-methamphetamine hydrochloride, m.p. 132-134°C (K & K Laboratories Inc., Plainview, New York, U.S.A.) were purchased.

The following compounds were obtained as gifts: Preludin, m.p. 177°C, (phenmetrazine hydrochloride, (±)-trans-2-phenyl-3-methyl morpholine hydrochloride), C.H. Boehringer Sohn, 6507 Ingelheim am Rhein, West Germany; Fenmetramide, m.p. 141-142°C ((±)-cis-2-phenyl-3-methyl-5-morpholinone), McNeil Laboratories Inc., Fort Washington, Pa., U.S.A.; (±)-amphetamine sulphate, m.p. 302°C (decomp.), S.K. & F. Laboratories, Philadelphia, Pa., U.S.A.; (+)-phendimetrazine bitartrate, ((±)-threo-2-phenyl-3,4-dimethyl-morpholine), m.p. 204-206°C, Ayerst Laboratories, Canada.

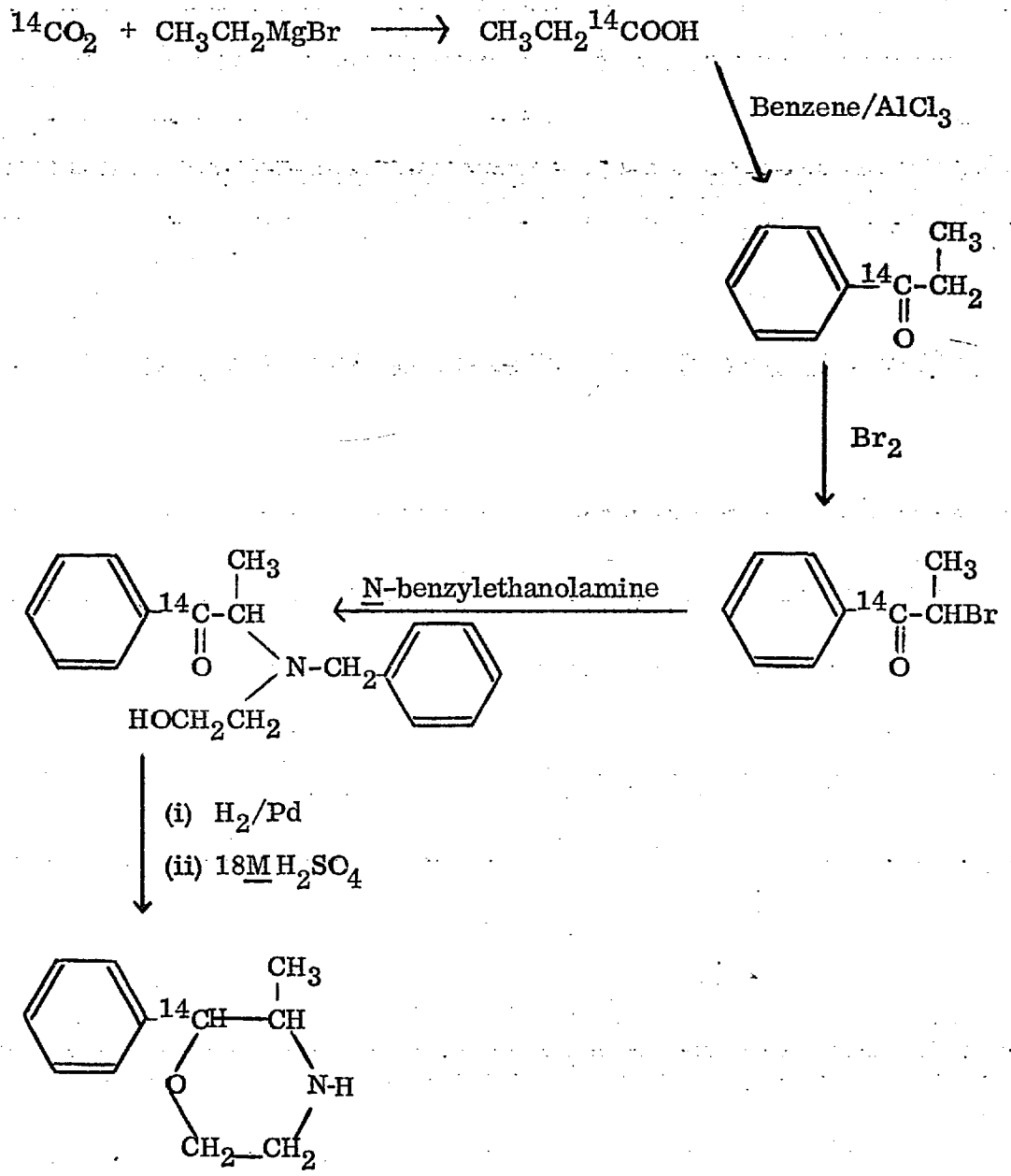
All melting points are uncorrected.

(±)-trans-2-Phenyl-[2-^{14}C]-3-methylmorpholine hydrochloride. [^{14}C]-Phenmetrazine

The synthesis of [^{14}C]-Preludin was carried out by two methods. The first method was basically that of Engelhardt and co-workers (1958) and the second followed essentially the method of Klosa (1963).

Method 1.

The sequence of reactions may be illustrated as follows (see Fig. 2.1).



Phenmetrazine

Fig. 2.1

[carboxy-^{14}C]-Propionic acid

Ethyl magnesium bromide (I) Magnesium turnings (2.4g) and one crystal of iodine were placed in the bottom of 500 ml round-bottomed flask and Na-dried diethyl ether (60 ml) was added. The suspension was continuously stirred and ethyl bromide (7.51 ml), previously pipetted into a dropping funnel containing dry diethyl ether (40 ml) was allowed to drip onto the ether/Mg suspension and slight heat was applied (approx. 35°C). After about two minutes the straw-colour of the iodine disappeared and the reaction mixture became grey and cloudy. Whilst the mixture was refluxed the ethereal solution of ethyl bromide was added dropwise (approx. 18 drops per minute) and the mixture became cloudier. After the reaction had ceased, more dry ether was added in order to obtain a 0.5M solution of the Grignard. The exact molarity was determined by titration (Calvin et al., 1949).

Carbonation of the Grignard (II) The apparatus for the incorporation of the ^{14}C from $^{14}CO_2$ (prepared by the action of 18M H_2SO_4 on $Ba^{14}CO_3$) into the Grignard, ethyl magnesium bromide was essentially as described by Calvin and co-workers (1949).

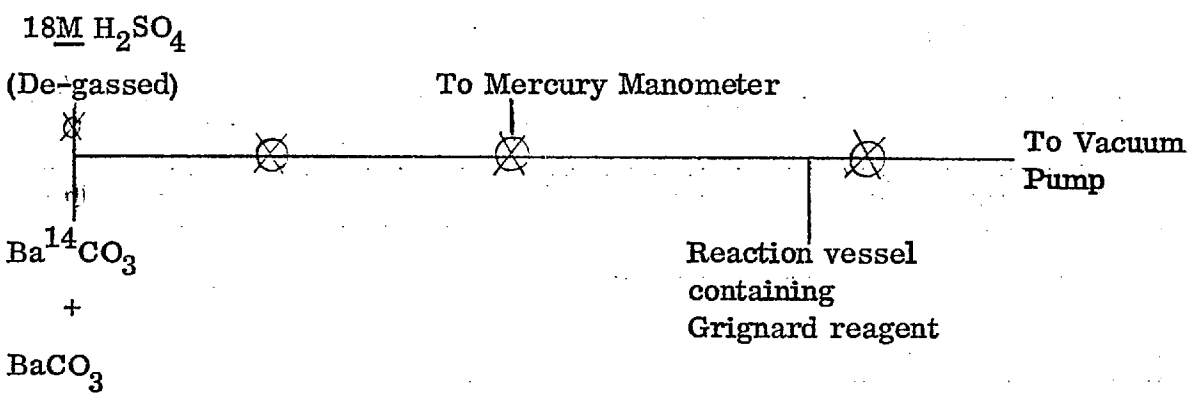


Fig. 2.2. Schematic Representation of Carbonation Apparatus

The Grignard solution was frozen with liquid N_2, the pressure within the apparatus reduced to 0.1 Torr and the temperature of the Grignard raised to -20°C using a mixture of acetone and solid CO_2. The $Ba^{14}CO_3$ (100 mg; 10.3 μCi/mg;

The Radiochemical Centre, Amersham, Bucks., U.K.) was triturated with $BaCO_3$ (3.4 g) and the whole treated dropwise with 25 ml 18M H_2SO_4, previously de-gassed. The $^{14}CO_2$ was admitted into the Grignard solution when the latter was mobile enough to be stirred. The $BaCO_3/H_2SO_4$ mixture was heated near the close of the reaction in order to obtain quantitative yields. On completion of the reaction, saturated ammonium chloride was added to the reaction mixture in order to decompose excess Grignard reagent, and the solution was allowed to stand overnight. The acidic solution was continuously extracted with diethyl ether for six hours to extract the [^{14}C]-propionic acid, however the acid was not actually isolated but was kept in ethereal solution for the next stage in the synthesis.

[^{14}C]-Propionyl chloride (III)

To the ethereal extract from the above synthesis, benzoyl chloride (10.65 g) was added. Subsequent heating and distillation gave [^{14}C]-propionyl chloride, b.p. 78°C (Brown, 1938).

1-Oxo-1-phenyl[1-^{14}C]propane. ([^{14}C]-propiophenone) (IV)

The Friedel-Craft reaction (Read, 1922) was carried out as follows. To the [^{14}C]-propionyl chloride (1.28 g) was added anhydrous benzene (2.0 ml). This solution was allowed to drop onto a suspension of aluminium chloride (2.5 g) in anhydrous benzene (3.0 ml), the temperature during the addition being kept below 20°C. After the addition, the solution was refluxed for two hours at 50°C, allowed to cool and poured into a separating funnel containing ice-cold water. The benzene layer was further washed with 2M NaOH (2 x 10.0 ml) and finally with water (2 x 10.0 ml). The benzene was dried over anhydrous Na_2SO_4 and finally subjected to fractional distillation at 80°C under reduced pressure (7.0 Torr) to give [^{14}C]-propiophenone (1.88 g).

2-Bromo-1-oxo-1-phenyl[1-^{14}C]propane (V)

Anhydrous benzene (10.0 ml) was added to the [^{14}C]-propiophenone (1.88 g) and the temperature raised to 70°C with stirring. Bromine (1.79 g) in anhydrous benzene (10.0 ml) was added dropwise, allowing the solution to decolourise before the addition of the next drop. After the addition was complete, the solution was refluxed for a further hour at 80°C, cooled, washed with water (2 x 2.0 ml) and the benzene dried over anhydrous Na_2SO_4.

(±)-1-Oxo-1-phenyl-[1-^{14}C]-2-N-benzylethanolaminopropane (VI)

To the benzene solution containing the brominated propiophenone, N-benzyl-ethanolamine (4.08 g) in anhydrous benzene (5.0 ml) was added with stirring. The temperature was raised to 80°C and refluxing was maintained for four hours after which the liquid was allowed to stand overnight. The resulting viscous material was washed with water (2 x 5.0 ml) to remove any N-benzylethanolamine hydrobromide and the benzene was evaporated under reduced pressure on a rotary film evaporator to leave an amber oil. This oil was dried in a vacuum dessicator over P_2O_5 and treated with ethanolic hydrogen chloride (7.5M) to yield crystals of the hydrochloride salt (m.p. 180°C; Engelhardt et al., 1958, give 180°C; yield 3.0 g).

(±)-1-Phenyl-[1-^{14}C]-2-ethanolaminopropan-1-ol hydrochloride (VII)

The compound (VI) (3.0 g) was dissolved in methanol (20.0 ml). To this was added 5% Palladium on charcoal catalyst (7.74 g; B.D.H., Poole, Dorset, U.K.) and the mixture hydrogenated at 210 kPa (30 lbin^{-2}) for 48 hours. On the release of pressure, the smell of toluene was apparent from the reaction vessel. The solution was filtered (Whatman No. 44) and evaporated under reduced pressure on a rotary film evaporator to give an oil which on treatment with acetone (2.0 ml) gave crystals of (±)-1-phenyl-[1-^{14}C]-2-ethanolaminopropan-1-ol hydrochloride (m.p. 158°C; Engelhardt et al., 1958, give 164°C). Several recrystallisations from methanol and

acetone raised the m.p. to 161°C.

(+)-trans-2-Phenyl-[2-^{14}C]-3-methylmorpholine hydrochloride (VIII)

To the hydrochloride of VII (1.0 g) was added 18M H_2SO_4 (2.0 ml) and the resulting dark green solution left in a stoppered tube in the dark for 48 hours at room temperature. The solution was then poured onto crushed ice in a separating funnel, made basic with 10M NaOH (5.0 ml) and extracted with diethyl ether (3 x 15 ml). The ether was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure on a rotary film evaporator, to leave a pale amber oil. The hydrochloride salt was prepared by adding an ethereal solution of hydrogen chloride and recrystallised from ethanol and diethyl ether to give [^{14}C]-phenmetrazine hydrochloride (m.p. 177°C; Stark et al., 1961, give 182°C; specific radioactivity 0.25 μCi/mg).

Purity was determined by reverse isotope dilution analysis carried out by dissolving 2.0 mg of [^{14}C]-phenmetrazine hydrochloride in water (100 ml). Authentic Preludin (200.0 mg) was dissolved in 50.0 ml of the radioactive solution. This solution was made basic with 2M NaOH, the free base extracted with diethyl ether (3 x 100 ml), and dried over anhydrous Na_2SO_4. The hydrochloride salt was prepared and recrystallised as above, to constant specific radioactivity. Using this method and chromatography, the [^{14}C]-Preludin was shown to be greater than 99% pure. Yield 302.0 mg.

Method 2.

The (+)-[^{14}C]-norephedrine hydrochloride ((+)-2-amino-1-phenyl-[1-^{14}C]-propan-1-ol hydrochloride) used for this synthesis was synthesised in this laboratory (for details see Sinsheimer et al., 1973).

(±)-1-Phenyl-[1-^{14}C]-2-ethanolaminopropan-1-ol hydrochloride (VII)

To the (±)-[^{14}C]-norephedrine hydrochloride (11.8 mg; 2.54 μCi/mg) was added (±)-norephedrine hydrochloride (188.2 mg). The two solids were mixed, dissolved in water and the pH adjusted to between 12 and 13 with 10M NaOH. The solution was saturated with NaCl and extracted with ethyl acetate (3 x 100.0 ml) which was pooled and dried over anhydrous Na_2SO_4. The ethyl acetate was evaporated in a stoppered 50 ml tube and redistilled ethylene chlorhydrin (2.5 ml) added and the mixture was heated for 10 hours on a water bath at between 85 and 95°C. On cooling, diethyl ether (15.0 ml) was added which caused the precipitation of the hydrochlorides present. These were filtered and recrystallised from ethanol (99.8% v/v) m.p. 159°C (Klosa, 1967, gives 163-168°C); Yield, 172 mg. Further recrystallisation from acetone and methanol yielded white crystals of (±)-[1-^{14}C]--N-(2-hydroxyethyl)-norephedrine hydrochloride (1-phenyl-[1-^{14}C]-1-oxy-2--ethanolaminopropane hydrochloride, m.p. 163-164°C). Yield, 166 mg.

(±)-trans-2-Phenyl-[2-^{14}C]-3-methylmorpholine hydrochloride (VIII)

Compound VII was cyclized in the same way as Method 1 to give [^{14}C]-phenmetrazine hydrochloride (m.p. 175-176°C; 78.3 mg). Yield with respect to the (±)-[^{14}C]-norephedrine hydrochloride was 39%.

Comparison of the (±)-[^{14}C]-N-(2-hydroxyethyl)-norephedrine (VII) obtained from the two syntheses showed the compounds to have identical infra-red (i.r.), mass spectra and chromatographic properties, implying that the products for the two synthetic routes were identical. Infra-red spectra and graphical representations of mass spectra for authentic Preludin, [^{14}C]-Preludin and (±)-N-(2-hydroxyethyl)-norephedrine may be found in the Appendix.

(±)-cis-2-Phenyl-3-methyl-5-morpholinone.((±)-cis-5-methyl-6-phenyl-3--morpholinone). (Clarke,1962) (IX)

(±)-Norephedrine base (12.0 g) was dissolved in dry benzene (1.2 l) and added to a stirred suspension of sodium hydride (2.0 g) (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) in dry benzene (30.0 ml) giving a pale yellow suspension. The reaction was allowed to proceed for 2 hours when H_2 was still being evolved, thus the suspension was heated to 50°C on a water bath until the evolution of H_2 had ceased (3 hours). The mixture was then cooled on ice to approximately 10°C and redistilled ethyl chloroacetate (10.0 g) was added dropwise over 15 minutes. Initially the yellow colour turned red, but eventually the yellow colour returned. The mixture was stirred for 15 minutes and refluxed for 2 hours and left to stand for 48 hours when the solution was washed with 2M HCl to remove unchanged norephedrine, then with water (20.0 ml) before finally being dried over anhydrous Na_2SO_4. The solvent was evaporated at reduced pressure on a rotary film evaporator to give a yellow oil. Partial crystallisation occurred overnight and was completed by the addition of boiling cyclohexane. Recrystallisations from benzene/cyclohexane gave a white powder m.p. 138-140°C (Clarke, 1962, gives 142-144°C). Yield 2 g.

The lactam ran as a single spot in several solvent systems on both paper and t.l.c. (see Table 2) and both the silylated and underivatised compounds gave a single peak on g.l.c. (see Table 1). A methanolic solution of the lactam showed no optical rotation. Infra-red, n.m.r. and the mass spectrum of this compound were all consistent with the proposed structure (see Appendix).

An authentic sample of the (±)-cis-2-phenyl-3-methyl-5-morpholinone (Fenmetramide) was obtained at a later date. The m.p. was identical to the above synthetic compound and showed no depression on a mixture of the two compounds. Using t.l.c. the R_F values of the two compounds were found to be the same as were

the infra-red spectra.

(±)-2-Phenyl-3-methyl-6-morpholinone,((±)-5-methyl-6-phenyl-2-morpholinone) (X)

(±)-N-(2-Carboxymethyl)norephedrine (XI)

(±)-Norephedrine base (28.0 g) was dissolved in redistilled ethyl chloroacetate (150.0 ml) and heated on a water bath between 85 and 95°C. A white precipitate was formed some minutes later and heating was continued for a further 2.5 hours, after which time diethyl ether (350 ml) was added causing a further precipitation of the impure (±)-N-(2-carboxymethyl)norephedrine ethyl ester hydrochloride. The solid was filtered off, washed with diethyl ether and dried in a vacuum dessicator over P_2O_5; m.p. 155-177°C. Further purification by recrystallisation from ethanol/diethyl ether and ethanol failed to yield a pure product. This material (12.5 g) was dissolved in water (95 ml) and 2M NaOH (95 ml) added. The mixture was heated to 40°C, left to stand for one hour and then extracted with ethyl acetate (100 ml) to remove any unchanged norephedrine. The pH of the remaining aqueous phase was adjusted to 6 with 10M HCl and left to stand overnight. The resulting (±)-N-(2-carboxymethyl)norephedrine was filtered off, washed with a little water and recrystallised from ethanol to give white crystals, m.p. 229°C (decomp.). Yield 2.6 g.

Found: C, 62.7; H, 7.1; N, 6.9%

$C_{11}H_{15}NO_3$ requires C, 63.1; H, 7.2; N, 6.7%.

(±)-2-Phenyl-3-methyl-6-morpholinone (X)

(±)-N-(2-Carboxymethyl)norephedrine (1.0 g) was dissolved in M HCl (20.0 ml) and refluxed for 15 minutes. The solution was then evaporated to dryness under reduced pressure. To remove excess acid, a further volume of water (10.0 ml) was added and the solution again evaporated to dryness under reduced pressure. This proceedure was repeated twice. The remaining white solid was dissolved in ethanol (5.0 ml) and dry diethyl ether added until no further precipitation of an oil

occurred. The oil soon crystallised and the solid was filtered off and re-crystallised twice from acetone/ethanol to give white crystals of (±)-2-phenyl-3-methyl-6-morpholinone hydrochloride, m.p. 159-161°C; Yield 0.6 g.

The compounds X and XI were further examined by i.r. and mass spectrometry and by n.m.r. spectroscopy as well. Infra-red, n.m.r. and the mass spectra of the compounds were all consistent with the proposed structures.

The R_F values in several solvent systems are to be found in Table 2.

A methanolic solution of the lactone (X) was found to exhibit no optical rotation of a beam of polarised light.

N-Hydroxyphenmetrazine

i) This synthesis was attempted on a number of occasions following exactly the steps quoted by Beckett and Salami (1972), namely N-oxidation with m-chloroperbenzoic acid, but under no conditions could a solid be obtained that had the properties quoted by the authors or that was capable of being recrystallised. Isolation of the suspected product was attempted by preparative thin layer chromatography but the compound consistantly broke down to give other components.

ii) Another synthetic route to the N-hydroxy compound was attempted and is represented below (Fig. 2.3).

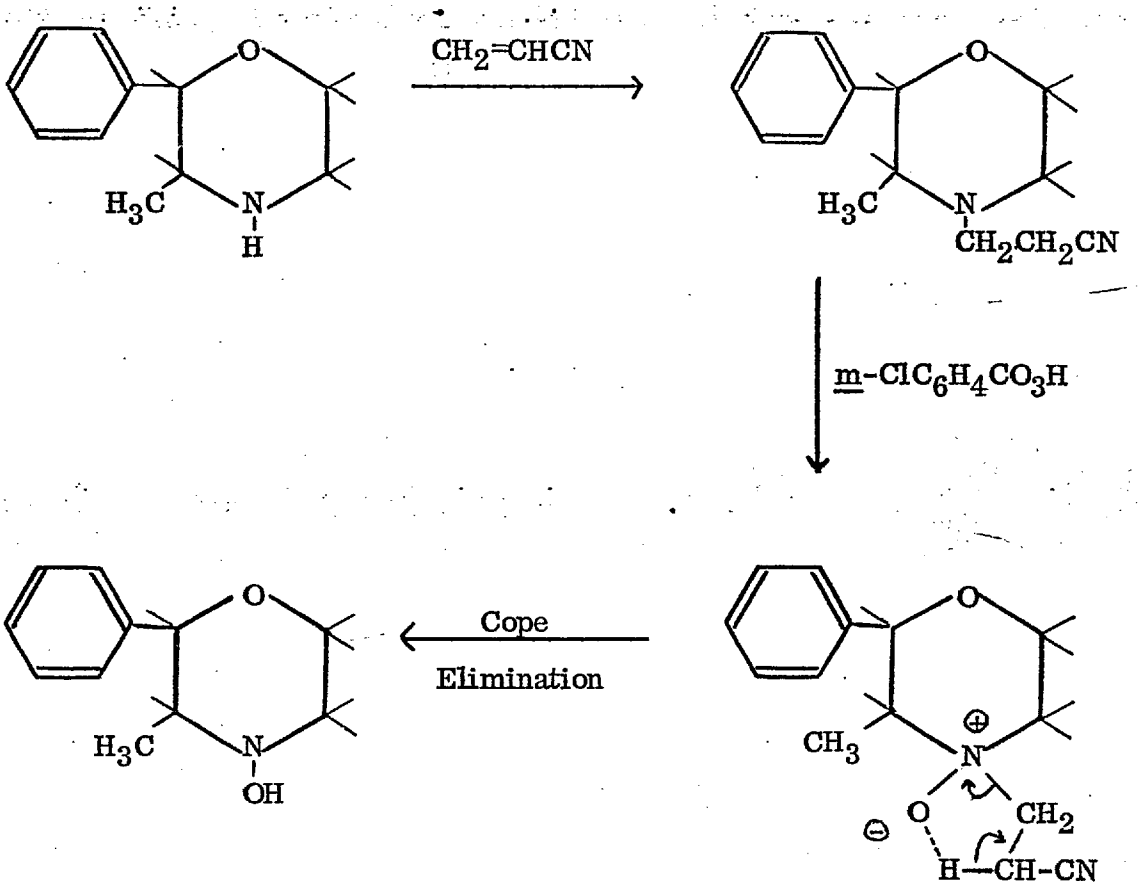


Fig. 2.3.

This reaction sequence was based on experiments carried out on the N-hydroxylation of proline, pipecolic acid and sarcosine by Nagasawa and co-workers (1972). The reaction was carried out on a small scale for Preludin and it became apparent that on this scale of synthesis, separation and purification of the intermediates provided a major drawback and time precluded a more thorough investigation of the synthesis.

Attempted synthesis of (±)-3-methylhydroxy-2-phenylmorpholine

An attempt to synthesise this compound was initiated since it represented a possible metabolite. The sequence of reactions anticipated for the reaction may be seen below (Fig. 2.4).

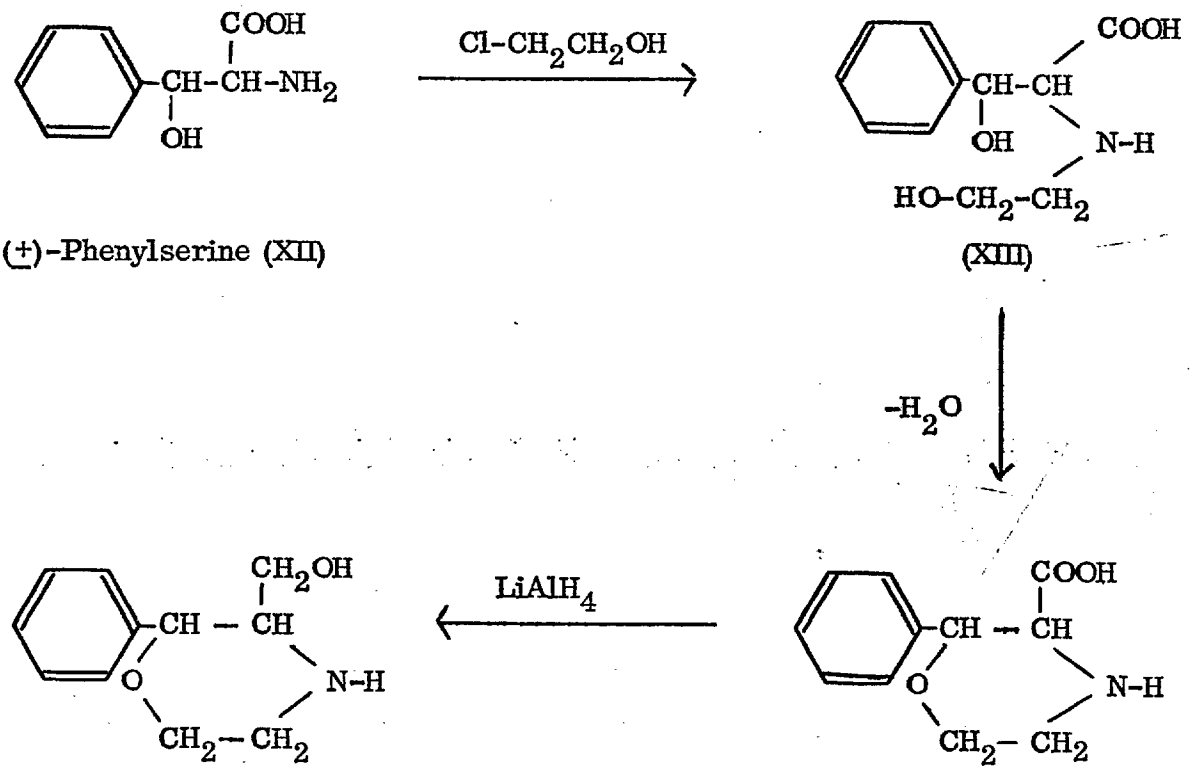


Fig. 2.4.

Thus, (±)-phenylserine (m.p. 192-194°C; Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) (20.0 g) was heated in a stoppered flask with redistilled ethylene chlorhydrin (70 ml; 50 ml initially and a further 20 ml added 10 hours later) on a water bath between 85 and 95°C for 13.5 hours. The mixture turned slowly brown and a strong smell of benzaldehyde was apparent. The cooled solution was treated with diethyl ether (400 ml) when a compound precipitated. The solid was filtered off, dissolved in boiling methanol, filtered and recrystallised to give white crystals, m.p. 238°C (decomp.). Further recrystallisation from ethanol/water gave white crystals, m.p. 238°C.

(±)-Phenylserine hydrochloride was prepared by dissolving some (±)-phenylserine in ethanolic hydrogen chloride (4.9M) and evaporating the solution to dryness in vacuo on the rotary evaporator. A white solid remained, m.p. 148°C (decomp.). The material was not recrystallised.

Cyclization of the supposed compound N-(2-hydroxyethyl)-phenylserine (XIII) was carried out by dissolving (XIII) (0.6 g) in 18M H_2SO_4 (6.0 ml) and leaving in a stoppered tube in the dark for 24 hours after which the mixture was poured onto crushed ice in a 50 ml tube and the pH adjusted to 6 with 10M NaOH. Some precipitation did occur over a period of 48 hours. The whole mixture was taken down to dryness *in vacuo* leaving a white/yellow solid, m.p. 234°C (decomp.). which when recrystallised from ethanol/water gave an m.p. of 238°C and an i.r. spectrum similar to the uncyclized material. Thin layer chromatography (Aluminium-backed plates; Silica gel 60 F_{254} 0.22 mm thickness; E. Merck A.G., Darmstadt, West Germany) in chloroform - methanol 9:1 v/v and benzene - methanol - diethylamine, 70:15:15, by vol., showed no difference between cyclized and uncyclized material. The i.r. spectrum showed a notable absence of peaks in the aromatic portion of the spectrum, thus it was postulated that the compound had broken down on reacting with the ethylene chlorhydrin to give benzaldehyde and glycine. The physical characteristics of authentic glycine were determined and found to resemble exactly the synthesised compound.

An alternative synthesis of (±)-2-phenyl-3-(methylhydroxy)morpholine using the selective brominating agent pyridinium bromide perbromide (see Fieser and Fieser, 1967) was attempted but the isolated product appeared to be non-aromatic, although mass spectrometry indicated a bromo-compound. (See Fig. 2.5 for proposed synthetic scheme).

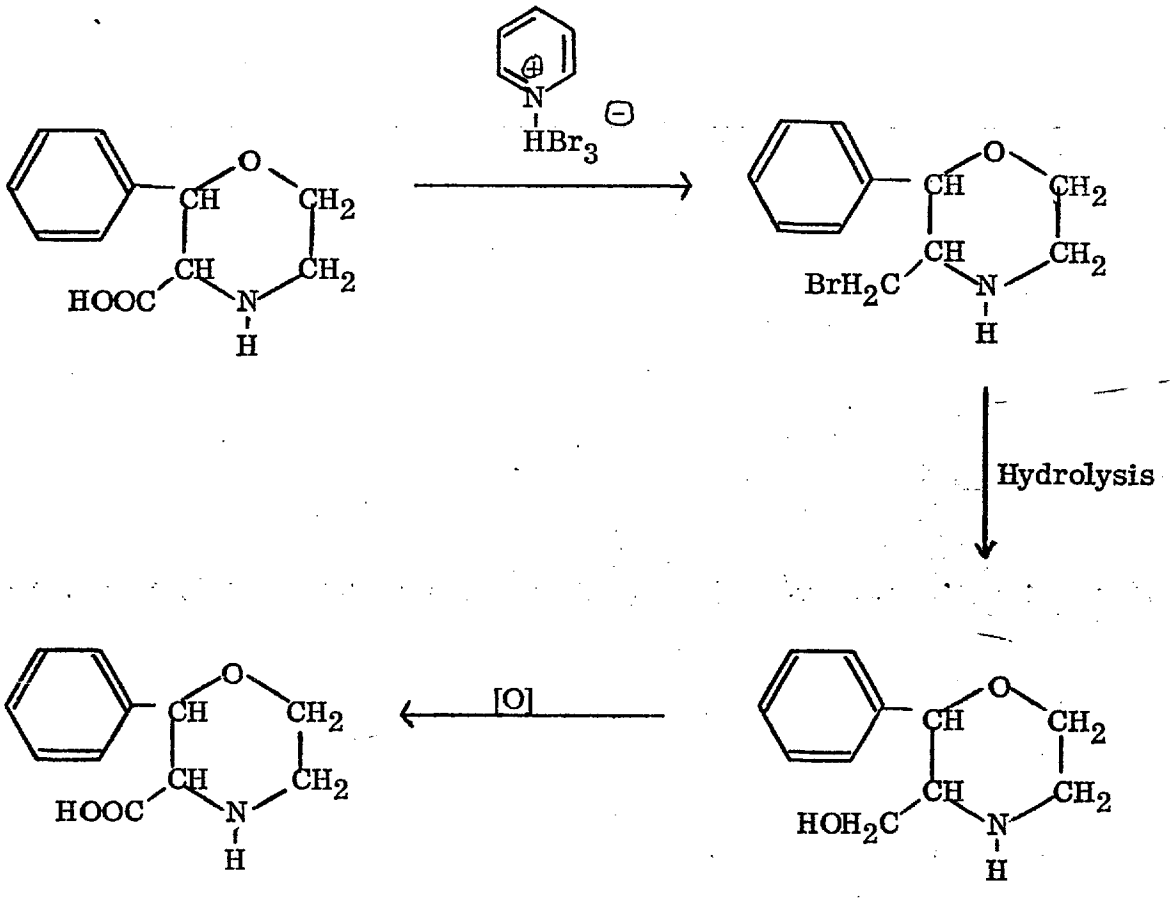


Fig. 2.5

Attempts were made to synthesise (a) 2-(4'-hydroxyphenyl)-3-methyl-morpholine and (b) 2-(4'-methoxyphenyl)-3-methylmorpholine, by the following reaction schemes (see Figs. 2.6 and 2.7 respectively).

Compounds XIV, XV and XVI were successfully synthesised and characterised by the methods of Dring et al., 1970 but compounds XVII, XVIII and XIX could not be satisfactorily isolated or identified.

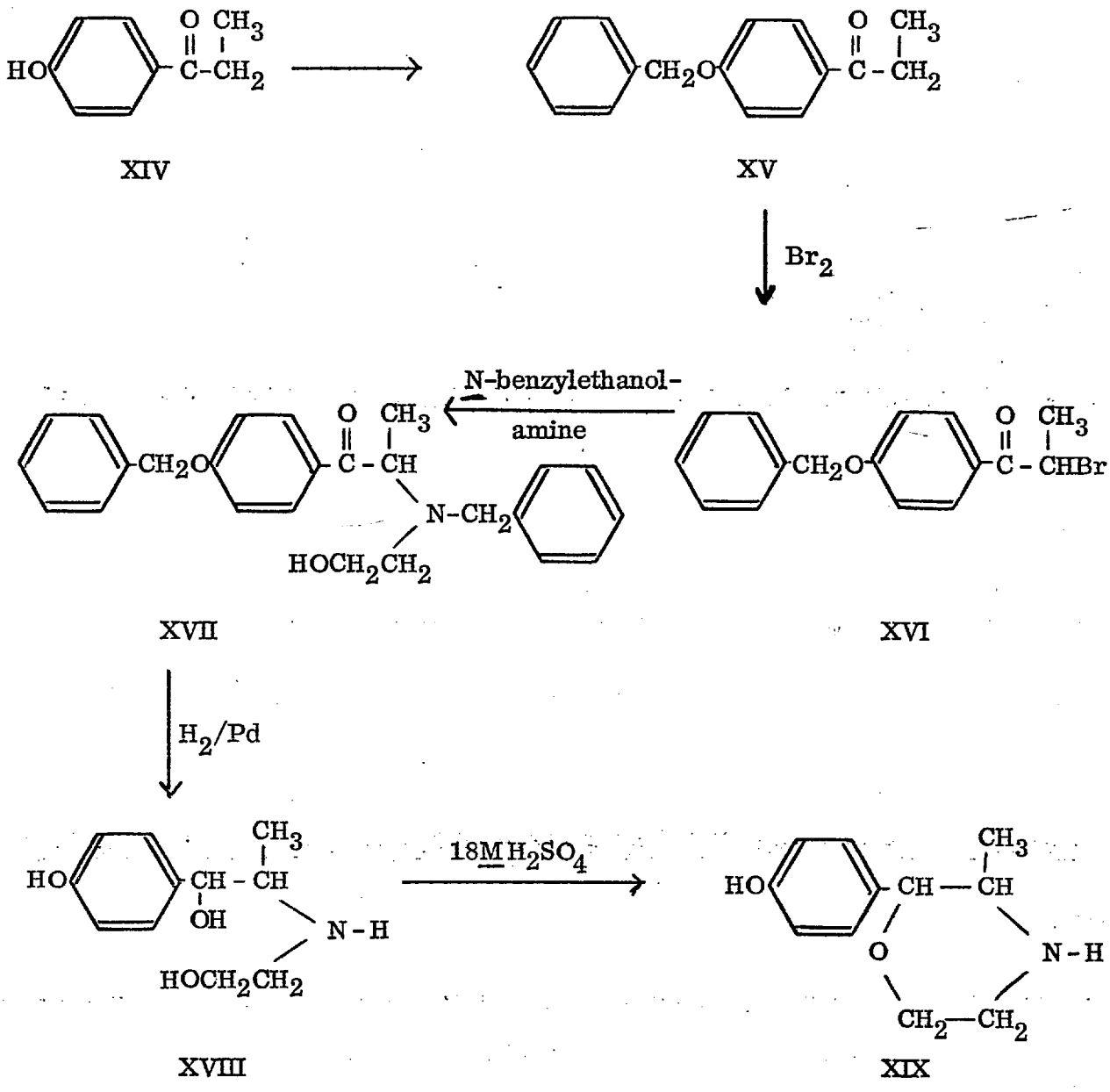
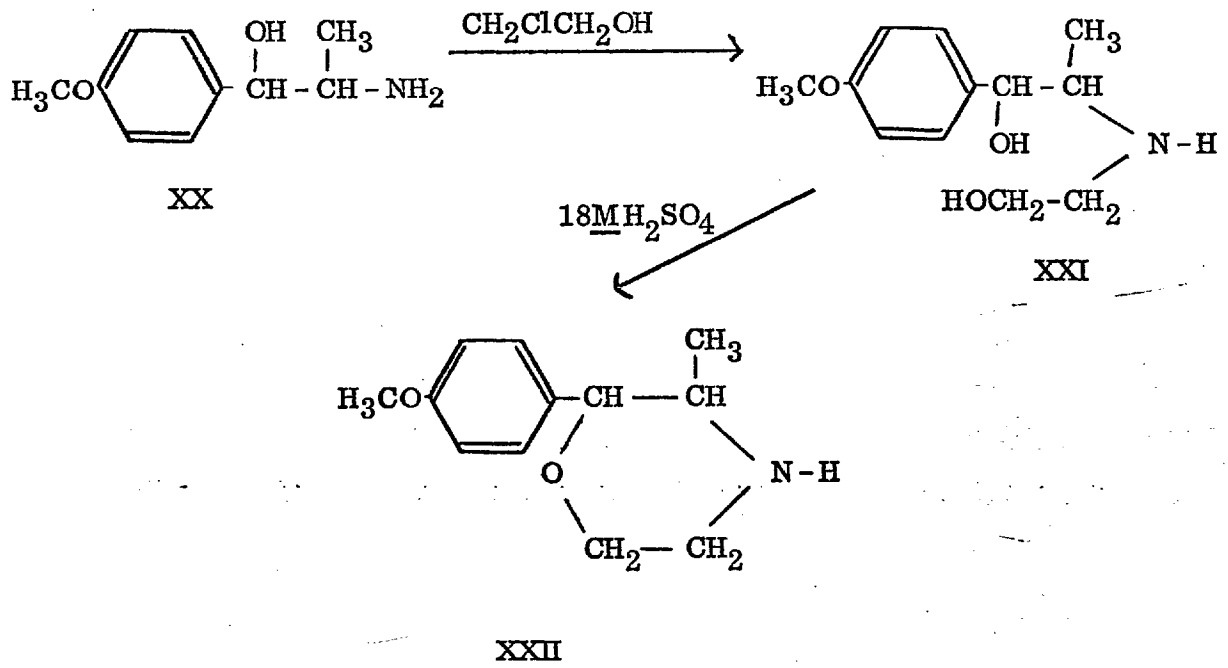

HO
O CH3
C-CH2
XIV
CH2O
XV
Br2
N-benzylethanol-
amine
CH2O
C - CH
N-CH2
HOCH2CH2
XVII
CHBr
XVI
H2/Pd
CH3
CH - CH
OH
N - H
HOCH2CH2
XVIII
18M H2SO4
CH — CH
O
N - H
CH2—CH2
XIX

Fig. 2.6



XX

XXI

XXII

Fig. 2.7

In this synthesis, compounds XXI and XXII could not be positively identified by the physical methods used, that is chromatography, infra-red spectroscopy or mass spectrometry.

Enzyme hydrolyses

To determine the presence of sulphate and/or glucuronide conjugates, a portion (0.1-0.25 ml) of the urine was incubated at 37°C with an equal volume of either a β-glucuronidase enzyme preparation (Ketodase, William R. Warner & Co. Ltd., Eastleigh, Hants., U.K.) or 500 units of sulphatase (Type H-2, Sigma Chemical Co., London, U.K.) plus an equal volume of pH 5 acetate buffer 0.2M for 48 hours. Controls containing boiled enzyme or saccharo-1,4-lactone were incubated at the same time.

Direct chromatography of urines hydrolysed with Ketodase was possible, however, in order to obtain satisfactory chromatograms from sulphatase incubations

methanol (10.0 ml) was added to precipitate the protein and the suspension centrifuged. The supernatant was kept to one side whilst the protein mass was re-extracted with methanol a further three times. The pooled supernatants were evaporated to dryness under reduced pressure, the residue taken up in methanol (0.2 ml) and chromatographed.

Metabolism of Preludin using microsomes from rat and guinea pig liver

The animals used in these experiments were female Wistar albino rats (200 ± 10 g) and female guinea pigs of the Dunkin-Hartley strain (1 kg ± 20 g).

Preparation of 10,000 x g supernatant and microsomes from rat and guinea pig liver

The animals were stunned by a sharp blow on the head and killed by cervical dislocation. The livers were quickly dissected out, rinsed in ice-cold 1.15% w/v KCl, blotted dry on filter paper and weighed. These livers were homogenised in ice-cold 1.15% w/v KCl using an Ultra Turrax homogeniser (Janke and Kunkel K.G., Staufen i. Breisgau, W. Germany) such that 1 g (wet weight) of liver was homogenised in 2 volumes of 1.15% w/v KCl. The homogenates were then centrifuged at 10,000 x g in an MSE '17000' centrifuge for 30 minutes at 4°C to give 10,000 x g supernatant. A portion of the 10,000 x g supernatant was centrifuged in an MSE 'Superspeed 40' centrifuge at 105,000 x g for 60 minutes at 4°C. The supernatant was decanted, the microsomal pellet re-suspended in 1.15% w/v KCl and re-centrifuged at 105,000 x g for a further hour. Removal of the supernatant left a microsomal pellet which was re-suspended in 1.15% w/v KCl such that the final concentration was 500 mg wet liver equivalent/ml. Microsomal protein was estimated by the method of Lowry and co-workers (1951) using Bovine Serum Albumin as the standard. For the rat 100 mg of liver was equivalent to 6 mg of microsomal protein and for the guinea pig 100 mg of liver was equivalent to 8 mg of microsomal protein.

Incubations

Incubation mixtures contained either 2.0 ml of whole liver homogenate or 10,000 x g supernatant, or the microsomal suspension plus 0.2 ml 0.1M $MgCl_2$, 1.0 ml of a $NADPH_2$ generation mixture (glucose-6-phosphate, 10 mg, NAD, 0.5 mg, NADP 1.0 mg, glucose-6-phosphate dehydrogenase, 2 units) and 1.0 ml Preludin (= 1 mg) with [^{14}C]-Preludin as a marker. The final volume was adjusted to 6.4 ml with 0.1M NaH_2PO_4/Na_3PO_4 buffer, pH 7.4. Control incubations were conducted using either aniline (1 µl) or 4-aminoantipyrine (1 mg) and semicarbazide hydrochloride (5 mg). These were added as aqueous solutions made up in 0.1M phosphate buffer, pH 7.4. Incubations were carried out in duplicate for 1 hour at 37°C in a Dubnoff metabolic shaker. Appropriate control flasks containing boiled liver fractions were run simultaneously. Viability of these liver fractions was tested by monitoring the 4-hydroxylation of aniline and the formaldehyde released by the N-dealkylation of 4-aminoantipyrine.

Detection of 4-aminophenol formed from aniline

This was determined by the method of Darby (1971), modified as follows. A portion (2.5 ml) of the incubation mixture was poured into 2.5 ml of 15% w/v trichloroacetic acid and the precipitated protein filtered off. To 2.5 ml of the filtrate was added 1% w/v phenol in 0.5M NaOH (1.0 ml) followed by 2M Na_2CO_3 (2.0 ml). The solution was allowed to stand at 37°C for 30 minutes. Investigations by Chabra and co-workers (1972) have shown that this method of detection of 4-aminophenol may be sufficiently accurate for quantification when using the microsomal fraction, but may be inaccurate for whole-liver homogenates and 10,000 x g determinations for which purpose they recommend the 'ether-extraction' technique of Kato and Gillette (1965). However, since only a qualitative estimation was required for the present experiments, the trichloroacetic acid precipitation method was used (Imai et al., 1966).

If required, the resulting blue colour could have been estimated at 630 nm.

Detection of formaldehyde from the N-demethylation of 4-aminoantipyrine

The incubation mixtures were poured into 15% w/v zinc sulphate solution (2.0 ml) and the protein further precipitated by the addition of saturated barium hydroxide solution (2.0 ml), followed by thorough mixing on a vortex mixer and and centrifugation. To the supernatant (5.0 ml) was added Nash reagent (2.0 ml) (Nash, 1953; 1 ml redistilled acetylacetone in 250 ml of 30% w/v ammonium acetate solution). The tubes were allowed to stand in a water bath at 60°C for 15 minutes when a yellow colour was formed. Since quantitation of the formaldehyde was not attempted, the solutions were not read at 412 nm.

Isolation of metabolites of Preludin

Methanol (15.0 ml) was added to the incubations to stop the reaction and also precipitate the proteins. The methanolic solutions were then centrifuged, the supernatants removed and taken down to dryness under reduced pressure on a rotary film evaporator. The protein mass was re-extracted with a further portion of methanol (15.0 ml) which was added to the former extracts and again taken down to dryness under reduced pressure. Methanol (0.5 ml) was added and the extract chromatographed on thin-layer plates (Silica gel G HF_{254}; 0.8 mm thickness, pre-eluted with methanol). Radioactivity on the plates was detected on the radiochromatogram scanner. The areas of radioactivity on the plates were eluted with ethyl acetate which was filtered (sintered glass) before being taken down to dryness under reduced pressure. The residual compound was redissolved in a small portion of methanol and examined by gas-liquid chromatography (g.l.c.) and combined g.l.c./mass spectrometry (m.s.). A portion of each of the extracts from the rat liver fractions was acetylated (acetic anhydride/pyridine) and a portion of each of the fractions from guinea pig liver was silylated (bis-trimethylsilyl-acetamide).

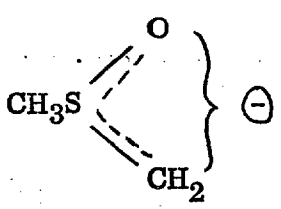
Biliary excretion of Preludin in rat bile

Female Wistar albino rats (190 ± 10 g, Allington Farm, Porton, Wilts. U.K.) were anaesthetised with thiopentone sodium (70 mg/kg, intraperitoneally; Intraval sodium, May and Baker Ltd., Dagenham, Essex, U.K.). The bile duct of each animal was exposed and cannulated with polyethylene tubing (i.d. 0.4 mm, o.d. 0.8 mm; Portex Plastics, Hythe, Kent, U.K.) and after sufficient time had elapsed for the bile flow to become regular, the animals were dosed with either Preludin or [^{14}C]-Preludin dissolved in normal saline (20 mg/kg; intraperitoneally).

Bile samples were collected at hourly intervals for six hours and finally after 24 hours. Portions (0.1 ml) of the first two hour bile samples were subjected to paper chromatography and radiochromatogram scanning.

Permethylation of rat bile containing Preludin metabolites

Permethylation was carried out with methyl iodide using the methylsulfinylmethide (XXIII) (see Fig. 2.8) carbanion as a catalyst (see Corey and Chaykovsky, 1962).



XXIII

Fig. 2.8

This was prepared in a manner similar to that of Corey and Chaykovsky (1962) by suspending powdered sodium hydride (48 mg) in dry dimethylsulphoxide (DMSO; 76°C/15 Torr). The suspension was heated at 65-70°C for one hour until rapid evolution of H_2 had ceased (Leclerq and Desiderio, 1971).

Initially, phenylglucuronide (Sigma Chemical Co., London, U.K.) was used to test the efficiency of the permethylation. Permethylation was accomplished essentially according to the method of Thompson and co-workers (1973) as follows. Phenylglucuronide (50 μg) was dissolved in dry DMSO (0.25 ml) and the tube was flushed with N_2. The $DMSO^{\ominus}$ carbanion catalyst (0.1 ml) was added, followed 15 minutes later by methyl iodide (0.05 ml). The reaction mixture was left at room temperature for 1.5 hours when water (1.0 ml) was added to stop the reaction and the whole extracted with chloroform (2.0 ml). This extract was washed with water (2 x 1.0 ml), followed by evaporation to dryness under a stream of N_2. The residue was taken up in methanol (25 μl) for g.l.c. and mass spectrometry (Thompson et al., 1973).

Phenyl glucuronide or Preludin were added to control bile (100 μl), permethylated and analysed as above. Bile from an animal dosed with Preludin was also permethylated as was a sample of control bile. Under these circumstances, the bile was evaporated to dryness under N_2 and then taken up in 0.25 ml of DMSO as before.

Combined g.l.c./m.s. was carried out on a Varian 1700 gas chromatograph equipped with a 1.52 m (5 ft) column (i.d. 3 mm) packed with 3% OV-101 on Celite AW-DMCS (100-200 mesh) in conjunction with a Varian MAT CH5 mass spectrometer. (see Table 1 for retention times and column temperatures).

Pharmacological activity of Preludin and 2-phenyl-3-methyl-5-morpholinone

A useful measure of the action of a compound on the central nervous system is the observation of the movements of an animal after administration of that compound (Jacobsen, 1964). A number of methods using this principle have been devised and one of the most recent is an activity meter capable of recording both fine and gross movements of an animal through changes in capacitance.

Fine and gross movements after the administration of Preludin to rats and

2-phenyl-3-methyl-5-morpholinone to mice were observed as follows.

Effect of Preludin

Ten female Wistar albino rats (200 ± 15 g) were used, the movements of each rat being recorded individually. Thus the timetable for each rat was as follows:

Day 1. The rat was dosed orally at 13.30 hours with water (1.0 ml) and placed in activity apparatus (Stoelting electronic activity monitor, Chicago, Ill., U.S.A.) for 4 hours and both fine and gross body movements were recorded over 10 minute intervals. After 4 hours the rat was returned to its original cage.

Day 2. The same rat was dosed orally with Preludin in water (20 mg/kg; 1.0 ml) at 13.30 hours and placed in the activity apparatus for 4 hours. At the end of this period, the rat was returned to its original cage.

Day 3. The same rat was dosed orally with water (1.0 ml) at 13.30 hours and placed in the activity apparatus for 4 hours.

The procedure for day 3 was carried out to investigate whether the animal displayed any indications of hypermotility 24 hours after dosing.

The results from each rat were subjected to statistical analysis.

The Effect of 2-phenyl-3-methyl-5-morpholinone

Ten male mice (Thielers Original strain) were used for the study.

Day 1. All ten animals were placed in the same cage and left in the room where the activity monitoring apparatus was housed from 09.30 hours at a room temperature of 23°C. At 14.00 hours the mice were all injected intraperitoneally with 0.2 ml of a mixture of 1% w/v carboxymethylcellulose (0.1 ml) and 0.1% v/v Tween 80 (0.1 ml). The gross and fine movements of the mice were recorded for 6 hours, when the mice were returned to the animal room for the night.

Day 2. The following morning at 09.30 hours the same mice were again brought into the same room as the activity apparatus at an ambient temperature of 23-24°C. At

14.00 hours the mice were weighed and dosed intraperitoneally (approx. 0.2 ml) with a suspension of 2-phenyl-3-methyl-5-morpholinone in 1% w/v carboxymethylcellulose and 0.1% v/v Tween 80, according to weight, such that each mouse received a dose of 100 mg/kg. Fine and gross movements were again recorded for 6 hours and statistically compared with the control results obtained 24 hours earlier.

Effect of 2-phenyl-3-methyl-5-morpholinone on hexobarbitone-sleeping time.

A further method available for the assessment of a potential psychotropic compound is the effect of this compound on the hexobarbitone-sleeping time.

Forty male mice (Thielers Original strain) (24 ± 2 g) were kept at between 20 and 23°C for at least 24 hours before being randomly divided into four groups of ten mice.

Hexobarbitone-sodium, freshly prepared from hexobarbituric acid (43.03 mg), M NaOH (0.194 ml) was diluted with water to a final volume of 5.0 ml.

Two dose levels of 2-phenyl-3-methyl-5-morpholinone were chosen, that is 10 mg/kg and 100 mg/kg intraperitoneally. The compound itself was triturated with 1% w/v carboxymethylcellulose to a thick paste and diluting the suspension with 0.1% v/v Tween 80 to give a mobile mixture capable of being drawn up easily into a syringe.

The method employed for this experiment approximately followed that described by Jacobsen (1964). Thus, the mice were dosed as follows. One group was dosed with the drug at the lower dose level and another group at the higher dose level and the two other groups acted as controls and were injected intraperitoneally with approximately 0.1 ml of a mixture of equal proportions of 1% w/v carboxymethylcellulose and 0.1% v/v Tween 80, according to weight. After exactly 30 minutes each mouse was injected with hexobarbitone sodium (100 mg/kg) in a volume of 0.20 ml per 20 g mouse. After 6 minutes the anaesthetised mice were turned onto their backs and the time noted at which the righting reflex returned to the animal (approx. 90 minutes).

The times for each group were subjected to statistical analysis to determine whether any alteration of sleeping time had occurred.

Animals for metabolic studies

Female Wistar albino rats (200 ± 20 g), female Dunkin-Hartley guinea pigs (300 ± 70 g) and male Tamarin monkeys (500 ± 20 g) were used. Both Preludin and [^{14}C]-Preludin were dissolved in water and administered by stomach tube to rats and guinea pigs. The [^{14}C]-Preludin given intramuscularly to the Tamarin monkeys was in 0.9% w/v sodium chloride and was sterilised in an autoclave maintained at 121-123°C, for 15 minutes at 0.70 kg/cm^2 (15 lb/in^2). Human subjects took [^{14}C]-Preludin dissolved in water.

The animals were individually housed in cages suitable for the collection of urine and faeces. They were allowed free access to water but were given no food for 24 hours after dosing to avoid contamination of the urine.

Apparatus

Spectrometry

Infra-red (i.r.) spectra were recorded on a Perkin-Elmer Infracord spectrometer and calibrated at 6.24 mμ with a polystyrene standard.

Ultra-violet (u.v.) spectra were recorded on a Pye-Unicam Recording u.v. spectrometer (SP 1800).

Mass spectra of compounds from direct insertion techniques and via the gas-liquid chromatography were recorded on a Varian MAT CH5 mass spectrometer.

Nuclear magnetic resonance (n.m.r.) spectra of protons were recorded on a Bruker HFX 90 spectrometer with Fourier transform (Bruker Magnetics, Burlington, Ma., U.S.A.) at King's College, University of London, and also on a Perkin Elmer R12 spectrometer with Fourier transform at the School of Pharmacy, University of London.

Polarimetry

Optical rotations of compounds were measured in methanol in a Perkin-Elmer Polarimeter Model 141 at both sodium and mercury wavelengths. The cell pathlength was 1 cm.

Measurement of pH

For determination of pH, a Pye-Unicam Model 291 pH meter was used. All pH measurements were recorded at room temperature.

Chromatography

For gas-liquid chromatography a Hewlett-Packard F & M Model 402 apparatus (Hewlett-Packard Inc., Pasadena, Calif., U.S.A.) with flame ionization detector was used. The glass column [1.83 m (6 ft.), internal diameter 3 mm] was packed with Chromosorb G AW-DMCS coated with 3% w/w SE-30.

A list of retention times at particular column temperatures may be found in Table 1.

For combined gas-liquid chromatography and mass spectrometry (g.l.c./m.s.), a Varian Aerograph 1700 gas chromatograph in conjunction with a Varian MAT CH5 mass spectrometer was used. (For details of g.l.c. column, see under Permethylation of rat bile).

Conditions for the Hewlett-Packard g.l.c. were either injection port temperature 240°C and oven temperature, 155°C, or injection port temperature 310°C and oven temperature 240°C. Nitrogen (carrier gas), air and H_2 pressures were 275, 165 and 140 kPa (40, 24 and 20 lb/in^2) respectively. For the g.l.c. in conjunction with the mass spectrometer, the injection port temperature was 230°C; oven temperature 155°C; the interface, 240°C; the Biemann-Watson type molecular separator 220°C; the line-of-sight inlet line, 190°C; the source 180°C. The electron beam energy was 70 eV and the helium carrier gas flow rate was 32 ml/min at 275 kPa (40 lb/in^2).

Silylation was carried out by dissolving or suspending the compound (3 mg) in benzene (0.4 ml) and bis-(trimethylsilyl)acetamide (0.1 ml) was added. The solutions, in a stoppered tube, were heated at 50°C for 30 minutes and the product (1 μl) was injected onto the column.

Acetylation was carried out according to the technique to be found under the heading 'Gas-liquid chromatographic assay for Preludin'.

Gas-liquid chromatographic assay for Preludin

Although isotope dilution procedures remain one of the most efficient and reliable methods of estimating radioactive substances, they are time consuming and tedious. Thus, in order to quantitate unchanged phenmetrazine excreted in the urine of rat, guinea pig and Tamarin monkey, by a method other than isotope dilution, a g.l.c. assay was developed, using methamphetamine as an internal standard. A calibration curve was constructed and acetylation was carried out as follows: To an aqueous solution of Preludin (0.2 mg/ml - 12 mg/ml, calculated as free base) was added a solution of methamphetamine (1.0 ml of a 1 mg/ml solution, calculated as free base) and the mixture diluted to 10.0 ml with water. The pH was adjusted to 14 with 10M NaOH (0.2 ml) and the aqueous phase saturated with solid NaCl before extracting with diethyl ether (2 x 20.0 ml). The diethyl ether was dried over anhydrous Na_2SO_4 and reduced almost to dryness under reduced pressure on a rotary film evaporator. Pyridine (0.5 ml) and acetic anhydride (0.5 ml) were added and the solution left overnight in a stoppered tube, after which time the contents of the tube were evaporated to dryness in vacuo and ethyl acetate (0.5 ml) added. Portions (1 μl) were then injected onto the SE-30 column at a temperature of 190°C (see Table 1 for retention time (T_R) values). The corresponding acetylated methamphetamine and phenmetrazine peaks were cut out from the recorder chart paper and a graph plotted of weight of acetylated phenmetrazine peak/weight of acetylated methamphetamine peak

Table 1. Retention Times (T_R) of Phenmetrazine and Related Compounds

Compound	Column	Oven temperature (°C)	Retention time (T_R) (min)
Phenmetrazine	SE-30	155	5.0
Phenmetrazine (acetylated)	SE-30	155	9.2
Phenmetrazine (silylated)	SE-30	155	12.25
Phenmetrazine (acetylated)	SE-30	240	2.0
Phenmetrazine (acetylated)	SE-30	190	7.5
Methamphetamine (acetylated)	SE-30	190	3.3
Phendimetrazine	SE-30	155	6.2
2-Phenyl-3-methyl-5-morpholinone	SE-30	155	20.5
2-Phenyl-3-methyl-5-morpholinone (silylated)	SE-30	155	16.0
2-Phenyl-3-methyl-5-morpholinone	SE-30	240	0.9
2-Phenyl-3-methyl-5-morpholinone (acetylated)	SE-30	240	0.9
2-Phenyl-3-methyl-6-morpholinone	SE-30	155, 240	N.D.
2-Phenyl-3-methyl-6-morpholinone (silylated)	SE-30	155	31.0
Rat major metabolite (acetylated)	SE-30	240	7.2
Guinea pig major metabolite	SE-30	155	20.5
Permethylated phenmetrazine	OV-101	200	1.0
Permethylated phenylglucuronide	OV-101	200	4.5
Permethylated bile from rat dosed with Preludin	OV-101	250	7.5

N.D. = not detected. See text for temperature of injection port and pressure of carrier gas.

against weight of phenmetrazine (μg). A linear relationship was found to hold between 0.2-12.0 μg of phenmetrazine.

The assay was then applied to the complete urine sample of either a rat, guinea pig or Tamarin monkey which had been dosed 24 hours previously with Preludin.

Paper and thin-layer chromatography

R_F values on both descending paper (Whatman 3 mm) and thin-layer chromatograph as well as colour reactions of the compounds are given in Table 2. The following solvent systems were used:

A. Butan-1-ol : formic acid (98-100%) : water (12:2:4, by vol.)

B. Propan-1-ol : NH_3 aq. (sp. gr. 0.88) (7:3, v/v)

C. Butan-1-ol saturated with 1.5M ammonia - ammonium carbonate buffer (Fewster and Hall, 1951).

D. 3-Methylbutan-1-ol : 2-methylbutan-2-ol : water : formic acid (5:5:10:2, by vol.) (Alleva, 1963).

Thin-layer chromatography was carried out on Silica Gel HF_{254} (E. Merck A.G., Darmstadt, Germany) plates of thickness 1.0 mm activated for 30 minutes at 110°C. All plates were pre-eluted with methanol. Aluminium-backed silica gel plates (Silica Gel 60 F_{254}, 0.2 mm thickness (E. Merck A.G.) were also used. Solvent systems used were as follows:

E. Chloroform : methanol (5:1, v/v)

F. Benzene : methanol : diethylamine (70:15:15, by vol.)

G. Chloroform : methanol (9:1, v/v)

The compounds were visualised with the following reagents:

1. Sodium nitroprusside spray

Sodium nitroprusside (5 g) was dissolved in 10% aqueous acetaldehyde. An

equal volume of 2% Na_2CO_3 solution was added just before use. Secondary amines show up as pale blue spots (Macek et al., 1956).

2. Iodine spray

Iodine (2 g) dissolved in methanol (100 ml). Most organic compounds were detected by this spray.

3. Potassium iodide and starch spray

Sodium hypochlorite solution (B.D.H., Poole, Dorset, U.K.; containing 10-14% available Cl_2) was diluted 1:1 with water. The chromatogram was sprayed with the diluted solution and dried in an oven in the presence of acetic acid vapour. The dry chromatograms were sprayed with potassium iodide/starch solution (approximately 1% w/v potassium iodide in saturated starch solution). Amides were detected as dark brown spots on a pale yellow background.

Diazotised 4-nitroaniline spray

The chromatograms were sprayed with diazotised 4-nitroaniline as described by Wickström and Salvesen (1952). Phenols gave strong purple spots which were not permanent.

Radiochemical techniques

The radioactivity in urine was estimated directly by counting an aliquot in a Packard Tri-Carb Scintillation Spectrometer, Model 3320 using Bray's scintillator (Bray, 1960). ^{14}C in faeces was estimated by one of two methods, either (i) the faeces were thoroughly frozen, ground in a mortar and a known weight of this powder was combusted in a Packard Tri-Carb Sample Oxidiser Model 320 and counted in the scintillation spectrometer, or (ii) the faeces were homogenised in water using a Waring Blendor and diluted to a known volume. Portions (0.3 ml) in 10M NaOH were left overnight to effect solution and then treated with H_2O_2 (100 vol., 6 drops), warmed

Table 2 R_F Values and Colour Reactions of Phenmetrazine and Related Compounds

Compound	Paper chromatography on 3MM paper				Thin layer chromatography						Colour with spray:		
	Solvent.... A	B	C	D	E		F		G		1	2	3
					Glass plate	Aluminium plate	Glass plate	Aluminium plate	Glass plate	Aluminium plate			
Phenmetrazine	0.71	0.87	0.93	0.49	0.29	0.32	0.51	0.55	0.21	0.32	blue	brown	grey
Norephedrine	0.61	0.85	0.82	0.39	0.24	0.23	0.35	0.33	0.21	0.16	blue v. pale	brown	grey fades
Phendimetrazine	0.76	0.89	0.96	0.52	0.51	0.54	0.60	0.68	0.44	0.50	N.D.	brown	N.D.
4-Hydroxynorephedrine	0.49	0.72	0.64	0.17	0.21	0.15	0.30	0.23	0.02	0.02	blue v. pale	brown	N.D.
2-Phenyl-3-methyl-5-morpholinone	0.87	0.71	0.82	0.81	0.59	0.59	0.61	0.62	0.48	0.57	N.D.	brown	brown
2-Phenyl-3-methyl-6-morpholinone	0.69	0.64	0.21	0.32	0.05	0.06	0.16	0.16	0.07	0.08	pale blue	brown	grey fades
N-(2-Hydroxyethyl)norephedrine	0.74	0.88	0.81	0.46	0.19	0.20	0.41	0.40	0.11	0.15	N.D.	brown	N.D.
N-(Carboxymethyl)norephedrine	0.68	0.66	0.01	0.41	0.01	0.01	0.02	0.01	0.00	0.00	N.D.	brown	N.D.
Amphetamine	0.77	0.92	0.88	0.51	0.02	0.05	0.64	0.53	0.02	0.05	N.D.	brown	brown
Benzoic acid	0.88	0.21	0.43	0.93	0.60	0.50	0.37	0.31	0.55	0.44	N.D.	straw	N.D.
Hippuric acid	0.83	0.19	0.30	0.83	0.45	0.28	0.15	0.19	0.42	0.16	N.D.	straw	brown
Unidentified metabolite	0.88	0.57	0.10	0.95	0.47	0.50	0.51	0.52	0.46	0.46	N.D.	brown	N.D.
Phenolic metabolite	0.68	0.79	0.18	0.59	0.44	0.47	0.46	0.47	0.30	0.30	Purple colour with diazotised 4-nitroaniline		

N.D. = Not detected

carefully to control the effervescence and the pH adjusted to 7 by the dropwise addition of 10M HCl. On cooling, Bray's scintillator was added and the mixture counted as for (i). A comparison of the two methods showed them to be equally efficient.

Scanning of paper chromatograms and thin-layer plates was carried out using a Packard Radiochromatogram Scanner (model 7200). In order to detect minor peaks, chromatograms were cut into 1 cm strips, counted in the liquid scintillation counter and histograms plotted.

Isotope dilution

Isotope dilution procedures were carried out on portions of urine (10.0 ml) obtained from rats and guinea pigs dosed with [^{14}C]-Preludin. Compounds searched for in this way were 4-hydroxynorephedrine (2-amino-1-(4'-hydroxyphenyl)propan-1-ol), hippuric and benzoic acids. Procedures for the former two compounds were according to the method of Dring and co-workers (1970) and estimation of total [^{14}C]-benzoic acid was carried out by refluxing the urine (10.0 ml) with an equal amount of 10M NaOH for 3 hours (Baldwin, 1961) and then adding carrier benzoic acid, acidified with 10M HCl before extracting with diethyl ether (3 x 100 ml) and evaporating. This isolated benzoic acid was recrystallised to constant specific radioactivity (see Dring et al., 1970).

Results

Rat

The results for the three rats dosed with [^{14}C]-Preludin may be found in Tables 3 and 4. Of the 85% of ^{14}C recovered, nearly 70% was accounted for in the first 24 hour urine. Thus it was the 24 hour urine which was examined for the presence of metabolite

Isolation and identification of metabolites

The urine was saturated with NaCl and continuously extracted at pH 8-9 with diethyl ether when it was found that about 90% of the radioactivity was extracted into the organic phase. The diethyl ether was dried over anhydrous Na_2SO_4, reduced in volume in vacuo and chromatographed on Whatman 3mm paper in systems A, B, C and D. On subsequent radiochromatographic scanning, the chromatograms showed the same pattern as the radiochromatograms of the original urine (see Appendix for radiochromatogram scans, and histograms of the 24 hour urine).

Enzyme hydrolysis with Ketodase followed by subsequent chromatography in system A showed that a peak at R_F 0.28 had disappeared and that one at R_F 0.68 had become more pronounced. It was inferred that the peak at R_F 0.68 might be the aglycone of a glucuronide conjugate of the metabolite at R_F 0.28. These two compounds accounted for approximately 40% and 28% respectively of the ^{14}C excreted in 24 hours (approximately 28% and 19% of the dose, respectively).

In order to identify the metabolite, eleven rats were dosed orally with Preludin (20 mg/kg) and a twelfth rat was dosed orally with [^{14}C]-Preludin (20 mg/kg). The urines were collected for 24 hours and that from the rat dosed with [^{14}C]-Preludin was mixed with the urine of the other animals. The pH of the urine was adjusted to 8-9 with 2M NaOH, saturated with NaCl and continuously extracted with diethyl ether. The diethyl ether was dried over anhydrous Na_2SO_4, reduced in volume under reduced pressure and chromatographed in system A on Whatman 3 mm paper, the ^{14}C being detected by radiochromatogram scanning. The area of ^{14}C representing the aglycone of the glucuronide (R_F 0.68) was cut out from several chromatograms and eluted with methanol over a period of 2 days. The methanol was reduced in volume in vacuo and chromatographed on Whatman 3 mm paper in system B where the metabolite had an R_F of 0.79. This area was cut out and eluted with methanol over two days,

reduced in volume as before and subjected to preparative thin-layer chromatography (t.l.c.) in system E on Silica Gel HF_{254} plates (20 x 20 x 0.1 cm thick). The area of ^{14}C (R_F 0.44) was detected by radiochromatogram scanning of a suitable plate (5 x 20 x 0.1 cm thick) and also under u.v. light (254 nm, Hanovia Chromatolite Ltd., Slough, Bucks., U.K.) where it was found that the metabolite quenched the fluorescence of the plates. The required area of radioactivity was scraped off and eluted with methanol overnight. The silica gel was filtered (glass sinter), the methanol reduced in volume under reduced pressure and subjected again to preparative t.l.c. run in system F. One distinct area of radioactivity was detected on the radiochromatogram scanner at R_F 0.46 and was eluted off with methanol as before. Further t.l.c. in system G showed one distinct area of radioactivity at R_F 0.30 which when sprayed with diazotised 4-nitroaniline gave a strong purple colour, indicative of a phenol. The u.v. spectrum in ethanol (see Appendix) showed a single peak with a λ_{max} of 277 nm which showed a bathochromic shift to 294 nm on adding one drop of 0.1M NaOH. (A similar shift was seen using 4-hydroxyamphetamine, λ_{max} 274 nm which exhibited a bathochromic shift in the presence of 0.1M NaOH, λ_{max} 291 nm). The visible spectrum of the diazotised metabolite was also recorded and gave a λ_{max} at 520 nm. Similar treatment of Preludin gave no such peak at this wavelength. A mass spectrum of the metabolite (see Appendix) by the direct insertion technique, gave a parent ion at m/e 193. The sample was then methylated using diazomethane (Vogel, 1961) resulting in a shift of the parent ion to m/e 207, corresponding to the addition of 14 mass units, that is a methylene group. Treatment of phenmetrazine (free base) with diazomethane failed to give a parent ion plus 14 mass units. A further sample of the metabolite was acetylated (see 'G.l.c. assay of Preludin' for method) to form the N,O-diacetylated derivative which had a retention time of 7.2 minutes on the g.l.c. (see Table 1). Nuclear

magnetic resonance spectroscopy showed a pseudo-AB coupling system in the region of the aromatic nucleus (J_{ortho} 8 Hz) which was characteristic of a para-substituted benzene ring. All this evidence indicated that the major metabolite in the rat was 2-(4'-hydroxyphenyl)-3-methyl morpholine.

Bile from a rat dosed with Preludin and permethylated (see Materials and Methods) showed an extra peak not present on the gas chromatogram of the control bile. The mass spectra of this peak indicated a small parent ion at m/e 439 (see Appendix for graphical representation of mass spectrum and proposed fragmentation pattern) which corresponded to the structure represented below (Fig. 2.9).

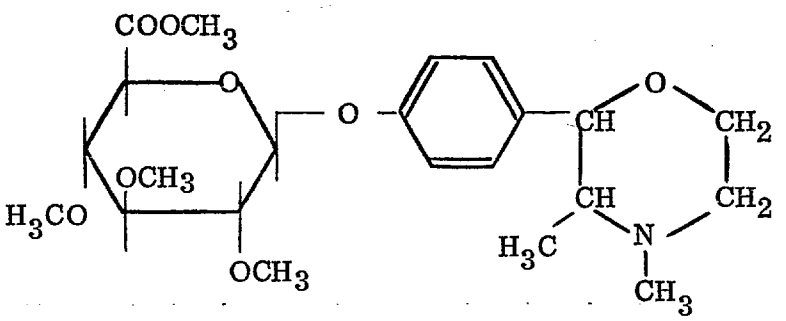


Fig. 2.9

The most important ions necessary for the identification of a permethylated glucuronide, according to Thompson and co-workers (1973) are 1) the m/e 101 and 201 ions, 2) the M^+ of the derivatised glucuronide and 3) the ion due to the aglycone, formed by glycosidic bond cleavage. Of the above criteria, certainly the ions of m/e 101 and 201 appeared to be present, having an intensity of 44% and 100% respectively. The parent ion was present at m/e 439, but with an intensity of only 2%. Cleavage of the glycosidic bond would be expected to give a fragment at m/e 206. This ion appeared to be present although only to the extent of 7%. The ion at m/e 408 represented a loss of 31 mass units from the parent ion which was probably $-CH_3O^{\cdot}$. The large ion at m/e 329 corresponded to a loss of 110 mass units from the parent structure or 79 mass units from m/e 408. The ion at m/e 408 could have lost

$-OOCH_3^{\bullet}$ to give m/e 361 and then lost a neutral molecule of CH_3OH to give m/e 329 in a manner similar to that described by Thompson and co-workers (1973). Unfortunately no metastable ions were present in the spectrum and so an accurate fragmentation pattern was not possible, but a theoretical one may be found in the Appendix. Thus the results indicated an O-glucuronide in the bile.

Paper chromatography of the bile on Whatman 3mm paper in systems A and B and subsequent radiochromatographic scanning showed the presence of 3 areas of radioactivity (System A: R_Fs 0.09, 0.29, 0.68; System B: R_Fs 0.07, 0.34, 0.79). Those areas at R_F 0.29 and 0.65 in system A corresponded to the glucuronide and aglycone respectively, found in the urine, as did those areas at R_F 0.34 and 0.79 in system B. The radioactivity associated with R_F 0.09 in system A and R_F 0.07 in system B was not present in the 24 hour urine. The chromatograms were sprayed with visualising agents, 1, 2 and diazotised 4-nitroaniline which gave a positive purple colour, indicative of a phenol, at R_Fs 0.68 and 0.79 in systems A and B corresponding in R_F to the aglycone. The ^{14}C found in the two systems used gave brown areas on spraying with 2, and a light blue colouration on spraying with reagent 1, indicating a secondary amino group.

Unchanged drug in the first 24 hour urine was estimated by the method described in the Materials and Methods section, under the heading 'Gas-liquid chromatographic assay for Preludin'.

In an attempt to isolate the metabolite at R_F 0.88 in system A, six rats were fed with Preludin and the labelled material in the same manner as for the isolation of the metabolite at R_F 0.68 (subsequently identified as a phenol). The urine was continuously extracted, chromatographed in system A and eluted with methanol (peak R_F 0.78) again as before. The volume of methanol was reduced *in vacuo* and chromatographed in system B. In this system two areas of radioactivity were

detected at R_Fs 0.57 and 0.71. The areas of ^{14}C were eluted (no attempt was made to elute the areas independently) with methanol and subjected to thin-layer chromatography in system E on Silica gel HF_{254} plates (20 x 20 x 0.1 cm) previously eluted with methanol. Radioactivity was detected on a suitable plate (5 x 20 x 0.1 cm) and showed two areas of radioactivity at R_Fs 0.47 and 0.59, the latter being the major. Again the metabolites were detectable by virtue of their ability to quench the fluorescence of the silica gel at 254 nm. Elution of both areas together from the plates, as above, and chromatography in system F gave two areas of radioactivity at R_Fs 0.51 and 0.61, clearly distinguishable as two bands under u.v. light (254 nm). Elution of these areas independently with methanol and further chromatography in system G gave R_Fs at 0.46 and 0.48 respectively. The areas of ^{14}C activity were eluted from the individual plates and subjected to mass spectrometry and also combined g.l.c./mass spectrometry. The spectra may be found in the Appendix and the retention times in Table 1. Thus it appeared that the compound isolated in the greater amount corresponded to the lactam, 2-phenyl-3-methyl-5-morpholinone. This was proved by comparison with the synthetic compound on paper chromatography, t.l.c., g.l.c. and mass spectroscopy. Too little of the other metabolite was isolated to give a meaningful trace on the g.l.c. but mass spectrometry by direct insertion indicated a compound very similar to the 2-phenyl-3-methyl-5-morpholinone, but with a slightly different fragmentation pattern and different base peak (see Appendix for graphical representations of the unidentified metabolite).

Quantitation of metabolites

Unchanged drug in the 24 hour urine was found to constitute approximately 10% of the dose. Metabolites in the urine were, (i) the phenolic metabolite, 2-(4'-hydroxyphenyl)-3-methylmorpholine, 28% of the dose, and (ii) the glucuronide conjugate of the phenol which accounted for 19% of the dose. The lactam metabolite, 2-phenyl-

-3-methyl-5-morpholinone was found to account for approximately 5% of the dose, and the unidentified metabolite also constituted 5% of the dose. Of the 68% of the radioactivity excreted in the urine over the 24 hour period, 67% of the dose was accounted for by the metabolites identified above.

Chemical oxidation of Preludin

The in vitro investigation of the metabolism of Preludin using the Udenfriend oxidation system (Udenfriend et al., 1954) proved to be unhelpful since paper chromatography, thin-layer chromatography and gas-liquid chromatography coupled with mass spectrometry all showed an excessive breakdown of the compound. None of the components could be identified.

Guinea pig

Excretion data for the three guinea pigs dosed with [^{14}C]-Preludin may be found in Tables 3 and 4. The total ^{14}C recovered was 97% and of that approximately 80% was found in the urine in the first 24 hours.

Isolation and identification of metabolites

A portion of the 24 hour urine (0.2-0.3 ml) was applied to Whatman 3 mm paper and chromatographed in systems A, B, C and D. Radiochromatogram scans and histograms may be found in the Appendix. The best separation of metabolites was obtained in system B where significant peaks occurred at R_Fs 0.57, 0.71 and 0.87.

In order to obtain the metabolites in a quantity large enough to carry out identification techniques, six guinea pigs were dosed orally with Preludin (20 mg/kg), one animal receiving [^{14}C]-Preludin. The urine was collected for 24 hours and the radioactive urine mixed with the urine of the other animals. The pH was adjusted to 8, saturated with NaCl and continuously extracted with diethyl ether which was dried over anhydrous Na_2SO_4, reduced in volume in vacuo and chromatographed in system B.

Areas of radioactivity were located by scanning the paper on the radiochromatogram scanner. Important observations on comparing the scans to those obtained for the rat (see Appendix) were that there was much more of the metabolite at R_F 0.71 excreted in the guinea pig, less unchanged drug and no indication of the phenolic metabolite at R_F 0.79 which was so prominant in the rat. The areas of radioactivity on the chromatograms run in system B were cut out, eluted with methanol as before, and further purified by chromatography in thin-layer systems E, F and G before finally being examined by g.l.c. and g.l.c./mass spectrometry. It was shown that the major metabolite in the 24 hour urine of the guinea pig was the lactam, 2-phenyl--3-methyl-5-morpholinone (about 50% of the dose) the next most abundant metabolite was the unidentified metabolite (10% of the dose) followed by unchanged material (6% of the dose) which was estimated by the g.l.c. method (see Materials and Methods). Incubation of a portion of guinea pig urine with the enzymes β-glucuronidase (Ketodase) and sulphatase gave equivocal results and although there was definitely a decrease in radioactivity at approximately R_F 0.2-0.4, reinforcement of the peaks corresponding to the metabolites mentioned above could not be detected. The unknown compound(s) corresponded to more than 10% of the dose and it was thought that these might be the result of N-oxidation of Preludin.

Metabolism of Preludin by in vitro liver preparations

Incubation of [^{14}C]-Preludin with whole liver homogenate, 10,000 x g supernatant, and microsomal fraction from rat liver as described in Materials and Methods showed that although the fractions were capable of N-demethylating 4-aminoantipyrine and hydroxylating aniline, they could not metabolize the [^{14}C]-Preludin. This was shown by extensive paper and thin-layer chromatography which in each case showed only one peak of radioactivity which corresponded to unchanged Preludin. The significance of this result is further expanded upon in the Discussion.

However, similar fractions of guinea pig liver showed metabolism of Preludin to have occurred. Thin-layer chromatography in systems E and G showed three areas of radioactivity. Practically, it was found that running the extract of the fraction first in system E, separately eluting the areas of ^{14}C and then carrying out further chromatography of the three areas in system G, gave a good separation. Further purification and examination of these areas by g.l.c. and g.l.c./mass spectrometry showed the metabolites to be the same as those found in the 24 hour urine of the guinea pig, namely the lactam, 2-phenyl-3-methyl-5-morpholinone, unchanged Preludin and the unidentified metabolite. The lactam and unidentified metabolite were present in approximately the same proportion as found in the urine (see Table 4). There was more unchanged Preludin present but this may be explained by the fact that the liver fraction was 'saturated' with Preludin as compared with the in vivo situation.

Tamarin monkey

From the excretion data, it may be seen that of the total amount of ^{14}C recovered (average 97%), approximately 70% was located in the first 24 hour urine. A portion of the urine (0.1 ml) was chromatographed in systems A, B, C and D and the resulting radiochromatogram scans and histograms may be found in the Appendix. Comparison between the radiochromatogram scans from the rat, guinea pig and Tamarin monkey urines in system B (see Appendix) showed that the lactam, phenolic metabolite and its glucuronide conjugate, unchanged drug and the unidentified metabolite were all present, but the majority of the radioactivity was associated with unchanged Preludin.

Identification of metabolites

The 24 hour urine and cage washings from one Tamarin monkey were adjusted to pH 8, saturated with solid NaCl and extracted with diethyl ether as for the rat and

guinea pig. The extract was chromatographed in system B and the areas of radioactivity detected on the radiochromatogram scanner. The ^{14}C areas on the chromatogram were eluted with methanol and run in systems E, F and G on aluminium-backed plates at the same time as standard compounds and a sample of the phenolic metabolite obtained from the rat. Thus the presence of the lactam, phenol, unchanged drug and unidentified metabolite was shown. A quantitative estimation of the metabolites was made by cutting up a chromatogram scan of the urine from both animals in system B and estimating the ^{14}C in the scintillation counter. The unchanged Preludin was further quantitated by using a sample of Tamarin monkey urine and using the g. l. c. method as explained in the section 'G. l. c. assay of Preludin' in the Materials and methods section.

Incubation of a portion of the urine with β-glucuronidase showed the presence of a glucuronide conjugate, the aglycone of which corresponded to that found and identified in rat urine as the phenolic metabolite.

A comparison between the excretion data and composition of the 24 hour urine for the rat, guinea pig and Tamarin monkey may be found in Tables 3 and 4.

Quantitation of the metabolites

From the 24 hour Tamarin urine, it was found that unchanged drug accounted for approximately 34% of the dose, the lactam, 2-phenyl-3-methyl-5-morpholinone, 12% of the dose, the phenol, 2-(4'-hydroxyphenyl)-3-methylmorpholine, 12% of the dose and its glucuronide conjugate, 3% of the dose. The unidentified metabolite corresponded to approximately 11% of the dose, thus leaving approximately 15% of the dose unaccounted for. This may be the result of N-oxidation of the material.

Man

The results of the excretion data from three men each given 25 mg [^{14}C]-Preludin orally may be found in Table 5. Of the ^{14}C excreted, most occurs (about 70%) in the first 24 hour urine.

Identification of metabolites

Chromatography in systems A and B were carried out on portions of the 24 hour urine voided by the three subjects. Radioactivity was detected on the radiochromatogram scanner (see radiochromatogram s and histograms in Appendix). The pattern of metabolite excretion was similar to that found in the rat and Tamarin monkey, namely the presence of the lactam, the phenolic metabolite and its glucuronide, unchanged drug and the unidentified metabolite. For more complete identification, a portion of the urine (800 ml) was adjusted to pH 8, saturated with solid NaCl and extracted with diethyl ether as before. A portion of the extract was subjected to thin-layer chromatography at the same time as standard compounds and run on both glass-backed and aluminium-backed Silica gel 60 F_{254} thin-layer plates in systems E, F and G. The plates were visualised using spray reagents 1, 2 and 3 (see Table 2 for colour reactions). A further portion was examined by g.l.c. using an OV-1 column (3%OV-1 on Chromosorb G AW-DMCS. Conditions used were injection port temperature 260°C; N_2, air and H_2 pressures were 275, 165 and 140 kPa [40, 24 and 20 lb/in^2] with a column temperature of 207°C). Unchanged phenmetrazine was detected (T_R 1.7 minutes) and so too was the lactam (T_R 5.0 minutes).

Incubation of a portion of the urine with the β-glucuronidase enzyme preparation (Ketodase) followed by paper chromatography in system A indicated a glucuronide conjugate of the phenolic metabolite.

Quantitative estimation of the metabolites was made by cutting up chromatograms of the urines run in systems A and B into 1 cm strips and estimating the radioactivity

Table 3. Excretion of Radioactivity by Various Species Receiving [^{14}C]-Preludin

Values are expressed as % of ^{14}C administered (averages with individual figures in parentheses)

Species		Rat	Guinea Pig	Tamarin Monkey	
Dose and route of administration		20 mg/kg orally	20 mg/kg orally	0.28 mg/kg intramuscular injection	
Number of animals		3	3	2	
Dose of ^{14}C (μCi/animal)		1.94	1.23	1.93	
				1	2
Urine	Day 1	68 (77, 65, 63)	82 (85, 78, 83)	74	69
	2	6 (5, 6, 7)	5 (5, 4, 6)	17	17
	3	2 (1, 2, 2)	1 (1, 1, 2)	4	6
	4	2 (1, 2, 3)	2 (2, 2, 1)	2	2
	5	1 (0, 1, 1)	<1 (0.2, 0.8, 0.4)	-	-
Total ^{14}C excreted		79	90	97	94
Faeces	Day 1	2 (1, 2, 3)	3 (4, 3, 2)		
	2	2 (1, 2, 2)	2 (2, 3, 2)		
	3	<1 (0.4, 1.1, 1.0)	2 (2, 1, 2)	1	2
	4	<1 (0.4, 1.0, 0.3)	-		
	5	2 (0.2, 3.0, 3.2)	-	-	-
Total ^{14}C excreted		6	7	1	2
Grand Total		85	97	98	96

Table 4. Metabolites of [^{14}C]-Preludin in the 24 hour urine of various species

Doses and animals were as in Table 3. Unchanged phenmetrazine was determined by the g.l.c. assay (see text). Other metabolites were determined by radiochromatogram scanning followed by cutting up the chromatogram into 1 cm strips and determining the radioactivity of each strip. Values are expressed as a % of the dose (averages with individual figures in parentheses).

Compound	Species... Rat	Guinea Pig	Tamarin Monkey	
			1	2
Phenmetrazine	10 (14, 7, 10)	6 (7, 4, 6)	31	37
2-Phenyl-3-methyl-5-morpholinone	5 (6, 4, 4)	50 (51, 56, 43)	14	9
2-(4'-Hydroxyphenyl)-3-methylmorpholine	28 (30, 29, 25)	N.D.	13	11
Glucuronide conjugate of 2-(4'-Hydroxyphenyl)-3-methylmorpholine	19 (22, 20, 17)	N.D.	3	3
Unidentified metabolite	5 (5, 4, 5)	10 (9, 9, 12)	12	9
Sum of above metabolites	67 (77, 64, 61)	66 (67, 69, 61)†	73	69
^{14}C in 24 hour urine	68 (77, 65, 63)	82 (85, 78, 83)	74	69

† Incubation of the urine with both a β-glucuronidase enzyme preparation and a sulphatase enzyme preparation followed by paper chromatography and radiochromatogram scanning showed the disappearance of some areas of radioactivity but there was no obvious indication as to which areas of radioactivity had been reinforced. It was thought that these areas of radioactivity might represent the products of N-oxidation

N.D. = not detected.

Table 5. Excretion of Radioactivity in Man Receiving [^{14}C]-Preludin Orally

Three subjects (Z. H. S., L. G. D. and R. B. F.) took orally 25 mg of [^{14}C]-Preludin in water. The dose of ^{14}C was 3.12 μCi/subject. Average values are given with ranges in parentheses.

Day after dosing	% of dose of ^{14}C found in urine
1	70 (62-82)
2	19 (17-21)
3	4 (2-6)
4	1.8, 1.21*
5	0.3, 0.2*
Total ^{14}C excreted	94 (87-100)

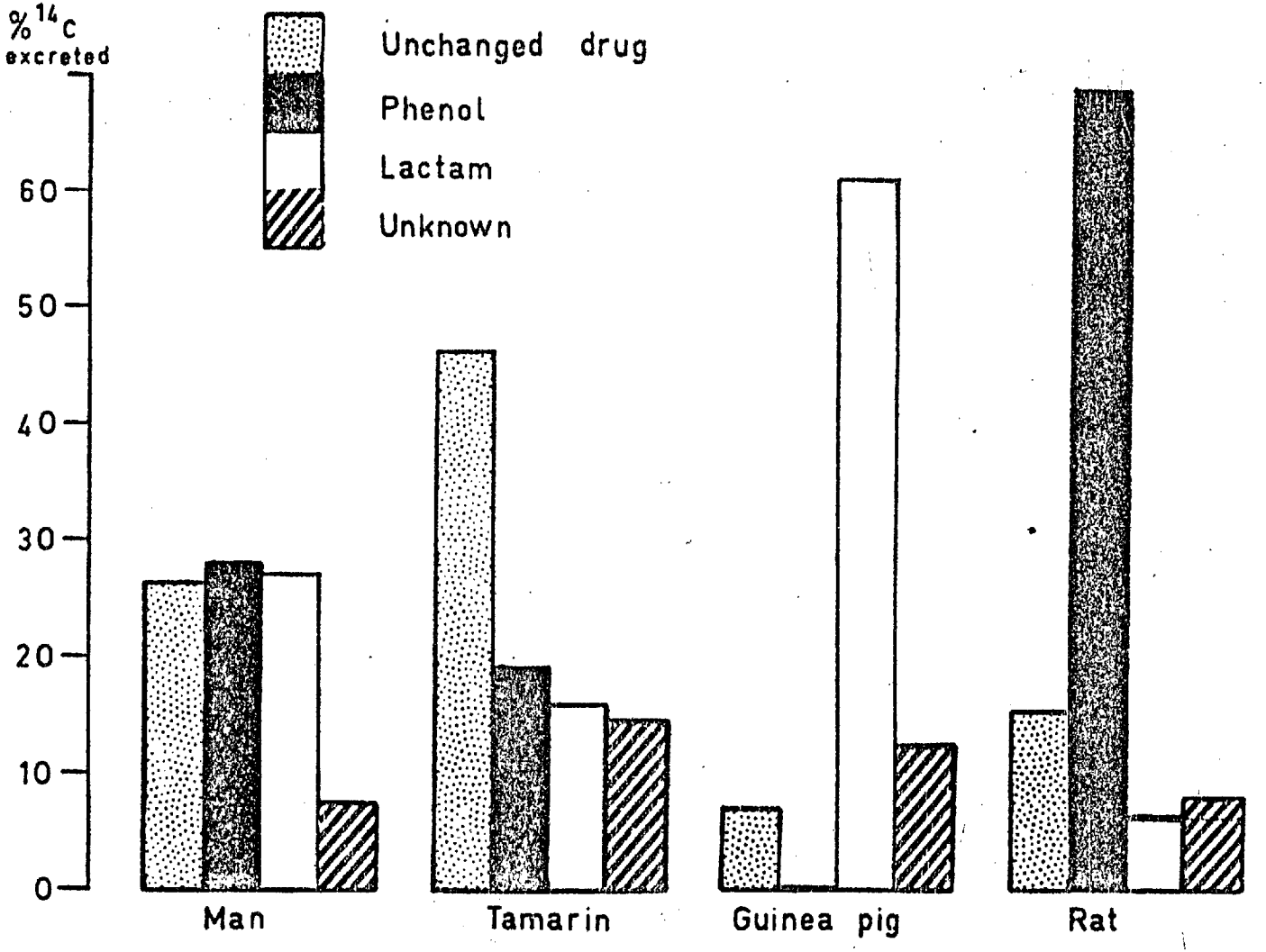
* Two subjects (Z. H. S. and R. B. F.)

Table 6. Metabolites of [^{14}C]-Preludin in Human Urine

Doses and subjects were as in Table 5. The urine analysed was the first 24 hour sample after dosing. Metabolites were determined by radiochromatogram scanning followed by cutting up the chromatograms into 1 cm strips and determining the radioactivity of each strip. Average values with individual figures in parentheses.

Compound	% of dose excreted in 24 hours as metabolite
Phenmetrazine	19 (21, 25, 11)
2-Phenyl-3-methyl-5-morpholinone	19 (17, 21, 19)
2-(4'-Hydroxyphenyl)-3-methylmorpholine	12 (10, 14, 12)
Glucuronide conjugate of 2-(4'-Hydroxyphenyl)-3-methylmorpholine	10 (6, 9, 14)
Unidentified metabolite	5 (6, 7, 5)
Sum of above metabolites	65 (60, 76, 61)
^{14}C in 24 hour urine	70 (62, 82, 66)

Comparison of composition of 24 hour urine from four species dosed with Preludin



in the scintillation counter. The composition of the 24 hour urine from the three subjects may be found in Table 6, whereby it was found that unchanged drug constituted approximately 19% of the dose and the lactam metabolite, 2-phenyl-3-methyl-morpholinone also 19% of the dose. The O-glucuronide accounted for 10% of the dose and its aglycone, 2-(4'-hydroxyphenyl)-3-methylmorpholine, for 12% of the dose. The unidentified metabolite was present to the extent of 5% of the dose leaving approximately 5% of the dose unaccounted for. This latter 5% may have been the result of N-oxidation.

Interpretation of mass spectra

Graphical representations of the mass spectra may be found in the Appendix. The major ions have been numbered according to their m/e value. Possible structures associated with these ions may also be found in the Appendix.

Pharmacological investigations

(i) Comparison of spontaneous activity in rat and guinea pig dosed orally with Preludin (20 mg/kg).

Using the Stoelting activity apparatus, the fine and gross movements of rats (10) and guinea pigs (10) were measured (see Materials and Methods section for more details). The results from rats dosed orally with Preludin and the same rats dosed with water were statistically compared using Students 't' test. A significant difference ($t = 4.2$, $p < 0.01$, $\emptyset = 8$) was found between the fine movements (for example, sniffing, twitching and head movements) of dosed and control rats, but no statistical difference was found on comparing gross locomotor movements, (see Appendix for graph). A similar comparison with guinea pigs at the same dose level showed no significant difference between fine and gross movements as compared with control values.

(ii) Investigation of the lactam metabolite of Preludin, 2-phenyl-3-methyl-5-morpholinone on the spontaneous activity of mice

Ten mice were dosed intraperitoneally on one day with 0.9% w/v NaCl solution in a suspension of 1% carboxymethylcellulose and 0.1% v/v Tween 80 and twenty four hours later the same mice were dosed intraperitoneally with an authentic sample of the lactam metabolite at a dose level of 100 mg/kg. The spontaneous activity was measured on the Stoelting activity apparatus and the data compared for control and dosed animals. No significant difference was found for either fine or gross movements of dosed and control animals over six hours (see Material and Methods section for more detail).

(iii) Effect of the lactam metabolite, 2-phenyl-3-methyl-5-morpholinone, on hexobarbitone sleeping-time

The time of restoration of the righting reflex was measured at two dose levels, 10 mg/kg and 100 mg/kg intraperitoneally, of authentic lactam. Control animals were dosed with 1% w/v carboxymethylcellulose and 0.1% v/v Tween 80 (see Materials and Methods section for the exact procedure followed). Statistical analysis of the results showed that there was no difference between control values and those obtained in dosed mice at 10 mg/kg. However, animals dosed at 100 mg/kg showed a significantly longer sleeping-time than the control mice ($t = 2.29$, $p < 0.05$, $\phi = 18$) indicating the lactam might have mild tranquillising properties. Also, at this higher dose level, ptosis developed some five minutes after dosing and lasted for approximately twenty minutes.

Discussion

Since its introduction in 1954, relatively few reports on the distribution and metabolism of Preludin have appeared. Engelhardt and co-workers (1958) studied the entero-hepatic circulation and distribution of [^{14}C]-Preludin in mice and rats and Quinn and co-workers (1967) followed the human plasma levels of Preludin using an in vitro radioactive complex with [^{3}H]-acetic anhydride. With regards to the metabolism of Preludin, Vidic (1957) detected a metabolite in human urine which gave a positive reaction with a ninhydrin spray, as well as unchanged material. In the rat, Engelhardt and his co-workers (1957) found two metabolites in the urine and three in the bile as well as unchanged drug in the urine of animals dosed at 100 mg/kg subcutaneously. Other workers (Beckett et al., 1967; Änggård, 1970) have detected unchanged drug in human urine by chromatographic and g.l.c. techniques and the binding of the drug to human plasma protein was reported by Franksson and Änggård (1970).

From the data summarised in Tables 3, 4, 5 and 6, it may be seen that there are species differences in the metabolism of Preludin, but it must be remembered that man and the Tamarin monkey received much smaller doses than the rat and guinea pig. Furthermore, Preludin was administered to the Tamarin monkeys by intramuscular injection which may affect the pharmacokinetics of drug absorption and elimination. Thus, in the latter species, the finding of more unchanged drug in the urine than in the other species where the dose was taken orally, may be explained by the fact that an intramuscular injection of the drug would result in an absorption into the systemic circulation and not necessarily into the portal vein for passage to the liver, thereby allowing more unchanged drug to be carried to the kidneys. On oral ingestion, absorption from the stomach into the blood stream ensures that the drug is presented to the liver very early in its systemic circulation.

Much of the drug may be metabolised by this 'first-pass' phenomenon and the remaining unchanged drug and metabolites (unless they are excreted in the bile) will then continue in the systemic circulation to the kidneys where excretion or reabsorption may occur.

Man and the Tamarin monkey appear to metabolise the drug in a similar fashion, both species producing the phenolic metabolite, the lactam and the unidentified metabolite. However, the Tamarin monkey excreted more unchanged material than man, 30% and 19% of the dose respectively, but man produced more of the phenolic and lactam metabolites than the Tamarin monkey, but excreted less of the unknown metabolite. The composition of the 24 hour urine of the rat and guinea pig differs considerably from man and the Tamarin monkey, and also from one another. In the rat, the major metabolite is the phenol, 2-(4'-hydroxyphenyl)-3-methylmorpholine, together with its glucuronide conjugate showing that the major route of metabolism is by aromatic hydroxylation and N-dealkylation to the lactam is a minor route. In the guinea pig however, the N-dealkylation is the major metabolic route whilst aromatic hydroxylation could not be detected by the techniques employed in this experiment, namely by cutting up strips of the chromatograms and estimating the radioactivity in a scintillation counter, as well as visual comparison of the radiochromatogram scans.

A further species difference has been noted using in vitro liver preparations of rats and guinea pigs. By using whole-liver homogenate, 10,000 x g supernatant and microsomal fractions of rat liver no metabolism of Preludin could be found. Similarly, amphetamine cannot be metabolised in vitro by rat liver microsomes (Axelrod, 1955; Dingell and Bass, 1969; Fuller et al., 1973) although metabolism does occur in the isolated perfused rat liver (Dingell and Bass, 1969) and rabbit liver microsomes (Daly et al., 1967; Parli et al., 1971). By using $^{18}O_2$-labelling

techniques Parli and co-workers have shown using rabbit liver microsomes, the production of benzylmethyl ketoxime and benzylmethyl ketone from amphetamine.

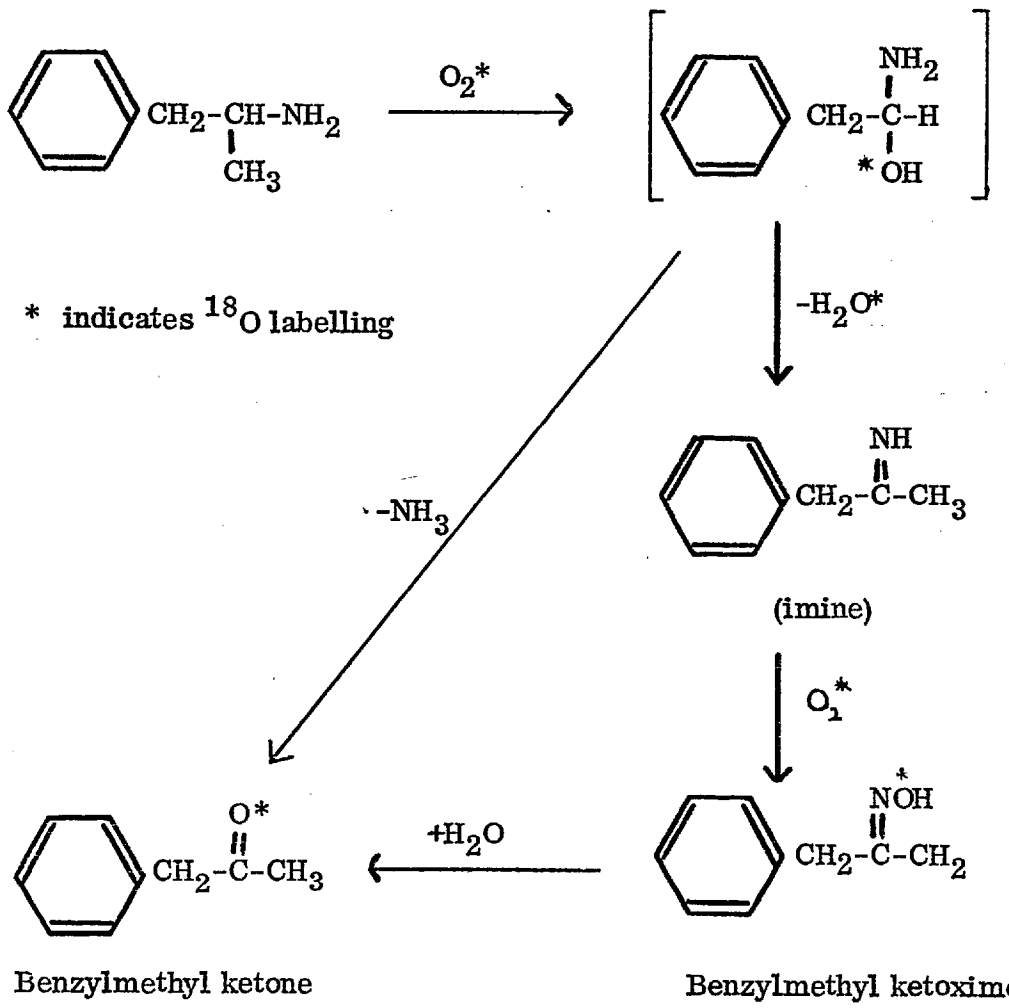


Fig. 2.10. In vitro metabolism of amphetamine by rabbit liver microsomes (Parli et al., 1971).

Axelrod (1955) suggested that the amphetamine was not deaminated by rat liver microsomes due to the presence of an inhibitory factor which is not present in rabbit liver microsomes, and he showed that addition of rat microsomes to rabbit microsomes inhibited the extent of deamination of amphetamine. Recently Werringloer and Estabrook (1973) have reported evidence for an inhibitory complex

of product and cytochrome P-450 generated during benzamphetamine metabolism by rat liver microsomes. Thus it is possible that amphetamine may produce a metabolite in the in vitro liver preparation which is inhibitory to further amphetamine metabolism. Franklin (1974a, b) has carried out some extensive studies on complexes of metabolites of amphetamines with hepatic cytochrome P-450 and suggests that the inhibitory intermediate might be a carbanion. Furthermore, amphetamine has been shown to inhibit the metabolism of hexobarbital in rat liver microsomes (Lal et al., 1970) and also to inhibit the demethylation of 4-chloro-N-methylaniline by 9,000 x g supernatant of rat liver (Louis-Ferdinand et al., 1972) possibly illustrating that an inhibitory intermediate complex is formed during amphetamine metabolism which may in some way interfere with the metabolism of other substrates. Similarly, the action of phenmetrazine in vitro may resemble that of amphetamine. However, incubation of Preludin with fractions of guinea pig liver in vitro gave the lactam, the unknown metabolite and also some unchanged drug. By using in vitro liver preparations Beckett and Salami (1972) claim to have isolated the N-hydroxy derivative of phenmetrazine, but they failed to unequivocally substantiate its structure. Unfortunately, no indication is given as to what species were studied.

Previous studies on the metabolism of amphetamine itself (Dring et al., 1970), methamphetamine (Caldwell et al., 1972a) and norephedrine (Sinsheimer et al., 1973) have shown that these amphetamines undergo extensive aromatic hydroxylation in the rat but not in the guinea pig. The present study shows this also to be the case for Preludin (see Table 4). Similarly in the guinea pig, amphetamine and methamphetamin are metabolised by transformation of the side chain and in Preludin, where the side-chain is incorporated into a ring structure, the major route of metabolism is N-dealkylation of the ring structure to give the lactam metabolite.

One of the reasons why the study of the metabolism of Preludin was initiated

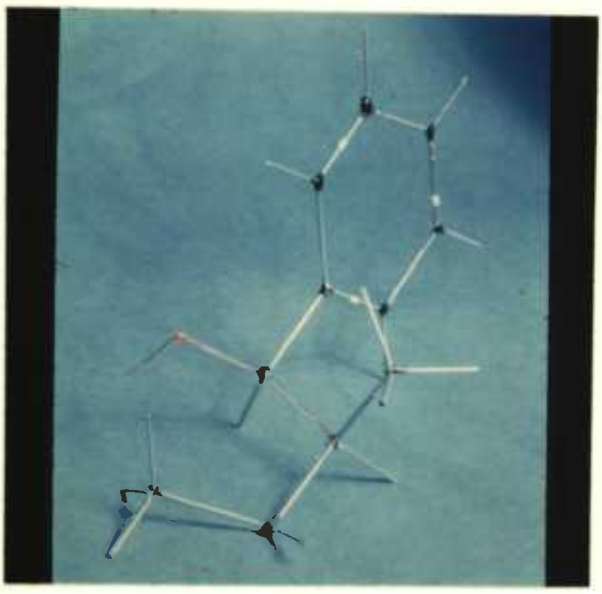
was to attempt to discover whether the compounds formed from Preludin by metabolism may be in any way responsible either for the intense central nervous system stimulation gained from a large dose of the drug or for the tolerance which quickly develops (Van Praag, 1968) to the anorexigenic and central stimulant effects. The compound 4-hydroxynorephedrine has been found to be a metabolite of amphetamine in the rat (Dring et al., 1970; Gropetti and Costa, 1969; Goldstein and Anagnoste, 1965; Lewander, 1970; 1971a,b; 1972) and to act as a putative 'false' transmitter at adrenergic nerve endings. Lewander (1971b) has also proposed that this compound might be involved in the tolerance to the peripheral but probably not to the central effects of amphetamine. The discovery of 4-hydroxynorephedrine as a metabolite of Preludin would come as no great surprise since metabolism of Preludin to amphetamine and hence to 4-hydroxynorephedrine is theoretically feasible. In fact it might be easier to form 4-hydroxynorephedrine from Preludin since the molecule is already substituted in the β-position, a reaction which, for amphetamine, is carried out in vitro and in vivo (Goldstein et al., 1964; Goldstein and Anagnoste, 1965; respectively) by dopamine-β-hydroxylase. The presence of 4-hydroxynorephedrine from Preludin would also help to explain the similarity of effects between Preludin and other amphetamines. However, in the species most likely to form the largest concentration of 4-hydroxynorephedrine, namely the rat, no such compound may be found. This has been checked additionally by reverse isotope dilution techniques on the 24 hour urine. A similar result is inferred in a paper from Costa and co-workers (1971). They found normal levels of noradrenaline, dopamine and 5-hydroxytryptamine in various brain parts, heart and lungs of rats receiving maximal tolerated doses of Preludin (5.6 μmol/kg, i.v.) whereas treatment with amphetamine caused a long lasting decrease of tissue noradrenaline, associated with the presence of 4-hydroxynorephedrine in the adrenergic neurones (Gropetti and

Costa, 1969). Therefore they concluded that Preludin must fail to form a metabolite capable of binding to the adrenergic nerve terminals. However, before the concept of 4-hydroxynorephedrine being a 'false' transmitter to explain some of the effects of Preludin is abandoned, it is interesting to postulate whether the major metabolite of Preludin in the rat, namely 2-(4'-hydroxyphenyl)-3-methyl-morpholine, may itself act as a 'false' transmitter. By using structural models, it may be seen in the photographs (over) that the stereochemistry of phenmetrazine and ephedrine in this case, are exactly the same. Therefore, it is perfectly feasible that the phenolic metabolite, 4-hydroxyphenmetrazine, may be taken up into adrenergic nerve terminals and produce similar pharmacological effects to that of 4-hydroxynorephedrine. But further work is necessary to see whether 4-hydroxyphenmetrazine fulfils the criteria for a transmitter within the sympathetic nervous system.

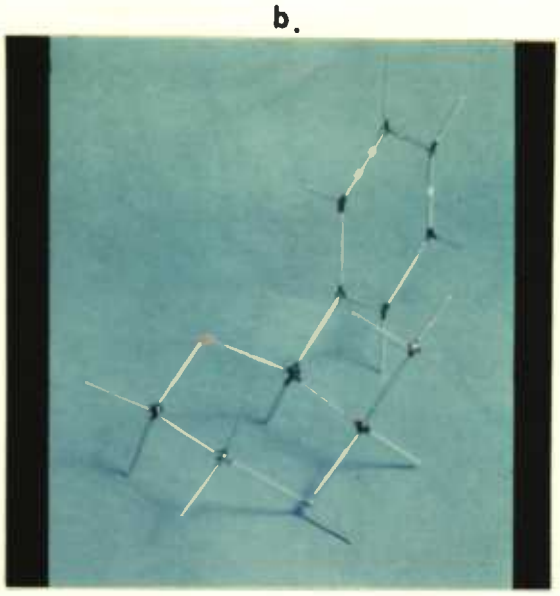
A further interesting find in this present study of Preludin metabolism is the formation of the lactam metabolite, 2-phenyl-3-methyl-5-morpholinone, in the four species studied. In man, this compound represents 19% of the dose excreted in 24 hours. This compound, also known as Fenmetramide, has been shown to exhibit muscle relaxant and tranquillising properties (Gannon and Poos, 1967) and it is interesting to speculate whether this metabolite is responsible for any of the effects of Preludin. For example, amphetamines including Preludin are capable of producing psychogenic dependence (Oswald and Thacore, 1963) and typical withdrawal symptoms are largely psychological and include fatigue, insomnia, lassitude and depression (see Van Praag, 1968). As Van Praag points out, these symptoms may be typical of the withdrawal syndrome or thay may be a relapse of the symptoms which led to the abuse in the first place, but it may be conceivable that on chronic ingestion of the drug there may be an accumulation of the lactam metabolite

Photographs illustrating the similarity in stereochemistry between a) ephedrine and b) phenmetrazine

a.



b.



which may then begin to exert its own therapeutic effect, an effect that may become more prominant on cessation or withdrawal of the drug. Further pharmacological experimentation on, for example, the half-life of the lactam in plasma, or protein-binding may be relevant here. It is therefore possible that the metabolism of Preludin may form two 'active' metabolites, namely the phenolic metabolite, 4-hydroxyphenmetrazine and also the lactam, 2-phenyl-3-methyl-5-morpholinone.

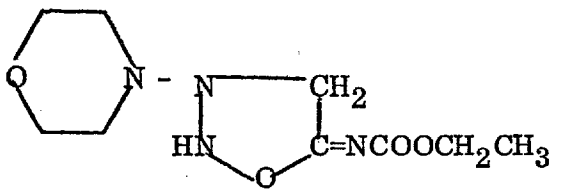
In all four species examined, one unidentified metabolite constantly recurs. Initally it was thought that this might be the lactone, that is 2-phenyl-3-methyl-6-morpholinone, however, synthesis of the lactone and comparison of R_F values in several chromatographic systems as well as mass spectra of the unknown and lactone showed them to be dissimilar. Comparison of the unknown metabolite with N-(2-hydroxyethyl)-norephedrine, a pre-cursor in the synthesis of Preludin, also proved unsuccessful using chromatography and mass spectrometry. The graphical representation of the mass spectrum of the unidentified product (to be found in the Appendix) shows the highest mass value at m/e 191; this may not in fact represent the parent ion because there are a limited number of compounds which can have their parent ion at this mass ion value, and also expect to exhibit a fragmentation pattern similar to the lactam metabolite. These alternatives are:-

i) the lactone, 2-phenyl-3-methyl-6-morpholine

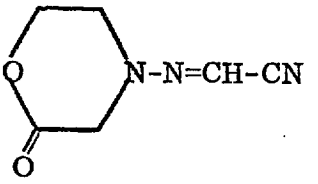
ii) N-methylated phenmetrazine, that is, phendimetrazine, and

iii) the lactam, 2-phenyl-3-methyl-5-morpholine.

As stated above, the identity of the unknown metabolite appears not to agree with the properties of the lactone, although a lactone metabolite of a morpholine compound (see Fig. 2.11) has recently been reported (Tanayama, 1974).



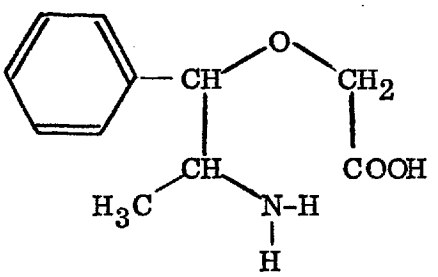
Molsidomine



Lactone metabolite

Fig. 2.11

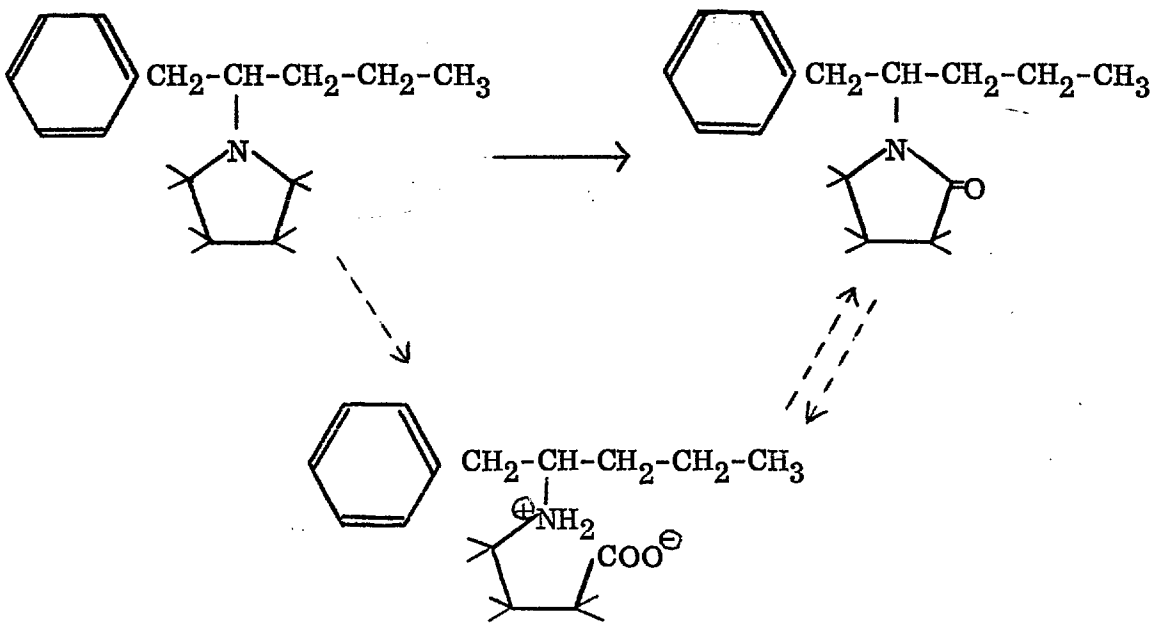
The mass spectrum of the unknown is different to that of the lactam and neither compound exhibits similar R_F values in all the chromatography systems used. This leaves phendimetrazine as the last alternative. N-Methylation by a non-specific methylase is possible and occurs in the case of normeperidine going to meperidine and pyridine being converted to N-methylpyridine (Parke, 1968). Comparison of the mass spectra shows that there is very little in common between the two compounds except the mass ion m/e 191. Thin-layer chromatography in systems E, F and G showed phendimetrazine and the unknown metabolite to have not too dissimilar R_F values, but paper chromatography, especially in systems B, C and D showed their respective R_F values to be very different. If the m/e 191 is not the parent ion, other possibilities as to the identity of the metabolite can be forwarded, for example, the compound shown below:-



m.w. 195

It may be remembered that earlier on in the discussion it was mentioned that Vidic, in 1957, found a metabolite in human urine which gave a positive reaction with ninhydrin. Certainly the above compound would be expected to show a colour with

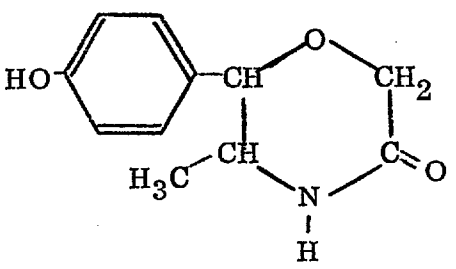
ninhydrin, perhaps not as intense as for an α-amino acid but certainly a colouration due to the primary amine function. A similar compound has been isolated from the urine of rabbits dosed with prolintane (Yoshihara and Yoshimura, 1972) and the authors postulated that the amino acid derivative might be an obligatory intermediate in the formation of the major metabolite, the lactam (see Fig. 2.12.)



(After Yoshihara and Yoshimura, 1972)

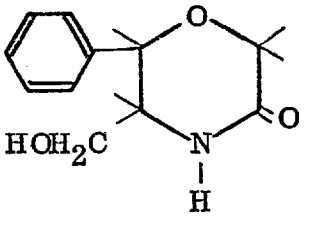
Fig. 2.12

Examination of the mass spectrum of the unknown compound fails to give an m/e 60 which would be indicative of the rearrangement ion found in carboxylic acids (Williams and Fleming, 1973), but even so, this amino acid cannot be entirely excluded. Another possible metabolite is the lactam phenol as represented by the structure below:-



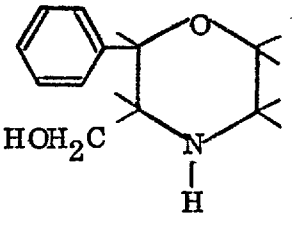
m.w. 207

It is possible that such a compound would not give a parent ion at m/e 207 but may show one at (M^+ - 18) mass units, that is m/e 189. This ion does not appear to be present in the mass spectrum of the unknown metabolite. Studying the metabolism of prolintane in rabbits, Yoshihara and Yoshimura (1972) have identified the lactam phenol by chromatography, i.r., u.v. and n.m.r. spectroscopy. They also found the lactam alcohol as well as the free alcohol; the equivalent structures in the phenmetrazine nucleus being shown below:-



m.w. 207

Lactam alcohol of phenmetrazine

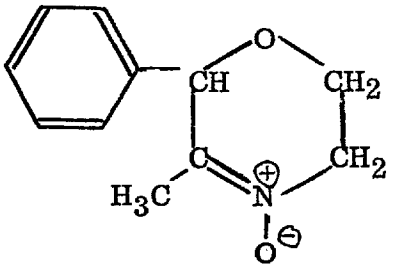


m.w. 193

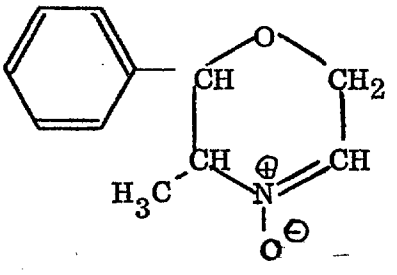
Alcohol of phenmetrazine

One other likely metabolite is that suggested by Beckett and Salami (1972), namely the N-hydroxy compound. As stated previously, the authors found no parent ion in the mass spectrum at m/e 193. Beckett and co-workers (1973b) on their studies of the identification of N-oxygenated products have obtained the N-hydroxy derivatives of some secondary amphetamines, for example, N-methyl-, N-ethyl-, N-propyl- and N-butyl- amphetamine. Under the conditions used two decomposition products were identified, namely the corresponding primary amine and the nitrone. A nitrone was also found to be a metabolite of Fenfluramine *in vitro* using guinea pig and rat liver preparations (Beckett *et al.*, 1973a). They conclude that the nitrone may arise from N and α-C metabolic oxidation of Fenfluramine to form a dihydroxy compound which dehydrates to form the nitrone. Alternatively the hydroxylamine metabolite undergoes oxidation on the α-C atom followed by dehydration. Thus, the nitrone may be considered to be a non-enzymic

product. For phenmetrazine, the nitrone may have two possible structures.



m.w. 191



m.w. 191

Referring back to the work of Beckett et al., (1973a), a mass spectrum of the nitrone of Fenfluramine produced an ion at $(M-15)^+$. If this were to apply to Preludin the $(M-15)^+$ would be m/e 176; this mass ion is indeed present in the mass spectrum of the unknown metabolite. Furthermore, the mass ion at m/e 69 in the mass spectrum of the unknown metabolite in keeping with the fragmentation pattern of Preludin may correspond to (CH_2=CH-NH-CH=CH_2^+). Final elucidation of the structure of the unknown metabolite will probably be brought about in two ways:

i) by obtaining a sufficient amount of the metabolite from biological sources, of which the use of in vitro guinea pig liver preparations may be the method of choice, or

ii) by chemical synthesis. Attempts at preparing the N-hydroxy derivative of Preludin by the direct action of m-chloroperbenzoic acid and also via a cyanoethyl derivative have both been shown to have their respective problems, however refinement of one or both of these methods may yet prove successful for obtaining the desired hydroxylamine (see Beckett et al., 1973c). From thence, the conversion to the nitrone could be carried out (Beckett et al., 1973a).

The evidence thus far would tentatively support a nitrone structure for the unknown metabolite. There are however two points to consider. The first is that so far no nitrones of amphetamine-like compounds have been found in the urine and the second

is that this nitrogen oxidation product accounts for a fairly large proportion of the dose in the 24 hour urine in all the species studied, that is, 5% in rat and man, 10% in guinea pig and 11% in Tamarin monkey.

It may be recalled that in the Introduction to Preludin under the heading of "Chemical aspects of Preludin", the different configurations of the phenmetrazine molecule were illustrated. One of the reasons for this may be explained now. The authentic Preludin obtained from Boehringer and the [^{14}C]-Preludin synthesised by two different methods for this present study all belonged to the threo-or trans-configuration. The erythro-or cis-conformer has been synthesised by Clarke (1962) and found to have a different i. r. spectrum and approximately a 30°C difference in melting point (trans - 180-181°C; cis- 152-154°C). The lactam metabolite synthesised for comparison with that found in the urine of rat, guinea pig, Tamarin monkey and man was the (±)-cis-conformer prepared by Clarke (1962), who also synthesised the (±)-trans-conformer. Unfortunately time precluded the synthesis of the (±)-trans-conformer but it may be that both the cis-and the trans-forms share identical chromatographic properties and give identical mass spectra. Certainly they would not give similar n. m. r. spectra. Thus, although the lactam has been obtained as a metabolite of Preludin in all species studied, the comparison has been with the cis-lactam. This implies that the phenmetrazine molecule has changed its configuration from trans-to cis-and the only way that this could be explained is by the morpholine ring opening and re-forming in a different configuration, which would be less dynamically stable, at least before further oxidation at position 5.

The dextrorotatory isomer of Preludin has been synthesised using the laevo-rotatory antipode N-(2-hydroxyethyl)-norephedrine (Stark et al., 1961) and has been claimed to be therapeutically more efficacious than the racemate (Preludin) or the laevorotatory isomer(Stark et al., 1961). Although the toxicity is the same, this

dextrorotatory compound has been claimed to have a far more powerful psychostimulant effect than the racemate, and also not to cause a rise in blood pressure. However, no mention is made of its anorexigenic effect compared with the racemate.

The antipodes of amphetamine exhibit different pharmacological activity and also show a difference in the extent of their metabolism in various species (Dring et al., 1970). No such metabolic studies appear to have been carried out for the antipodes of phenmetrazine, but it is feasible that, like amphetamine, a difference in the extent of metabolism occurs with this compound too.

Some of the effects of dextrorotatory phenmetrazine (dexphenmetrazine) have been studied in humans, by Bartak and Skranc (1972) who measured oxygen intake, ventilation, heart rate and lactic acid levels in students at rest and on a bicycle ergometer, and by Bohdanecký and co-workers (1972) who studied the effect of (+)-phenmetrazine on rat reticular unit activity.

In conclusion, it would appear that Preludin is metabolised in much the same way as other amphetamines, and there is a species variation. Aromatic ring-hydroxylation predominates in the rat, side-chain oxidation in the guinea pig and a combination of both pathways in man and Tamarin monkey. Two of the hitherto unidentified metabolites have been isolated and characterised, namely the phenol and the lactam. These compounds may warrant further pharmacological investigation to see if they are in any way responsible for some of the adverse reactions seen on the prolonged administration of Preludin. It would also be most useful to investigate the metabolism of the individual antipodes and to see whether this relates in any way to their pharmacology. The identity of the unknown metabolite is at present not certain, however, the data so far collected would indicate that it is most probably a nitrone.

CHAPTER THREE

Investigation into the Fate of [^{14}C]-(±)-Amphetamine in the Tamarin Monkey

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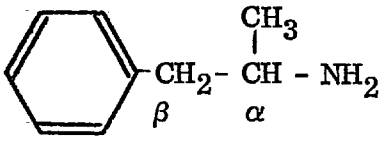
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INTRODUCTION

Amphetamine, (±)-2-amino-1-phenylpropane, was first prepared by Edeleano in 1887. There are a number of methods of synthesis (see Hider, 1969, for a review) including the incorporation of tritium, 14-carbon and deuterium into the molecule. The amphetamine molecule contains one asymmetric centre, namely the α-carbon atom. (see below in Fig. 3.1).



Amphetamine

Fig. 3.1

There are two isomers of amphetamine and therefore three forms in all. Namely (+)- or d- amphetamine, which has the 2S configuration (Cahn et al., 1956), otherwise known as dextroamphetamine, (-)- or l- amphetamine, which has the 2R configuration, and (±)- or dl- amphetamine. The racemic form was resolved by Alles in 1939. In 1933, Alles observed that amphetamine produced an arousing action on animals under anaesthesia. This prompted its use as a respiratory stimulant in cases of barbiturate intoxication. Amphetamine, as free base or carbonate, was introduced under the name Benzedrine as a drug suitable for administration by inhalation to produce shrinking of the nasal mucosa in head colds and sinusitis (Krantz and Carr, 1965).

Amphetamine is a sympathomimetic agent with mainly indirect effects on adrenergic receptors. The (+)-antipode has a more potent central nervous system stimulatory effect than the (-)-antipode, although this latter antipode is slightly more potent in producing vascular effects such as an increase in both systolic and

diastolic blood pressure and pulse pressure (Aboul-Enein, 1971). The involvement of endogenous brain amines has been discussed under the heading 'Amphetamine, Phenmetrazine and Norephedrine: Effects on catecholamines and false transmitter theory.' Suffice it to say that the wide spectrum of effects caused by amphetamine is not due to the interaction with one particular amine, but rather it is dependent on 5-hydroxytryptamine (5-HT), noradrenaline and dopamine. Indeed, treatment with the catecholamine-depleting agent reserpine and the subsequent effects of amphetamine administration have led to equivocal results. Some investigators found an increase in the effect of amphetamine following reserpine treatment whilst others found no change and yet others found a decrease (Van Rossum, 1972). But treatment with α-methyl-para-tyrosine (which inhibits the biosynthesis of catecholamines) in combination with reserpine completely blocks the central effects of amphetamine (Van Rossum, 1972).

The toxic dose of amphetamine may vary considerably from one individual to another but it is reasonably well tolerated in therapeutic doses. Acute toxicity may manifest itself in a number of ways which may be conveniently subdivided into (i) central effects, ii) cardiovascular effects and (iii) gastro-intestinal effects.

(i) Central effects:- These include agitation, disorientation, restlessness, tremor, anorexia, insomnia, euphoria, confusion, delirium, hallucinations, panic states, convulsions, fever and coma. Suicidal or homicidal tendencies may occur.

(ii) Cardiovascular effects:- These include effects such as headache and palpitation. Particularly with high doses, the systolic and diastolic pressure may be increased and tachycardia, anginal pain or cardiac arrythmias may occur.

(iii) Gastro-intestinal effects. Amongst these effects are dryness of the mouth, metallic taste, nausea, difficulty in micturation, constipation, vomiting, diarrhoea and abdominal cramps.

Aplastic anaemia and panhaemocytopenia have occasionally occurred after prolonged use. Chronic amphetamine toxicity produces symptoms similar to those of acute toxicity. However, weight loss may be severe and accompanied by psychotic reactions and hallucinations.

Amphetamine has been administered for its anorexigenic effect in the treatment of obesity but its precise mode of action is not yet fully understood. A role of 5-HT in the mediation of the effects of anorexigenic drugs has been proposed by some workers (Opitz, 1967; Jespersen and Scheel-Krüger, 1970; Goldman et al., 1971), but the results of these authors have been equivocal. Other studies have concluded that catecholamines, especially noradrenaline, play an important effect in the anorexigenic action of amphetamine (Goldman et al., 1971; Leibowitz, 1970; Abdallah, 1971; Berger et al., 1971; Holtzmann and Jewett, 1971). The anorexigenic effect of (+)-amphetamine has been blocked by α-methyl-para-tyrosine (Abdallah, 1971; Holtzmann and Jewett, 1971; Frey and Schulz, 1973) and also by disulfiram, an inhibitor of dopamine-β-hydroxylase (Frey and Schulz, 1973). Further explanations for the anorexigenic action of amphetamine have included a reduction in smell and taste thresholds for food, decreased gastric motility and an inhibitory effect of elevated concentrations of plasma free fatty acids (produced by amphetamine) on the 'feeding centres' or 'hunger centres' in the hypothalamus (Turner, 1969). Amphetamine does not significantly increase basal metabolism or nitrogen excretion and Modell (1960) thought that the anorexia may be due to elation, enabling the patient to adhere more readily to a restricted diet.

Nelson and Forfar (1971) have reported that amphetamine causes a low incidence of teratogenic effects when administered to pregnant mothers. But there was a definite increase in the number of congenital abnormalities in infants born to mothers receiving amphetamine during pregnancy.

The first clinical application of amphetamine was in the treatment of narcolepsy (Prinzmetal and Bloomberg, 1935) and has been used for postencephalitic Parkinsonism and in conjunction with phenobarbitone and other agents in the treatment of epilepsy. It has also been used in the treatment of mild depressive neuroses, orthostatic hypotension and as an adjuvant in the treatment of chronic alcoholism and poisoning by depressant drugs. Amphetamine has been reported to stop persistant hiccup and to be of value in the treatment of urinary incontinence and nocturnal enuresis (Martindale, 1972a). However, the non-medical use of amphetamine both with respect to 'doping' in sport and drug abuse by addicts has far exceeded the therapeutic use of the drug. In the case of 'doping', suppression of fatigue is the desired effect, whilst euphoria is the aim in addicts. At very high dose levels (Oswald and Thacore, 1963, have reported the intravenous administration of ten times the therapeutic dose of amphetamine), the phenomenon of stereotyped behaviour may also be observed, involving such actions as continuous chewing or teeth-grinding movements with rubbing of the tongue along the inside of the mouth and lower lip often resulting in ulcers of the tongue and lip (Ashcroft et al., 1965). On repeated administration, tolerance to some of the effects of amphetamine may occur, for example, tolerance to the central stimulation and anorexigenic effects. However, not all parts of the central nervous system become tolerant at the same rate, so that when the doses are increased or become more frequent in order to obtain the same effect as with previous doses, nervousness and insomnia may persist as the dose is increased. Addicts dependent on amphetamine are prone to accidents, aggressive anti-social behaviour and, particularly after intravenous administration, to psychotic episodes. The sudden withdrawal of amphetamine does not cause physiological disruption or physical withdrawal symptoms, yet fatigue, prolonged sleep and hyperphagia are observed (Kramer et al., 1967; Kalant, 1966). Lewander (1971a, b; 1972) has suggested that 4-hydroxynorephedrine

a metabolite of amphetamine, may be responsible for the induction of tolerance to some of the effects of amphetamine in rats. This substance can deplete noradrenaline (Daly et al., 1966) and replace it in the neurosecretory stores, suggesting that it may interfere with adrenergic neurone function by acting as a false transmitter. Lewander concludes (1971b) that 4-hydroxynorephedrine might be involved in the tolerance developed to peripheral but probably not to the central effects of amphetamine.

Absorption, Distribution, Metabolism and Excretion of Amphetamine

Amphetamine, when administered orally, is well absorbed from the gastrointestinal tract and is distributed in most organ tissues. High levels may be found in the brain and cerebrospinal fluid (Aboul-Enein, 1971). In man and rat, the extent to which amphetamine is excreted unchanged is correlated with its lipid solubility, some 20-30% of unchanged amphetamine appearing in the urine of man (Williams et al., 1973). An extensive study on the metabolism of amphetamine in man and six other species has been reported by Dring and co-workers (1970) and Fig. 3.2 shows the major pathways of metabolism. The nature and amounts of the metabolites were found to vary from one species to another. Further details concerning the study may be found in the Discussion where the results of the administration of amphetamine to the Tamarin monkeys has been interpreted and compared to the results from the studies by Dring and co-workers (1970) and Caldwell and co-workers (1972b).

Urinary excretion of amphetamine is greatly influenced by urinary pH. In acidic urine 80% of the drug is excreted unchanged, whilst under conditions of alkalinity the drug is reabsorbed by the renal tubules and only 2-3% is excreted (Beckett and Rowland, 1965; Rowland and Beckett, 1966). Advantage is taken of this fact when dealing with cases of amphetamine poisoning, the urine being

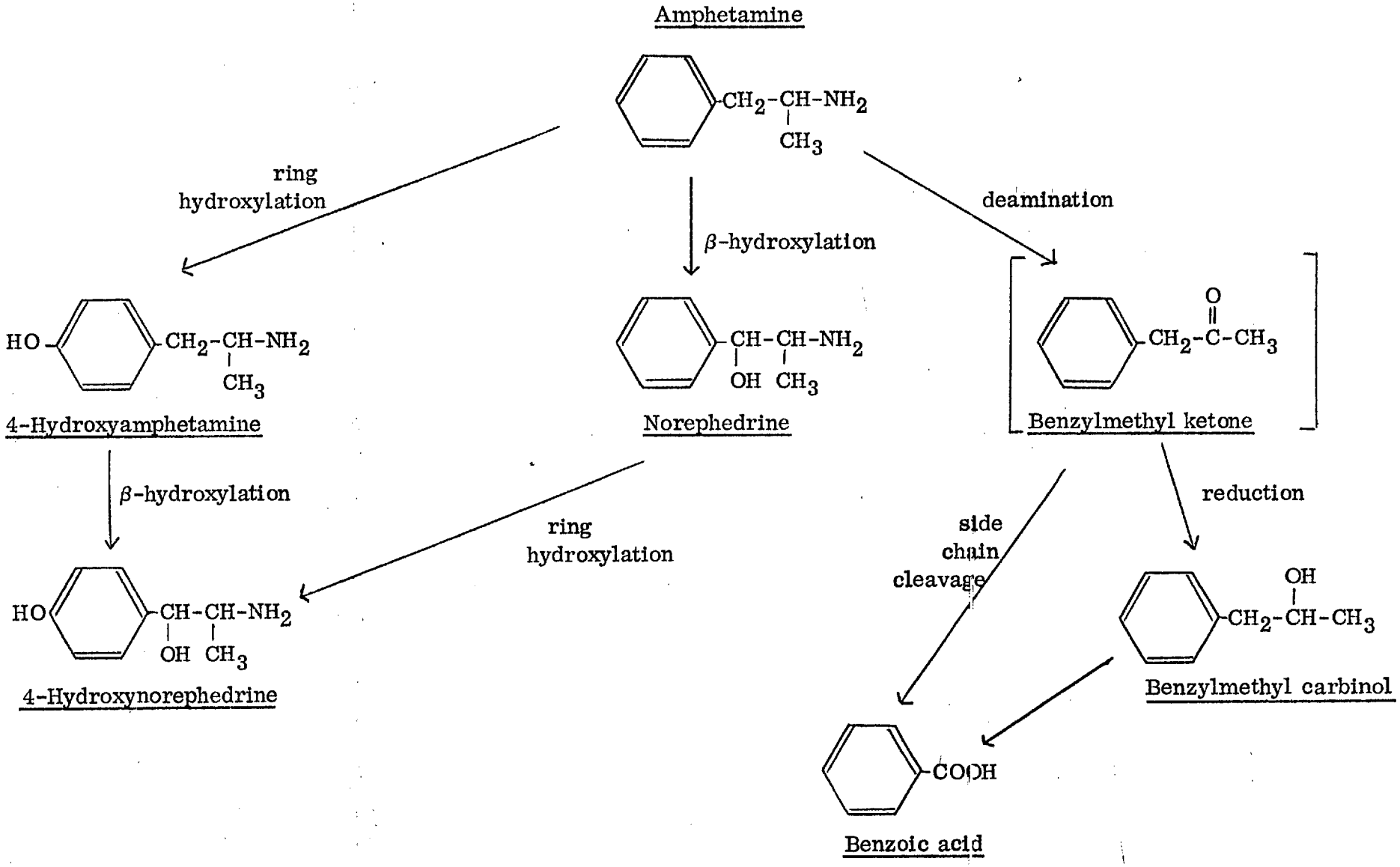


Fig. 3.2. Pathways of Amphetamine Metabolism.

All compounds, plus conjugates where appropriate, have been detected in urine after administration of amphetamine (Dring et al., 1970).

maintained at an acidic pH by the administration of ammonium chloride. Rowland and Beckett (1966) further warned that care should be exercised when amphetamines are given to patients on carbonic anhydrase inhibitors or to other patients liable to have a high urine pH. Recently it has been reported that a group of subjects receiving a balanced protein diet and producing an acidic urine excreted much more unchanged amphetamine than a group receiving a low protein diet and producing an alkaline urine (Wesley-Hadzija, 1971). The stimulant effect of amphetamine might be prolonged and increased under conditions of urine alkalinity (Smart and Turner, 1966). Cannabis may also enhance the stimulatory effect of amphetamine (Whyte, 1967).

Investigation of the Metabolic Fate of [^{14}C]-(±)-Amphetamine Sulphate in Tamarin Monkey

The Tamarin monkey is a species of marmoset which has not been extensively used for drug metabolism studies. By using this species for an investigation into the metabolic fate of amphetamine, norephedrine and Preludin, it was hoped that the metabolism would prove to be similar to that found in man and thereby justify the further use of these animals in relation to human drug metabolism and toxicity studies. This could eliminate the use of the rhesus monkey, the primate of choice, which is much larger, more expensive and more difficult to house satisfactorily.

Materials and Methods

Compounds

[^{14}C]-(±)-Amphetamine sulphate, ((±)-2-amino-1-phenyl[^{14}C]propane) m.p. 302°C (decomp.), Paredrine, ((±)-2-amino-1-(4'-hydroxyphenyl)propane) hydrobromide, m.p. 190-192°C were the gifts of S.K. & F. Laboratories,

Philadelphia, Pa., U.S.A. (±)-4-Hydroxynorephedrine (2-amino-1-(4'-hydroxyphenyl)propan-1-ol) m.p. 198°C, was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

The [^{14}C]-(±)-amphetamine sulphate (1.96 μCi in Water for Injection (0.5 ml)) was prepared by dissolving a known weight of compound (1.64 mg) in Water for Injection, and under sterile conditions, dividing the solution into ampoules and sterilising them in an autoclave maintained at 121-123°C for 15 minutes at 0.70 kPa.

Animals

Two male Tamarin monkeys (500 ± 20 g), obtained from Animal Suppliers, London, U.K., were housed individually in perspex metabolism cages of dimensions (31 x 45 x 38)cms. The animals received no food for the 24 hours immediately after dosing, but were allowed free access to water. Thereafter, the monkeys were returned to their normal diet.

Classification of Tamarin monkey

Order: Primate; Sub-order: Anthropoidea; Family: Callitrichidae; Genus: Saguinus; Species: Oedipus.

Radiochemical techniques

The counting of ^{14}C in urine and the preparation and counting of radiochromatograms and isotope dilution procedures were carried out as described by Dring and co-workers (1970) and Caldwell and co-workers (1972a), except that in order to estimate the total [^{14}C]-benzoic acid content, the urine (1.0 ml) was refluxed with an equal amount of 10M NaOH for 3 hours (Baldwin, 1961) and then carrier benzoic acid added in the normal manner (Dring et al., 1970).

Radioactivity in the faeces was estimated by homogenising the faeces in water (30 ml) in a Waring Blendor and diluting to 50 ml with washings from the homogeniser. Portions (0.3 ml) were pipetted into scintillation vials, 10M NaOH

(0.2 ml) was added to each vial and left overnight. Six drops of H_2O_2 (100 vol.) were added and the vials were carefully heated to control the effervescence. The pH was adjusted to 7 with 10M HCl. On cooling, the vials were filled with scintillator fluid (Bray, 1960) and counted in either the Packard Tri-Carb Scintillation Counter, Model No. 3214, or the Packard Tri-Carb Scintillation Counter, Model No. 3320.

Chromatography

Descending paper chromatography was used on Whatman No. 1 paper. The R_F values in the two solvent systems chosen, together with colour reactions of amphetamine and other known metabolites with diazotised 4-nitroaniline are listed in Table 7.

Ion Exchange Chromatography

A convincing separation of 4-hydroxyamphetamine and 4-hydroxynorephedrine from the same biological source, for example urine, proved to be very difficult by classical paper and thin layer chromatographic methods. Since 4-hydroxynorephedrine has been reported to be a metabolite of (±)-amphetamine in man (Caldwell et al., 1972b), using a modified method of Lewander (1971a), the urine from the Tamarin monkeys was examined for the presence of this compound as well as 4-hydroxyamphetamine. To the 24 hour Tamarin urine (1.0 ml) was added β-glucuronidase (1.0 ml; Ketodase, William R. Warner & Co. Ltd., Eastleigh, U.K.) and the solution incubated at 37°C for 48 hours. At the end of this period 2M NaOH (0.4 ml) was added to the incubate and the solution extracted with toluene/isoamyl alcohol 16% v/v (10 ml) with continuous shaking for 15 minutes. The organic phase (containing the non-phenolic aromatic amines) was withdrawn and put aside for further analysis. Radioactivity remaining in the aqueous phase (containing 4-hydroxyamphetamine, 4-hydroxynorephedrine, hippuric and benzoic acids)

was counted in the scintillation counter before being taken down to dryness under reduced pressure on a rotary film evaporator. The remaining residue was extracted with ethanol (5.0 ml), filtered, evaporated to dryness, and the remaining solid taken up in water (2.0 ml), a portion (0.1 ml) being withdrawn for counting. Carrier 4-hydroxyamphetamine and 4-hydroxynorephedrine (100 μg of each) were added to the aqueous solution, the pH adjusted to 4.5 and the aqueous extract placed on a cation exchange resin (Dowex 50-W 200-400 mesh $Na^{\oplus}$form; column 5 x 0.5 cm i.d.). The column was washed with water (5.0 ml), then 1M sodium acetate buffer, pH 6, containing 0.1% w/v ethylenediaminetetramine and again with water (10.0 ml). The amines were eluted from the column with 1M HCl and collected in 2 ml fractions on a Central fraction collector (Aimer Products, London, U.K.). The fluorescence of each fraction was measured on an Aminco-Bowman spectrophotofluorimeter (American Instrument Co. Inc., Silver Spring, Maryland, U.S.A.) at an activating wavelength of 275 nm and an emission wavelength of 320 nm. Calibration curves over the range 1-10 μg/ml were constructed for 4-hydroxyamphetamine and 4-hydroxynorephedrine in 1M HCl. Recoveries from the column for the two compounds were 80.2% and 97.5% respectively. Portions (0.5 ml) of the 2ml eluates were counted in order to estimate the radioactivity per tube to see if any ^{14}C activity was associated with the fluorescent measurements of the two 4-hydroxylated amines. Paper chromatography was carried out on the remainder of the 2 ml eluates.

The organic phase separated earlier was back-extracted into 0.1M HCl (1.0 ml), evaporated to dryness, the residue taken up in ethanol (0.2 ml) and chromatographed in system II.

Table 7. R_F Values of Amphetamine and Possible Metabolites

Whatman No. 1 paper was used for descending chromatography using the following solvents: I, Butan-1-ol saturated with 1.5M ammonia-ammonium carbonate buffer (Fewster and Hall, 1951); II, 3-Methylbutan-1-ol : 1,1-dimethylpropanol : water : formic acid (98-100%) 5:5:10:2 by volume, (Alleva, 1963).

Compound	System I	System II	Colour with diazotised 4-nitroaniline
Amphetamine	0.87	0.53	Purple/red
4-Hydroxyamphetamine	0.77	0.33	Purple
4-Hydroxynorephedrine	0.64	0.19	Purple
Norephedrine	0.81	0.40	Purple/red
Hippuric acid	0.31	0.80	Red/brown
Benzoic acid	0.42	0.91	Red/brown
Benzoyl glucuronide	0.07	0.60	None
4-Hydroxybenzoic acid	0.08	0.61	Brown

Preparation of diazotised 4-nitroaniline spray (after Wickström and Salvesen, 1952).

4-Nitroaniline (0.25 g) was dissolved by gentle heating in M HCl (25 ml) and this solution was diluted to 50 ml with ethanol and cooled. To a portion of this solution (10.0 ml) was added sodium nitrite (0.1 g). Immediately after the nitrite had dissolved the chromatogram was sprayed with the solution. Five minutes later the chromatogram was sprayed with a 0.5M solution of NaOH in ethanol. Amines and phenols appeared as coloured spots but were not permanent.

Enzyme hydrolysis

Urine portions (0.5 ml) were mixed with an equal volume of a β-glucuronidase enzyme (Ketodase) and incubated at 37°C for 48 hours. Controls containing boiled enzyme and saccharo-1,4-lactone (Sigma Chemical Co., London, U.K.) were incubated at the same time. Similarly, hydrolyses using a Sulphatase enzyme (Type H-2, Sigma Chemical Co., London, U.K.) were set up using 500 units of enzyme per ml of urine.

Results

Identification of metabolites

By using paper chromatography and plotting histograms of the distribution of radioactivity (see Appendix) it was apparent that the vast majority of radioactivity excreted in the urine over 24 hours was the unchanged drug at R_Fs 0.87 and 0.53 in systems I and II respectively (53 and 58% of the dose). The hydroxylated metabolite, 4-hydroxyamphetamine was present at R_Fs 0.77 and 0.33 in systems I and II respectively (4.7 and 6.2% of the dose). Hippuric acid (R_F 0.31 in system I; R_F 0.80 in system II) was also present (about 2% of the dose). Either incubations with the β-glucuronidase enzyme or hydrolysis of the urine (0.5 ml) with 10M NaOH for three hours followed by chromatography in system I, showed a glucuronide to be present, the aglycone of which produced a radioactive peak at R_F 0.42 in system I, corresponding to benzoic acid. Thus, the conjugate appeared to be benzoyl glucuronide.

Isotope dilutions carried out by the methods of Dring et al. (1970) confirmed the presence of 4-hydroxyamphetamine and hippuric acid (see Table 8).

Discrepancies between isotope dilution figures and chromatographic figures

Table 8. Metabolites of [^{14}C]-Amphetamine found in the 24 hour urine of three Primates

Species	Man		Tamarin Monkey	Rhesus Monkey	
Isomer of amphetamine	(±)*	(±) †	(±)	(+)†	(-)†
Dose and route	0.29 mg/kg Oral	0.07 mg/kg	0.3 mg/kg Intramuscular injection	0.7 mg/kg Oral	
% Dose of [^{14}C] excreted in 24 hour urine	57, 64	61, 64, 73	72, 78	57, 80	59, 82
% Dose excreted unchanged in 24 hour urine	18, 20	35, 23, 32	50, 58	3.8, 31	23, 34
% Dose excreted as benzoic acid (total)	28, 27	24, 22, 17	5, 6	38, 31	20, 27
% Dose excreted as hippuric acid	n.d.	19, 17, 14	5 (2ǂ), 3 (2ǂ)	20, 0	6, 8
% Dose excreted as 4-hydroxyamphetamine (total)	2, 4	1, 6, 2	6 (4ǂ), 4 (3ǂ)	0, 11	1, 6
% Dose excreted as 4-hydroxynorephedrine (total)	0.3, 0.4	n.d.	0.2, 0.4	n.d.	n.d.

* Data from Caldwell et al., (1972b) † Data from Dring et al., (1970) ǂ Isotope dilution values n.d. = not determined.

Table 9. Excretion of Radioactivity by Tamarin Monkey receiving [^{14}C]-(±)-Amphetamine Sulphate

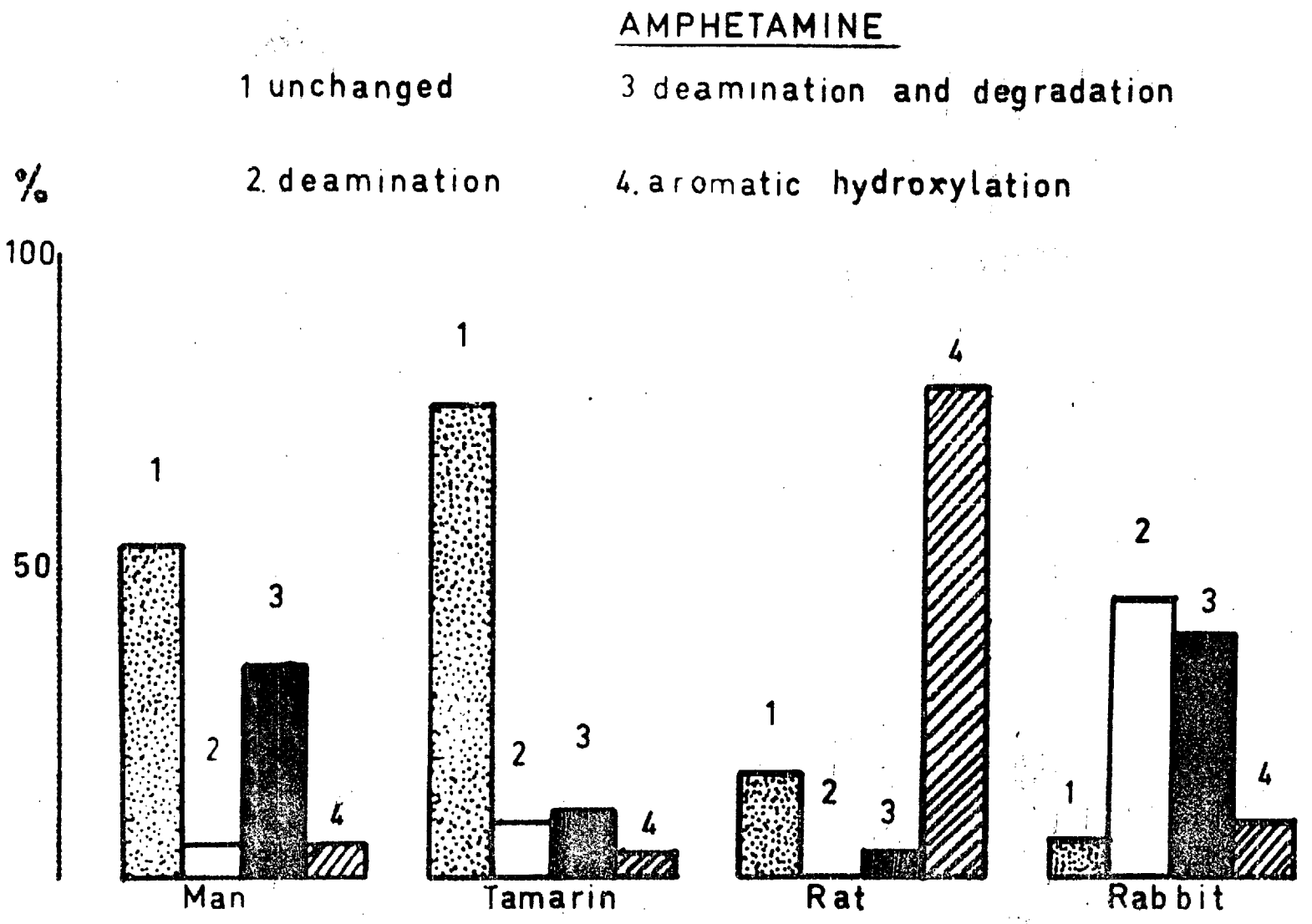
Each animal was dosed with [^{14}C]-(±)-Amphetamine sulphate (0.3 mg/kg, 1.9 μCi) intramuscularly. Values are expressed as a % ^{14}C administered.

	Time after dosing (hours)	Tamarin Monkey 1	Tamarin Monkey 2
Urine	24	71.8	78.1
	48	11.8	12.5
	72	2.4	2.1
	96	0.8	0.7
Faeces	96	2.4	1.9
	Total ^{14}C recovered (%)	89.2	95.3

Table 10. Metabolites of [^{14}C]-(±)-Amphetamine sulphate in the 24 hour urine of the Tamarin Monkey

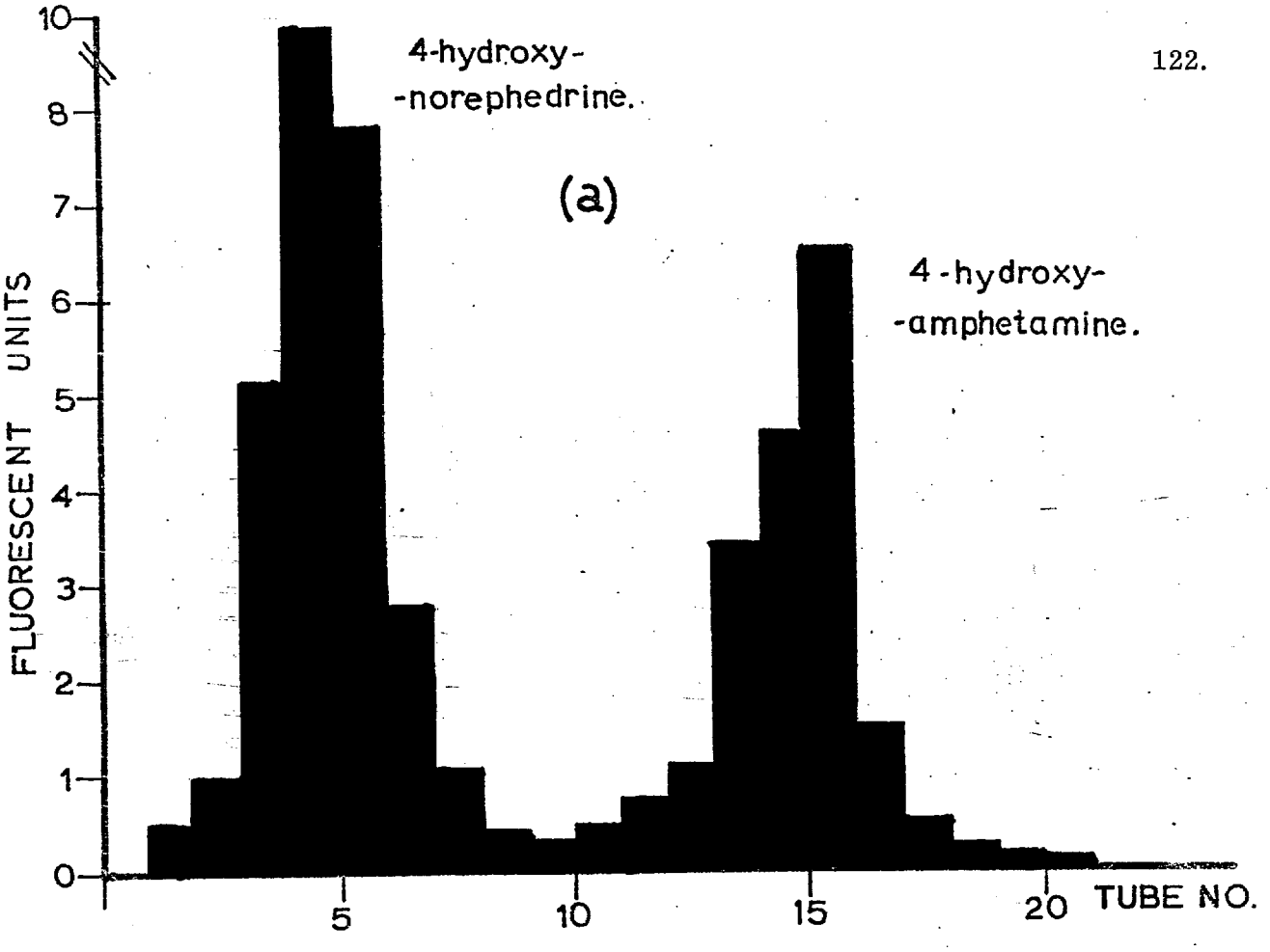
Dose used was as in Table 8. Figures in parentheses indicate those values obtained by reverse isotope dilution procedures (see text).

Compound	% dose in 24 hour urine	
	1	2
Amphetamine	50.4	58.0
4-Hydroxyamphetamine	5.7 (4.2)	4.7 (3.1)
4-Hydroxynorephedrine	0.2	0.4
Hippuric acid	4.5 (2.0)	2.9 (2.1)
Total benzoic acid	4.5	5.9
Unidentified	3.2	4.6
Sum of metabolites	68.5	76.5
% ^{14}C excreted in 24 hour urine	71.81	78.14



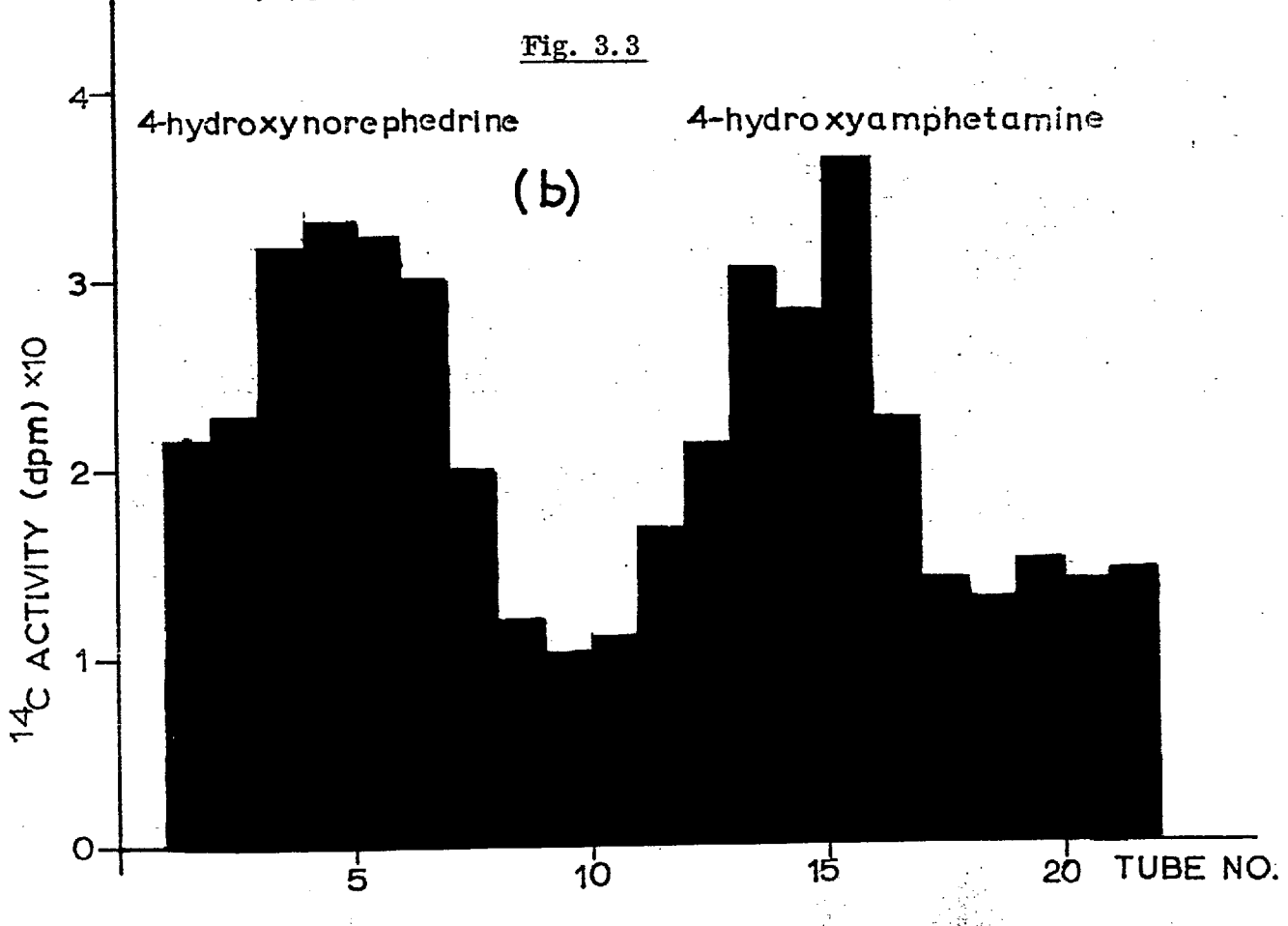
Comparison of Metabolic Pathways of Amphetamine as Illustrated by the Composition of the 24 hour Urine Samples of Four Species

(after Williams, 1974)



Histograms of (a) fluorescent units vs. tube number containing 2.0 mls of eluate obtained by treating 24 hour Tamarin monkey urine as in the text and passing through a Dowex 50W 200-400 mesh Na^+ form ion-exchange resin and (b) radioactivity (dpm) vs. tube number.

Fig. 3.3



may be due, in some part, to the small number of counts being dealt with in the urine which was a result of the low specific radioactivity of the [^{14}C]-amphetamine sulphate.

Excretion data for the two Tamarin monkeys, and the composition of the 24 hour urine from both animals may be found in Tables 9 and 10 respectively.

Ion-exchange chromatography further substantiated the presence of 4-hydroxyamphetamine to be approximately 3% of the dose excreted in 24 hours. There was also some evidence for the presence of 4-hydroxynorephedrine, accounting for 0.2 and 0.4% of the dose.

No sulphate conjugates were apparent by enzyme hydrolysis followed by paper chromatography.

Discussion

From the results it may be seen that approximately 75% of the administered radioactivity is excreted in the first 24 hours in the urine, and of this, approximately 55% in unchanged material. The significance of this may be examined by comparing it with the figures obtained by Dring and co-workers (1970) and Caldwell and co-workers (1972b) from their studies on man. By using the figures of Dring and co-workers (1970) it may be seen (Table 8) that the percentage of unchanged drug in the urine of man is greater than that found in the urine of the rhesus monkey after oral dosing of either the (+)- or (-)-isomers of amphetamine.

The percentage of unchanged drug excreted in the urine in 24 hours by the Tamarin monkeys appears to exceed that of man and rhesus monkey (see Table 8).

An evaluation of the degree of aromatic ring hydroxylation in man, Tamarin monkey and rhesus monkey indicates that there is a close correlation in the amount of 4-hydroxyamphetamine and 4-hydroxynorephedrine excreted in the 24 hour urine of these species (see Table 8).

Further scrutiny of the data shows no further similarity between the amounts of metabolites formed by man and the Tamarin monkey. Although man and Tamarin monkey both appear to form free benzoic acid and its glycine conjugate, the amounts excreted by the two species differ widely (Table 8).

In conclusion, more unchanged amphetamine is excreted in the Tamarin monkey urine over a period of 24 hours than in that of man over the same period. The aromatic ring hydroxylated metabolites, 4-hydroxyamphetamine and 4-hydroxynorephedrine were produced in approximately the same amounts in the 24 hour urine of man and Tamarin monkey dosed with the (±)-antipode. Products of the side-chain degradation of amphetamine in Tamarin monkey urine were much lower than that found in the 24 hour urine of man and rhesus monkey (Table 8).

It is important to emphasise that the dose administered to the Tamarin monkeys was injected by the intramuscular route, thereby possibly leading to a difference in the pharmacokinetics of absorption, distribution and excretion of unchanged drug and metabolites, whereas in man and rhesus monkey, the dose was administered by the oral route.

CHAPTER FOUR

Investigation into the Fate of [^{14}C]-(±)-Norephedrine in the Tamarin Monkey

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INTRODUCTION

Norephedrine, erythro-2-amino-1-phenylpropan-1-ol, (see Fig. 4.1) has two asymmetric centres, namely the α and β carbons and there are therefore four possible isomers. Those forms belonging to the erythro-series are known

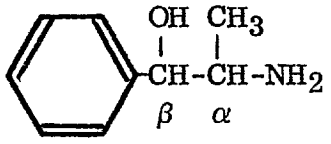


Fig. 4.1

as the norephedrines, and those with the threo-configuration are known as the nor-pseudoephedrines. The (±)-erythro-racemate was resolved by Jarowski and Hartung in 1943. The erythro-racemate has been synthesised in this laboratory with the incorporation of a ^{14}C-label on the β-carbon atom (Sinsheimer et al., 1973).

Norephedrine is a sympathomimetic agent with a similar locus of action to ephedrine (the N-methylated form of norephedrine), but it is somewhat more active as a vasoconstrictor and less active as a central nervous system stimulant and on responses mediated through β-receptors. Norephedrine is usually administered orally for the symptomatic treatment of allergic conditions such as bronchial asthma or hay fever. It has also been given to reduce the appetite in obesity (Martindale, 1972b). Given intramuscularly or by slow intravenous infusion, norephedrine has been used to raise the blood pressure in hypotensive states such as those occurring in surgery or with spinal anaesthesia. A 1-3% solution or nasal jelly has been used for nasal decongestion in rhinitis and sinusitis (Weisberg and Breslow, 1966). Toxic effects have included diffuse abdominal pain, throbbing headache, nausea, elevated blood pressure, restlessness, incoherent speech and vomiting. Kane and Green (1966) reported temporary acute psychotic episodes on using norephedrine as a nasal decongestant. An enhancement of the effects of norephedrine when given in conjunction

with tranylcypromine have been reported by Cuthbert and co-workers (1969).

Absorption, Distribution, Metabolism and Excretion of Norephedrine

Norephedrine hydrochloride is readily absorbed from the gastrointestinal tract although the compound has poor lipid solubility. Axelrod (1953) studied the metabolism of (-)-norephedrine in the dog and rabbit, and Beckett and Wilkinson (1965) carried out their studies in man. In 1973, Sinsheimer and co-workers, using [^{14}C]-(±)-norephedrine, studied the metabolism in rabbit and rat as well as man. (The results of these studies will be compared with the results obtained from dosing Tamarin monkeys with the [^{14}C]-(±)-norephedrine and may be found in the 'Discussion').

The study of the metabolism of norephedrine has taken on a greater significance since a metabolite of norephedrine, namely 4-hydroxynorephedrine has been cited as a putative 'false' transmitter within the central nervous system (Costa and Gropetti, 1970; Lewander, 1970). 4-Hydroxynorephedrine has been found as a urinary metabolite of amphetamine in the rat (Lewander, 1971a,b), in man (Caldwell et al., 1972b), both in amphetamine-dependent and naive subjects (Sever et al., 1973a) and also in in vitro studies by Goldstein and Anagnoste (1965).

The compound has also been found as a urinary metabolite of 4-hydroxy-amphetamine in man (Sever et al., 1973b; Sjoerdsma and Von Studnitz, 1963). Lewander (1971a,b) has outlined a possible mechanism for the induction of tolerance to some of the effects of amphetamine, invoking the 'false' transmitter properties of 4-hydroxynorephedrine in his argument.

The study undertaken in the Tamarin monkey was initiated in order to compare the elimination of norephedrine and its metabolites primarily with that in man and also with other species. Further, structural similarities exist between norephedrine and Preludin and an examination of norephedrine in a species which has also been

used to investigate Preludin might indicate any similarities between the metabolism of the two drugs.

Investigation of the Metabolic Fate of [^{14}C]-(±)-Norephedrine in Tamarin Monkeys

Materials and Methods

Compounds

(±)-Norephedrine hydrochloride (2-amino-1-phenylpropan-1-ol), m.p. 190-192°C was obtained from Sigma Chemical Co., London, U.K.). (±)-4-Hydroxynorephedrine (2-amino-1-(4'-hydroxyphenyl)propan-1-ol), m.p. 198°C was obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.

The [^{14}C]-(±)-norephedrine hydrochloride was synthesised in this laboratory by Sinsheimer and co-workers (1973), the final product having a specific activity of 2.45 μCi/mg.

The [^{14}C]-(±)-norephedrine hydrochloride was prepared for injection by dissolving a known weight (1.2 mg) in Water for Injection (3.0 ml) and the solution was sterilised by filtration through a Millipore filter. The filtrate was divided into 0.5 ml ampoules.

Animals

See amphetamine studies, Chapter 3.

Radiochemical techniques

See Chapters 2 and 3.

Chromatography

Descending paper chromatography was used on Whatman No. 1 chromatography paper. The R_F values in the three solvent systems chosen, together with the appropriate colour reactions with diazotised 4-nitroaniline for norephedrine and other related compounds are listed in Table 11.

Table 11. R_F Values of Norephedrine and Related Compounds

Whatman No. 1 paper was used for descending chromatography using the following solvent systems: III, butan-1-ol saturated with 1.5M ammonia - ammonium carbonate buffer (Fewster and Hall, 1951); IV, 3-methylbutan-1-ol : 2-methylbutan-1-ol : water : formic acid (98-100%) 5:5:10:2 by volume (Alleva, 1963); V, butan-2-ol : formic acid (98-100%) : water 100:2:10 by volume (Matsuda, 1971).

Compound	Solvent system: III	IV	V	Colour with diazotised 4-nitroaniline
Norephedrine	0.64	0.81	0.37	Purple/red
4-Hydroxynorephedrine	0.34	0.61	0.17	Purple
4-Hydroxybenzoic acid	0.81	0.00	0.85	Brown
4-Hydroxyhippuric acid	0.80	-	0.71	Purple
Benzoic acid	0.93	0.31	0.88	Red/brown
Hippuric acid	0.83	0.18	0.79	Red/brown
1,2-Dihydroxy-1-phenylpropane	0.92	-	0.84	-
1-Hydroxy-2-oxo-1-phenylpropane	0.94	0.71	0.80	-
1,2-Dioxo-1-phenylpropane	-	-	0.81	-

Enzyme hydrolysis

See Chapters 2 and 3.

Results

Identification of metabolites

By chromatographic evidence in all systems, undoubtedly the most abundant excretion product in the 24 hour urine of the Tamarin monkey was unchanged norephedrine (R_Fs 0.64, 0.87 and 0.37 in solvent systems III, IV and V respectively). Chromatography solvent system V was less definitive than was hoped, the most significant point being the lack of radioactivity in the area where 4-hydroxynorephedrine was expected (R_F 0.17).

In system IV there appeared to be some benzoic acid (R_F 0.31), some hippuric acid (R_F 0.18) and some radioactivity associated with the origin which may have indicated either 4-hydroxybenzoic acid or benzoylglucuronide. The enzyme hydrolysis with the β-glucuronidase preparation appeared to affect this area, but exactly which peak was reinforced was difficult to elucidate, probably because the peak at the origin was associated with so little radioactivity.

System III illustrated the presence of hippuric and benzoic acids (R_Fs 0.83, 0.93 respectively). This is better illustrated on the histogram plot of the chromatogram.

The presence of hippuric acid was confirmed by reverse isotope dilution. The value obtained, namely 3% of the dose, compared very favourably with the value estimated chromatographically. Unchanged norephedrine was estimated by cutting up the chromatograms into 1 cm strips and estimating the radioactivity in the scintillation counter. Figures of 75% and 78% were obtained for the two Tamarin monkeys dosed.

Table 12. Excretion of Radioactivity by Tamarin Monkeys receiving [^{14}C]-(±)-Norephedrine Hydrochloride

Each animal was dosed with [^{14}C]-(±)-norephedrine hydrochloride (0.3 mg/kg; 0.4 μCi) intramuscularly. Values are expressed as a % of ^{14}C administered.

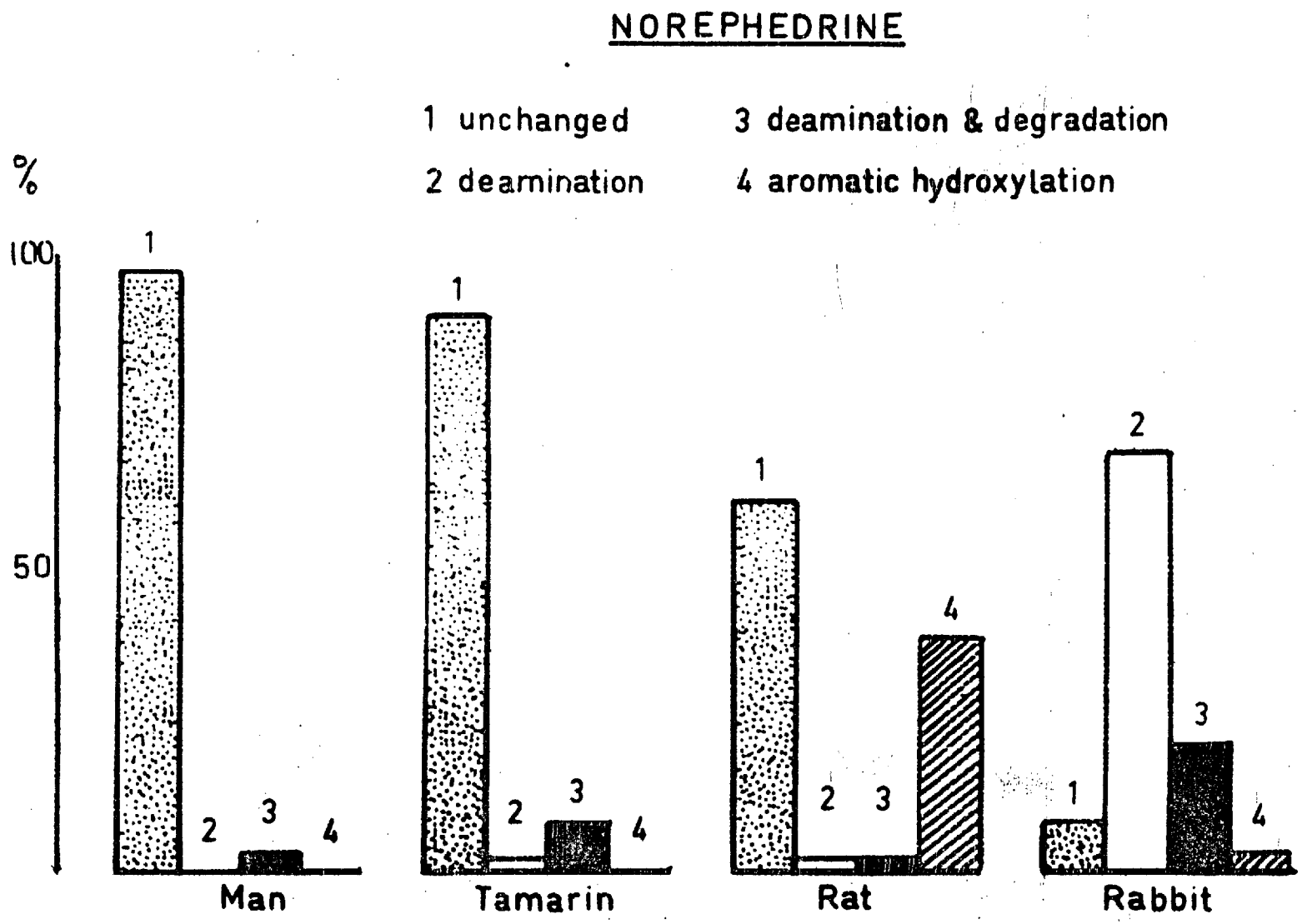
	Time (hours) after dosing	Tamarin Monkey 1	Tamarin Monkey 2
Urine	24	86.0	88.7
	48	7.8	5.9
	72	0.7	0.9
	96	0.3	0.1
Faeces	96	1.3	2.1
Total ^{14}C recovered (%)		96.1	97.7

Table 13. A Comparison of the Percentage of [^{14}C]-(±)-Norephedrine Metabolites found in the 24 hour Urine of Four Species of Animal

Species	Man†	Tamarin monkey	Rabbit†	Rat†
Dose and route	0.36 mg/kg oral route	0.3 mg/kg intramuscular route	12 mg/kg oral route	12 mg/kg oral route
% dose of [^{14}C] excreted in 24 hour urine	94 (92-96)	86, 88	89 (83-96)	80 (78-81)
% dose excreted unchanged in 24 hour urine	86.3 (84.4-88.8)*	75.2, 78.4	8.4 (2.6-15.2)	48.1 (44.0-54.7)
% dose excreted as hippuric acid	2.5*	3.17, 4.2	21.1*	0.2*

† Data from Sinsheimer et al., 1973. For the data of Sinsheimer et al., 1973, mean values are given with ranges in parentheses

* Figures obtained by reverse isotope dilution



Comparison of Metabolic Pathways of Norephedrine as Illustrated by the Composition of the 24 hour Urine Samples of Four Species

'after Williams 1974

A table of the excretion data obtained from the two Tamarin monkeys dosed with [^{14}C]-(±)-norephedrine may be found in Table 12.

Discussion

To place these results obtained from the Tamarin monkeys in perspective, they have been compared with the data of Sinsheimer and co-workers (1973) who gave [^{14}C]-(±)-norephedrine to the rat, rabbit and man. An important point to be considered during this comparison is that Sinsheimer and co-workers administered their doses orally, whereas the doses given to the Tamarin monkeys were given intramuscularly.

A table comparing the amount of ^{14}C excreted in 24 hours in the urine and the composition of urine has been constructed and is illustrated in Table 13.

The excretion values for unchanged norephedrine and hippuric acid are similar in man and Tamarin monkey. However, the Tamarin monkeys appeared to give two other biotransformation products, namely a glucuronide conjugate (possibly benzoylglucuronide) which constituted 1.7-1.9% of the dose and benzoic acid, which constituted 2.03-3.2% of the dose both estimated chromatographically. Sinsheimer and co-workers (1973) gave no figures for benzoic acid in the 24 hour urine of man, but stated that 0.1% of the dose in the rat and 3.8% of the dose in rabbit was benzoic acid.

4-Hydroxynorephedrine (0.8% of the dose) was detected in the 24 hour urine of man (Sinsheimer et al., 1973) but the true figure for the total biotransformation of norephedrine to 4-hydroxynorephedrine may have been higher, due to the long half-life of the latter metabolite in the body (Gropetti and Costa, 1969). In the urine of the Tamarin monkeys there appeared to be none of the ring-hydroxylated material when estimated chromatographically. The metabolite 4-hydroxynorephedrine is

formed extensively in the rat, accounting for approximately 30% of the dose in the 24 hour urine (Sinsheimer et al., 1973), more than half of this being conjugated with glucuronic acid. Less aromatic ring hydroxylation of norephedrine was found in the 24 hour urine of rabbit (1% of the dose) and this occurred in its free form and not conjugated with either glucuronic or sulphuric acid.

Sinsheimer and co-workers (1973) found the [^{14}C]-(±)-norephedrine to be extensively metabolised in the rabbit and reported the finding of three intermediate compounds in the metabolism of the drug, namely 1,2-dihydroxy-1-phenylpropane, found free and conjugated as a sulphate, 1-hydroxy-2-oxo-1-phenylpropane, found free and conjugated as a glucuronide and 1,2-dioxo-1-phenylpropane. However, none of these compounds appeared to be present in the 24 hour urine of man and only the diol could be detected in the rat (0.1% of the dose). None of these compounds could be detected chromatographically in the 24 hour urine from the Tamarin monkeys.

Thus, in conclusion, both man and Tamarin monkey appeared to excrete the vast majority of administered [^{14}C]-(±)-norephedrine unchanged (77% for the Tamarin monkey, 86% for man) and both species formed approximately the same amounts of hippuric acid (3.17% for the Tamarin monkey, 2.5% for man). Man appeared to have ring-hydroxylated 0.8% of the dose (Sinsheimer et al., 1973) which appeared conjugated in the urine. Although a conjugate was detected in the 24 hour urine of the Tamarin monkey, there appeared to be no 4-hydroxynorephedrine. No free benzoic acid appears to have been found in the 24 hour urine of man whereas approximately 2.5% of the dose appeared in the Tamarin monkey urine as free benzoic acid.

The finding that the majority of the drug was excreted unchanged in the first 24 hours is in agreement with human excretion data of Beckett and Wilkinson (1965); Heimlich et al., (1961) and Sinsheimer et al., (1973).

CHAPTER FIVE

Variations of Drug Metabolism in Primates and Other Species

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INTRODUCTION

Toxicity testing of a potentially new drug for use in humans is primarily carried out in laboratory animals such as cats, dogs, mice, rabbits, rats and monkeys, before being tested in man. Such a scheme of events implies that the data obtained from these species may be directly extrapolated to man. This optimistic approach may be equally counter-balanced by the pessimistic concept that man is unique in his response to drugs (Brodie and Reid, 1967). A given chemical may have a profound effect in one animal species but have little or no effect in a second species. These differences in drug action may be the result of species differences in the rates of drug metabolism, pathways of metabolism or in the response of the animal to the drug (Conney, 1967), but the problem of species variation may be further exacerbated by other factors such as strain, environment and sex.

The species of choice

Although there are approximately 1.5 million known species of animals and over 50,000 of these are vertebrates, rarely more than a few dozen of these are used in laboratories (Koelle, 1967). There are very many factors to be examined in deciding whether a certain species of animal is a suitable model from which to extrapolate the results of toxicity-testing to man. Initially, as well as having a fairly large number of animals to work with, it is inadvisable to choose an animal which has a peculiar characteristic. "The unusual qualities of an animal are sometimes a surprise and often a nuisance" said Schmidt-Nielsen in 1967 and in his article he has quoted 'unusual' animals such as the fat sand rat which has as its peculiar characteristic only long-looped nephrons. In its natural sparse and highly saline habitat this rat manages to survive quite well,

but when the animal was fed with a controlled protein diet it acquired diabetes mellitus which often proved fatal. The diabetes mellitus was found to display the full pathology of adult human diabetes. Further examples are the blowfish, 'fugu', which has in its spines the poison known as tetrodotoxin, a specific inhibitor of sodium movement. The species chosen for comparative toxicology should resemble man as closely as possible with regard to both its pharmacological response to the drug and also to the rate and extent of biotransformation of the compound. It is also advantageous to know facts about the husbandry of the animal since relaxed animals rather than stressed ones, are usually needed. Carr (1967) has listed four aspects of research that are likely to reveal man as fairly separate from other species. These are (i) his diseases, (ii) certain of his adverse reactions to drugs, (iii) his highly developed central nervous system and (iv) his unique ethical claims on the investigator. Concerning point (i), Carr emphasised the point that drugs are developed for use in abnormal rather than normal man and therefore prediction of the behaviour of drugs in man is often best accomplished by studies in animals that have model diseases rather than in healthy animals. Such a model disease is the arthritic one, which is induced in rats by intra-dermal injections of tubercle bacilli into, for example, the foot pad. This kind of model may be used in a screening test for antiinflammatory drugs. Morton and Chatfield (1970) have shown that such 'arthritic' rats display decreased drug metabolic activity as determined by the in vitro metabolism of propoxyphene.

To illustrate some of man's adverse reactions to drugs, Carr (1967) quoted the examples of drugs which cause a cholestatic reaction in man, such as

chlorpromazine and anabolic steroids. However, although anabolic steroids have been shown to affect hepatic canaliculi in rats, chlorpromazine-treated rats do not show any hepatic lesions.

Having accepted the philosophy that it is better to test drugs on animals before trying them out on humans, an ability to predict the routes of metabolism of such drugs is desirable. However, a review of the literature, especially the extensive reports of Williams (1964; 1967a,b; 1969) is enough to demonstrate the diversity of metabolism between various species. For example, synthesis of glucuronides in the cat is at a lower level compared with other species, although not entirely absent (Robinson and Williams, 1958) and this is due to a deficiency of glucuronyl transferase (Dutton and Greig, 1957). The dog appears unable to acetylate primary amines (Muenzen et al., 1926). The matter is further complicated when a compound may be metabolised by more than one pathway and the relative importance of one particular pathway can vary from one species to another as for example with ephedrine (Axelrod, 1953) and amphetamine (Axelrod 1954a,b; Dring et al., 1970). Further difficulties may also arise within a given species, for example, in mice dosed with hexobarbital, a sevenfold difference in range for the duration of action has been reported (Jay, 1955).

Brodie and Reid (1967) have commented on the fact that compounds with low activity in animals are rarely selected for clinical trials in man. As evidence against this method of screening, Brodie and Reid have given examples of drugs that would not have been discovered by animal screening. For example, phenylbutazone which showed a negligible antiinflammatory action in animals and had a very short half-life in the body, was shown to be potent in man when, quite by chance, the drug was used as a solubilising agent for the parenteral injection of aminoantipyrine (Gsell and Muller, 1950). Another example was imipramine which

in rabbits and mice failed to accumulate appreciable amounts of the active metabolite, desimipramine (Brodie, 1965; Dingell et al., 1964).

General principles of drug metabolism

Metabolism of drugs and other chemicals is necessary in order to terminate or lower the toxicity of their actions in the body. During metabolism the chemical is converted to a more polar, less lipophilic compound which may be eliminated from the body through, for example, the kidneys.

The general patterns of drug metabolism in all species of animal can be illustrated as follows:-

Drug $\xrightarrow[\text{asynthetic reactions}]{\text{Phase I}}$ Oxidation reduction and/or hydrolysis products $\xrightarrow[\text{synthetic reactions}]{\text{Phase II}}$ Conjugation products

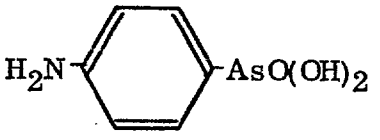
(After Williams, 1959).

Since the reactions of these two phases may be catalysed by enzymes, it is to be expected that these enzymes will vary from species to species in amount and nature and thus provide variations in the rate and route of drug metabolism between different species. Metabolism in the animal may fail to reach completion and therefore the products of both Phase I and Phase II metabolism may be found as well as the original compound. Also some compounds which are highly polar may not be metabolised at all (see Table 14).

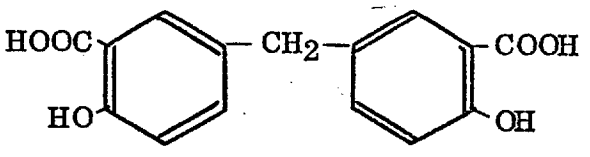
Species variations in the metabolism of chemicals may be due to (i) variations in the rate of transformation along a common route of metabolism or (ii) variations due to different routes of drug metabolism (Williams, 1964). The rate of biotransformation may be due to (a) a variation in the amount of enzyme within a species of animal, (b) the amount of natural inhibitor to the enzyme may vary, (c) the amount of enzyme reversing a reaction may vary and (d)

Table 14. Polar Compounds which are not Metabolised

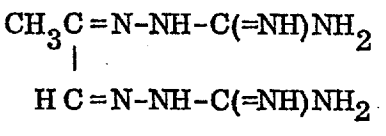
Arsanilic acid*



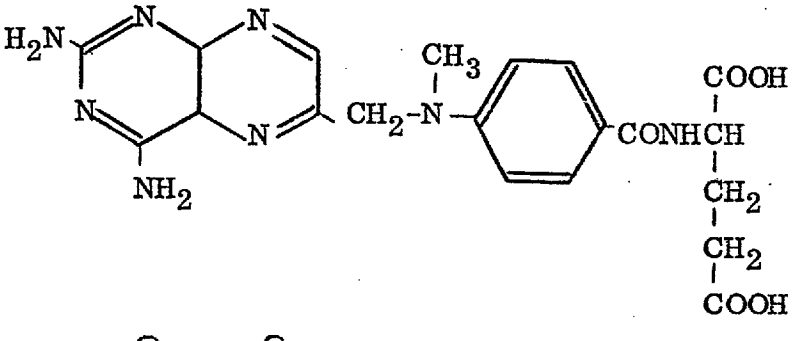
5-5-Methylenedisalicylic acid*



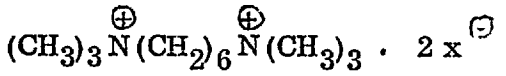
Methylglyoxal-bis-guanylhydrazine*



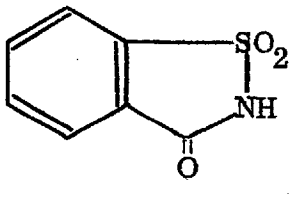
Methotrexate*



Hexamethonium salts*



Saccharin†



* After Williams, 1967 a † After Ball et al., 1974.

there may be a species variation in enzymic control (Williams, 1967b).

The use of Primates for drug metabolism studies

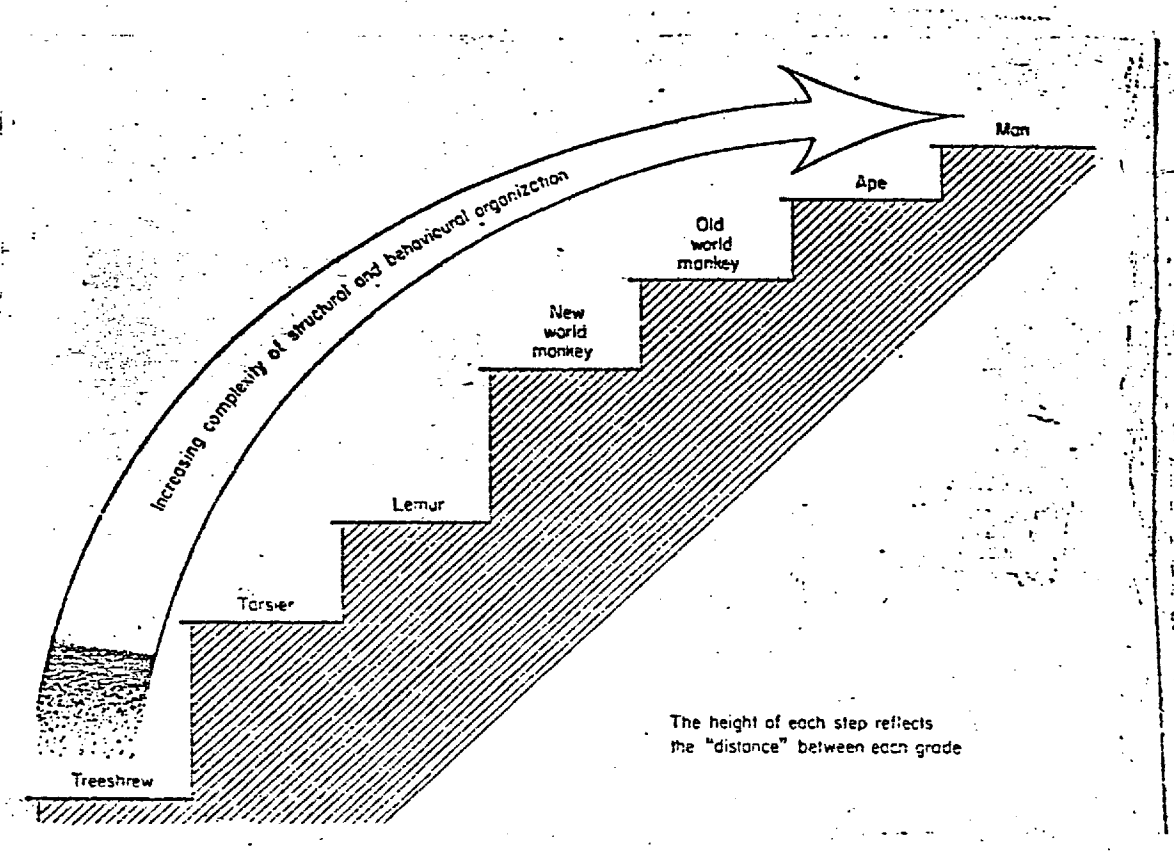
It is only comparatively recently that the metabolic fate of drugs in subhuman primates has become of major interest, the premise underlying this interest being that drug metabolism patterns in man may be better predicted in a subhuman primate than in studies carried out in less closely related species such as dogs and rats (Smith, 1967). Smith (1966) concluded that patterns of metabolism were more predictable using monkeys than by using dogs (see Table 15). Scrutiny of different metabolic pathways within man, monkey and dog, showed a number of instances in which the dog exhibited divergent metabolic pathways from those found in the other two species (see Table 16).

Smith (1967) also tabulated the data presented by Williams (1967a) to illustrate the closer similarity of Old World monkeys to man and the apes rather than New World monkeys and Prosimians (Table 17).

Williams (1967a) has in his paper showed the extent of hydroxylation of amphetamine in mammalia and compared the results of Dring and co-workers (1966) with that of Ellison and co-workers (1966) (see Table 18).

Dring and co-workers (1970) have also published data concerning the metabolism of the (+)- and (-)- antipodes of amphetamine in the Rhesus monkey and a comparison with man indicates that the monkey appears to hydroxylate the (+)-antipode to a greater extent than man, whilst the (-)-antipode, although not so extensively hydroxylated, is also metabolised more extensively in the monkey.

Similarly, Williams (1967a) has quoted the N-hydroxylation and 7-hydroxylation of N-2-fluorenylacetamide in man and Rhesus monkey (see Table 19).



A Staircase of Primate Evolution, illustrating the Morphological and Behavioural Grades Found Among Living Primates

(After Napier and Napier, 1967)

Table 15. Similarity of Metabolic Patterns in Dogs and Monkeys to Man

	Degree of similarity to human metabolic patterns		
	Good	Fair	None
Dog	5	1	4
Monkey	4	4	1

Numbers refer to compounds tested. (After Smith, 1967).

Table 16. Presence or absence of certain enzyme pathways

Enzyme pathway	Man	Rhesus Monkey	Dog
Glutamine conjugation	+	+	(uses glycine)
Acetylation of aromatic amines and hydrazines	+	+	-
Mercapturic acid formation	slow	- or slow	rapid
N-Methylation of amantadine	-	-	+
Phosphate conjugation of 2-naphthylamine	-	-	+
Aromatisation of quinic acid	+	+	-
Sulfadimethoxine glucuronide formation	+	+	-

(after Smith, 1967).

Table 17. Presence of Certain Metabolic Pathways in Primates

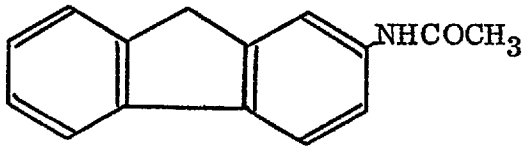
	Man and Apes	Old World Monkey	New World Monkey	Prosimian
Glutamine conjugation	+	+	?	-
Aromatisation of quinic acid	+	+	-	-
Glucuronide formation of sulfadimethoxine	+	+	+	+

Data from Williams, 1967a (After Smith, 1967).

Table 18. Extent of Hydroxylation of Amphetamine in Man and Squirrel Monkey

		% dose hydroxylated found in urine	
Order	Species	Dring et al.	Ellison et al.
Anthropoidea	Man	0.5 - 9	2 - 3
	Squirrel monkey	—	2 - 3

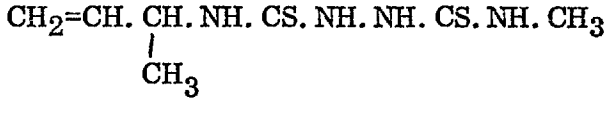
Table 19. Hydroxylation of N-2-Fluorenylacetamide in Various Species



		% dose		
Order	Species	N-Hydroxylated	7-Hydroxylated	Carcinogenicity
Anthropoidea	Man	4-14	25-30	?
	Rhesus monkey	2-3	9-18	?

Smith (1967) has collected data showing that the serum albumin of apes and Old World monkeys have a closer similarity to human serum albumin compared with New World monkeys, Prosimians and non-primates. A similar pattern emerged with the carboxylic esterases and carbonic anhydrases of primate erythrocytes. Smith's conclusion was that data from Old World monkeys, especially the macaques, was the most similar to that obtained from man and amongst the New World monkeys, only the owl monkey, Aotes, looked like a candidate for further attention. Whilst attempting to summarise data from a symposium on comparative pharmacology, Koelle (1967) tried to answer the self-imposed question, "do species patterns of drug metabolism exist?" He replied, "the answer, unfortunately, is yes and again no! " His tentative conclusions were that 1) orangutans and baboons more closely resemble man than the New World monkeys, and 2) "the whole matter was probably irreparably confused some 15,000 or 20,000 years ago by some visitor from the East who insisted on taking along his pet owl monkey when he made the Grand Tour across the Bering Strait and down to South America! "

Brodie and Reid (1967) were not so optimistic about the comparison of data from primates and man, since although the patterns of drug metabolism were similar in man and monkey, the rates of metabolism were very different. They quoted as examples the compound ICI-33828 which was metabolised twenty times



ICI 33828

more rapidly and aminoantipyrine, phenylbutazone, amidopyrine and oxyphenbutazone, six to ten times more rapidly in Rhesus monkey than in man.

Conclusions on the Validity of Use of the Tamarin Monkey in Metabolic Studies

The Tamarin monkey is a species of marmoset belonging to the Family, Callitrichidae. The Rhesus monkey (Macaca) belongs to a higher order, Cercopithecidae and it is members of this Family that are usually used as the final species in the investigation of drug action, toxicity and metabolism before the drug is tested in human clinical trials. The mature Rhesus monkeys are usually quite large (5-10 kg) and also expensive both to buy and to house satisfactorily. The Tamarin monkeys, on the other hand, are comparatively small (300-510 g), cheaper to buy than the Rhesus monkeys and also easier to accommodate. Therefore it would appear, at first sight, to be advantageous to use Tamarin monkeys for toxicity testing prior to human use, providing that this species exhibits a similar toxicological spectrum to man.

The metabolism of Preludin, amphetamine and norephedrine in the Tamarin monkey has been investigated and compared with the data from other species. For Preludin, this data may be found in Chapter 2, Tables 3, 4, 5 and 6, for amphetamine in Chapter 3, Tables 8, 9 and 10 and for norephedrine in Chapter 4, Tables 12 and 13. A comparison of the amount of unchanged drug excreted in the 24 hour urine of each species shows that for Preludin, more unchanged material is to be found in the urine of the Tamarin monkey than in that of man (34% of the dose in the Tamarin monkey and 19% in man). For amphetamine a similar conclusion may be drawn (approximately 54% of the dose in the Tamarin monkey and between 19 and 30% in man). However, with norephedrine, more compound is excreted unchanged in the 24 hour urine of man than in the Tamarin monkey (approximately 86% of the dose in man and 76% of the dose in Tamarin monkey). Aromatic ring hydroxylation in both species after administration of Preludin has occurred to approximately the same extent (12% of the dose in man, 13% in the Tamarin monkey), although man forms more of the glucuronide conjugate

than the Tamarin monkey (about 10% of the dose in man and 3% of the dose in the Tamarin monkey). Aromatic ring hydroxylation of amphetamine accounts for 3% of the dose in man and 4% of the dose in the Tamarin monkey. Very little of the aromatic hydroxylated metabolite of norephedrine, 4-hydroxynorephedrine, appears to be found in the 24 hour urine of man dosed with norephedrine (Sinsheimer et al., 1973, give a value of 0.8% of the dose, calculated by reverse isotope dilution), but none of this compound could be detected chromatographically in the urine of the Tamarin monkeys. The lactam metabolite of Preludin, 2-phenyl-3--methyl-5-morpholinone is found to the extent of approximately 19% of the dose in man and 12% in the Tamarin monkey. Side-chain degradation of amphetamine in both species shows it to be far more extensive in man than in the Tamarin monkey for both benzoic acid and its glycine conjugate. With norephedrine, however, the amount of hippuric acid excreted in the 24 hour urine of both species is very similar (approximately 3% of the dose for man and nearly 4% for the Tamarin monkey). Free benzoic acid in man after a dose of norephedrine has not been recorded by Sinsheimer and co-workers (1973).

The results of this present investigation using the Tamarin monkey compare quite favourably with those obtained in man, except in the extent of the side-chain degradation of amphetamine to benzoic and hippuric acids. It is most important to recall that in every case, the drug was administered to the Tamarin monkey by intramuscular injection whilst in man the compounds were presented orally (as were the (+)- and (-)- antipodes of amphetamine to Rhesus monkeys, Dring et al., 1970). Such a difference in route of administration might result in a difference in the pharmacokinetics of drug absorption, distribution and elimination. To nullify this variable, oral dosing of the Tamarin monkeys was attempted by injecting a solution of the drug into a rusk (a favourite delicacy of the animal) and leaving it on a plate in the animal's cage. However, the

Tamarin monkeys were most uncooperative and showed a marked suspicion of the food, regardless of who presented it. After several attempts at oral dosing, it was decided to inject the drug in order to be certain of administering the whole dose to the animal. As well as interfering with the pharmacokinetic properties of the drug, administration of a drug by injection also subjects the animal to stress. The restricted means of drug administration, together with the difficulties in handling the Tamarin monkeys may prove to be factors against the use of this species as the one of choice over the Rhesus monkey. Although the latter are no better tempered they can at least be dosed orally. However, as mentioned previously, the Tamarin monkeys are cheaper to buy than Rhesus monkeys (approximately £35 to £50 for a Tamarin monkey compared with approximately £100 for a Rhesus monkey). Tamarin monkeys are smaller in size and consequently less compound is needed to dose them; also they are easier to house than the Rhesus monkeys although they need an ambient temperature of between 25-27°C.

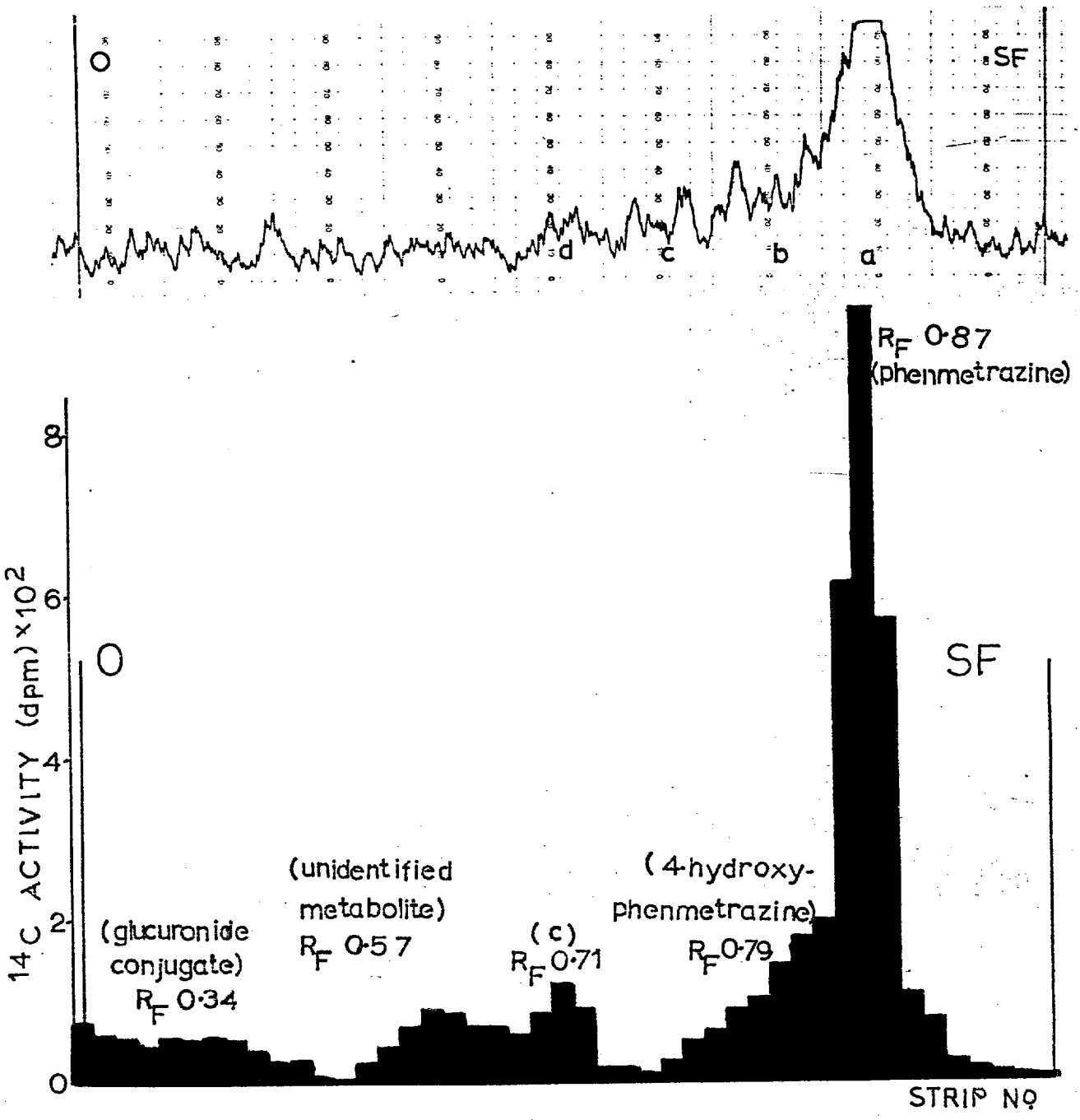
APPENDIX 1

RADIOCHROMATOGRAM SCANS AND HISTOGRAMS

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Tamarin Monkey 1.

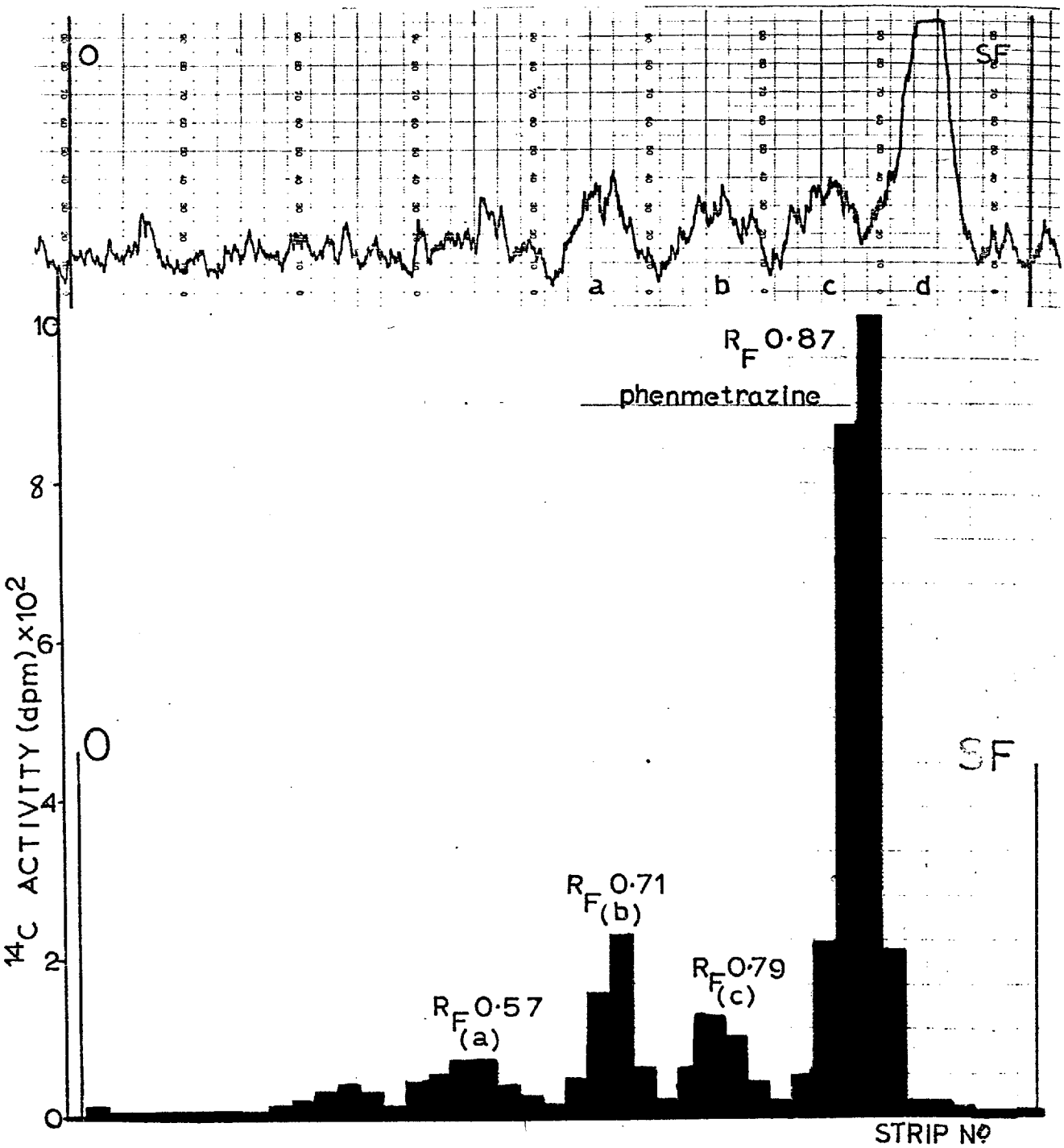


Radiochromatogram scan and histogram of 24 hour urine from Tamarin monkey dosed intramuscularly with [^{14}C]-Preludin (0.28 mg/kg; 1.93 μCi) and chromatographed on Whatman 3 mm paper in system B.

O = Origin. SF = Solvent front

a = phenmetrazine, b = 4-hydroxyphenmetrazine, c = 2-phenyl-3-methyl-5--morpholinone, d = unidentified metabolite.

Tamarin Monkey 2.



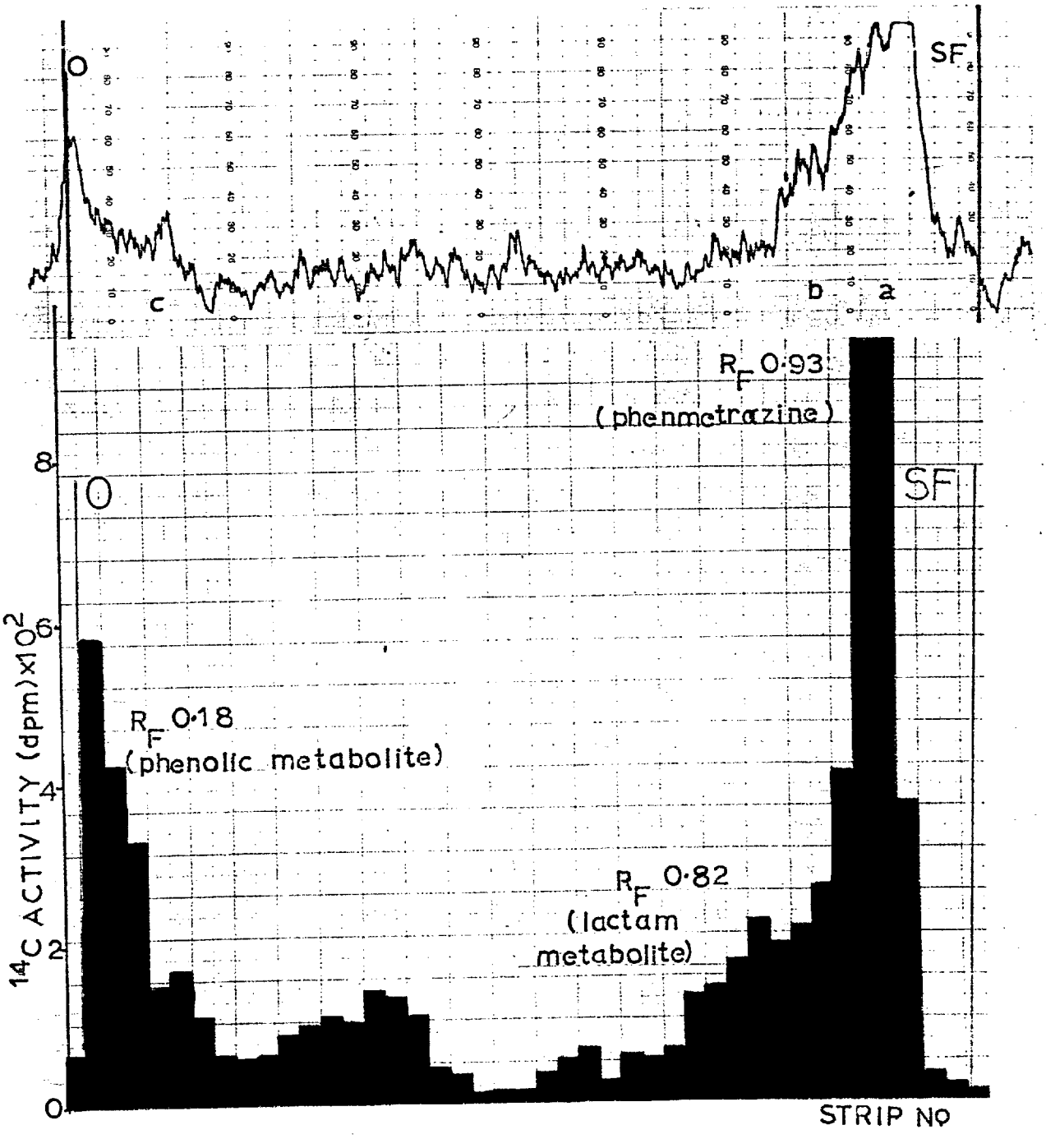
Radiochromatogram scan and histogram of 24 hour urine from Tamarin monkey dosed intramuscularly with [^{14}C]-Preludin (0.28 mg/kg; 1.93 μCi) and chromatographed on Whatman 3 mm paper in solvent system B (see Chapter 2, Materials and Methods).

O = Origin. SF = Solvent front.

a = Unknown metabolite, b = 2-phenyl-3-methyl-5-morpholinone,

c = 2-(4'-hydroxyphenyl)-3-methylmorpholine, d = phenmetrazine.

Tamarin Monkey 2.

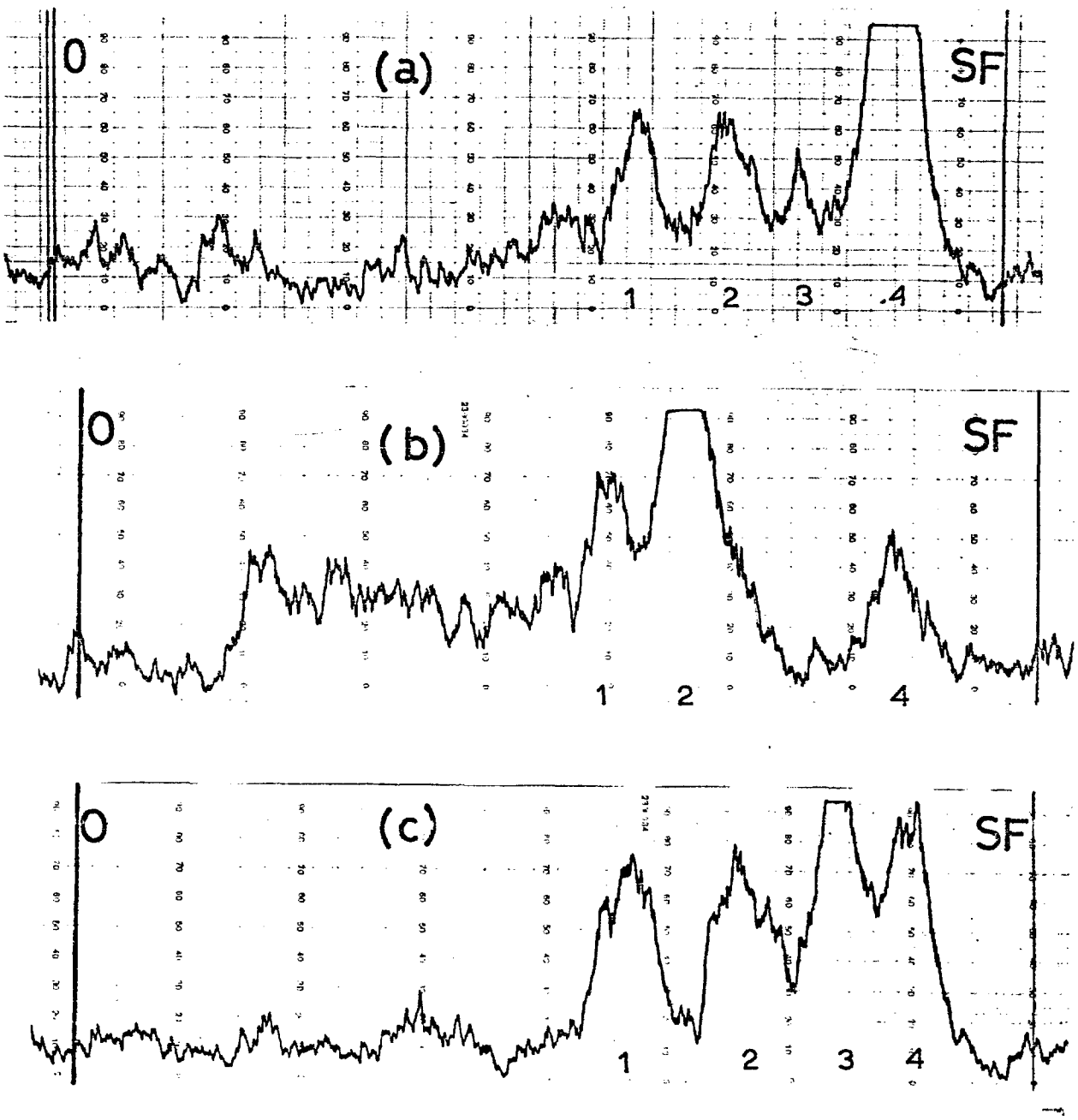


Radiochromatogram scan and histogram of 24 hour urine from Tamarin monkey dosed intramuscularly with [^{14}C]-Preludin (0.28 mg/kg; 1.93 μCi) and chromatographed on Whatman 3 mm paper in solvent system C (see Chapter 2, Materials and Methods).

O = Origin. SF. = Solvent front

a = phenmetrazine, b = 2-phenyl-3-methyl-5-morpholinone,

c = 2-(4'-hydroxyphenyl)-3-methylmorpholine.

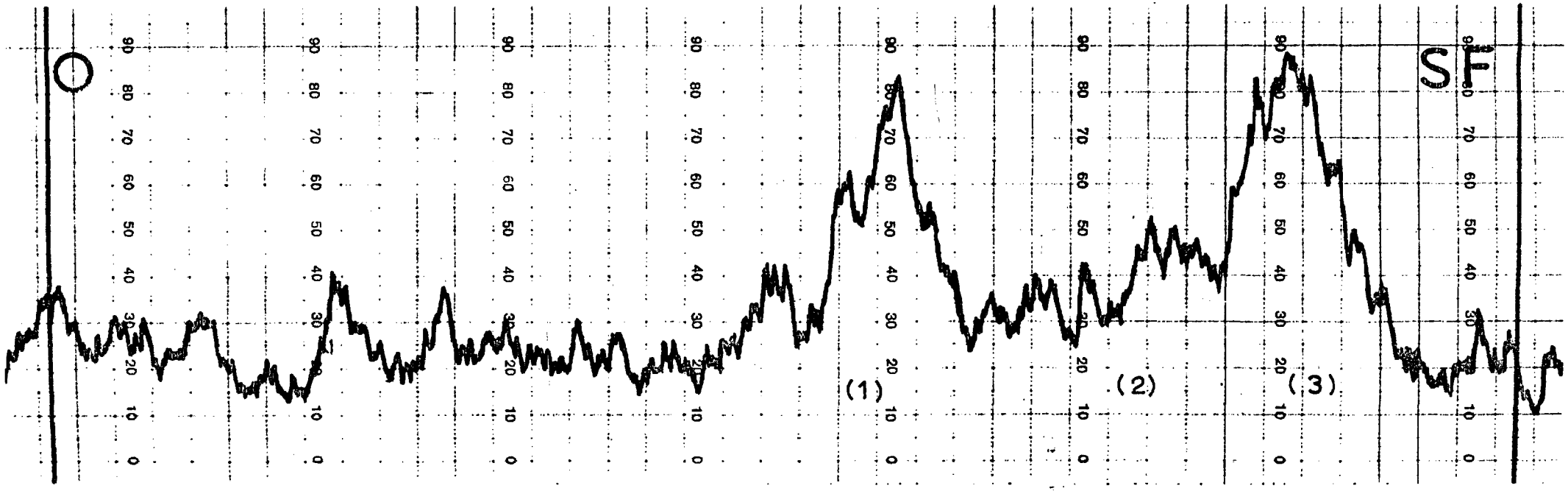


Radiochromatogram scans comparing the 24 hour urine samples obtained from (a) Tamarin monkey, (b) guinea pig and (c) rat, dosed with [^{14}C]-Preludin

The Tamarin monkey was dosed intramuscularly with [^{14}C]-Preludin (0.28 mg/kg) and the rat and guinea pig were dosed orally with [^{14}C]-Preludin (20 mg/kg). Chromatography was carried out on Whatman 3mm paper in solvent system B.

O = Origin. SF = Solvent Front.

1 = unidentified metabolite, 2 = lactam metabolite, 3 = phenolic metabolite, 4 = phenmetrazine.

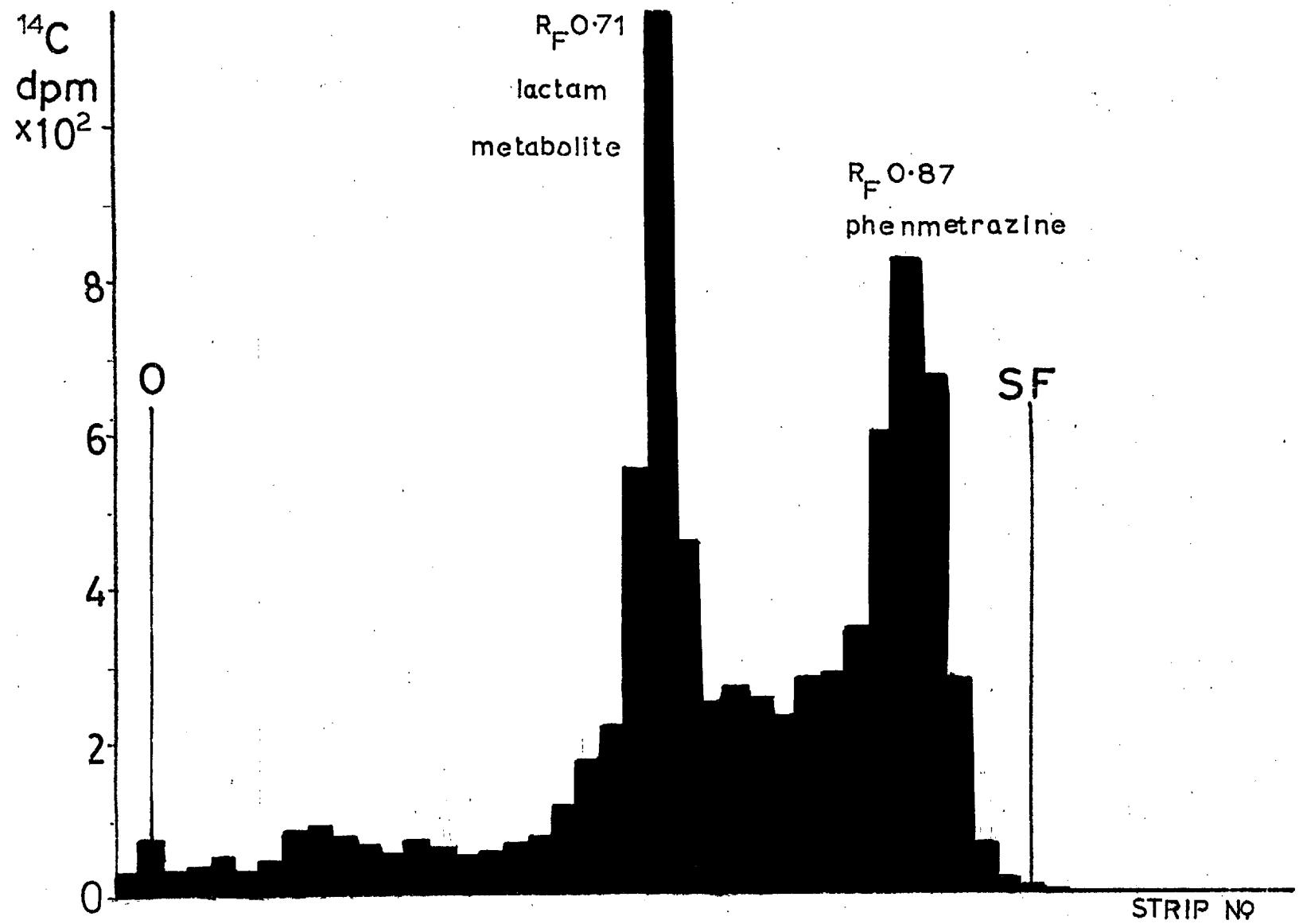


Radiochromatogram scan of 24 hour urine from human subject (Z. H. S.) given 25 mg Preludin orally (3.12 μCi). The urine was chromatographed in Solvent system B on Whatman 3mm paper.

O = Origin. SF = Solvent Front

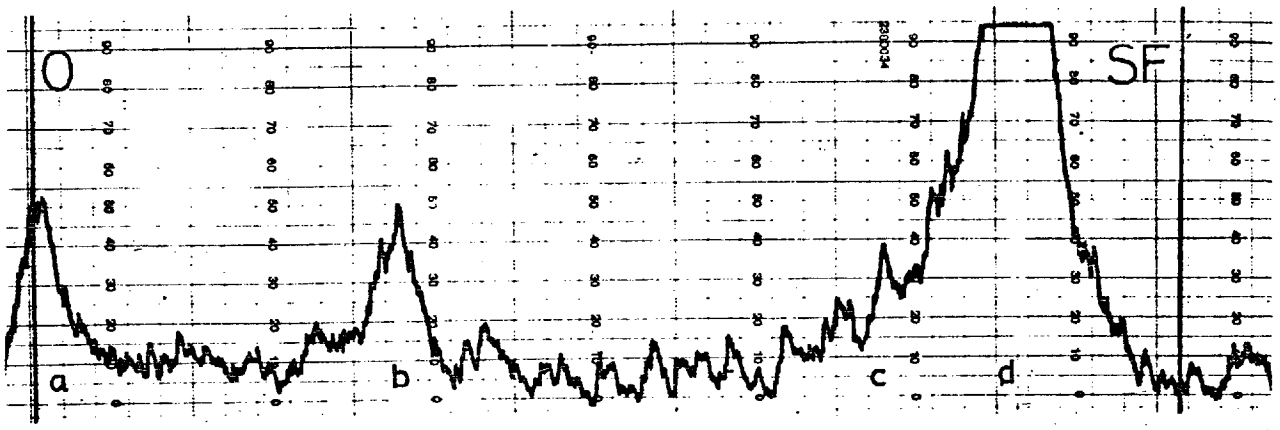
(1) = lactam metabolite, 2-phenyl-3-methyl-5-morpholinone, (2) = phenolic metabolite, 2-(4'-hydroxyphenyl)-3-methylmorpholine

(3) = phenmetrazine.

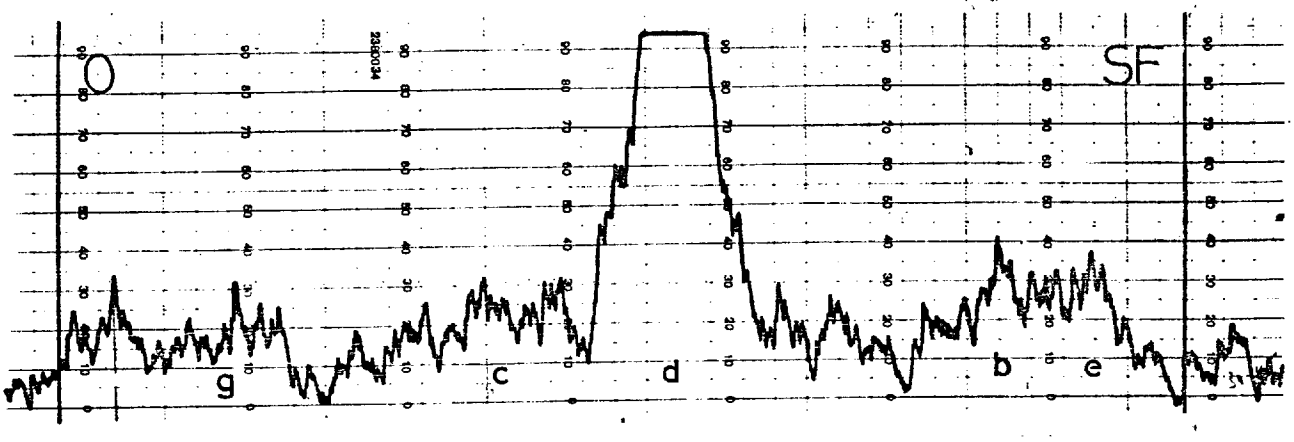


Histogram of 24 hour urine from human subject (Z. H. S.) given 25 mg of [^{14}C]-Preludin (3.12 μCi) subjected to paper chromatography on Whatman 3 mm paper and chromatographed in solvent system B (see Chapter 2, Materials and Methods).

O = Origin SF = Solvent front.



SOLVENT SYSTEM I



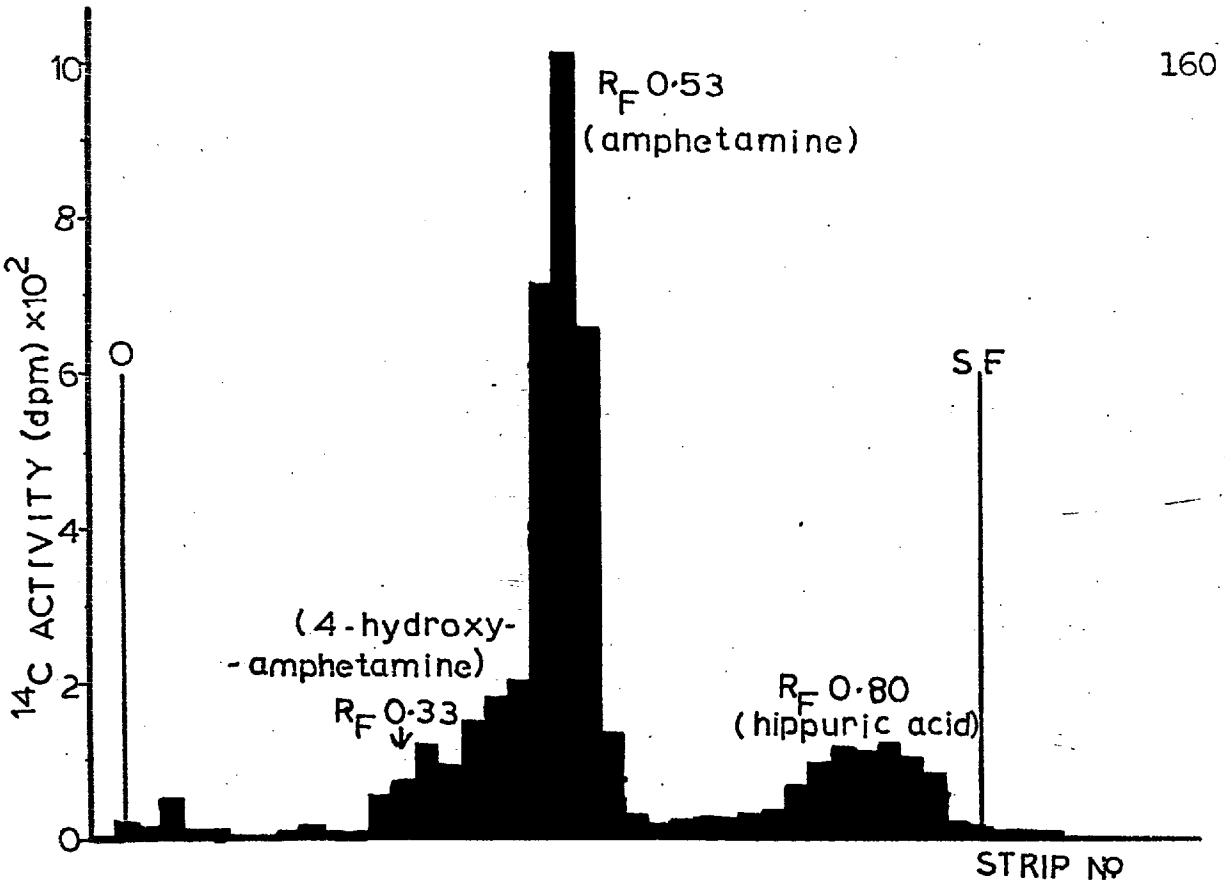
SOLVENT SYSTEM II

Radiochromatogram scans of 24 hour urine from Tamarin monkey dosed intramuscularly with [^{14}C]-(±)-amphetamine sulphate at 0.3 mg/kg (1.9 μCi) and chromatographed on Whatman No. 1 paper in solvent systems I and II (see Chapter 3, Materials and Methods).

O = Origin. SF = Solvent front.

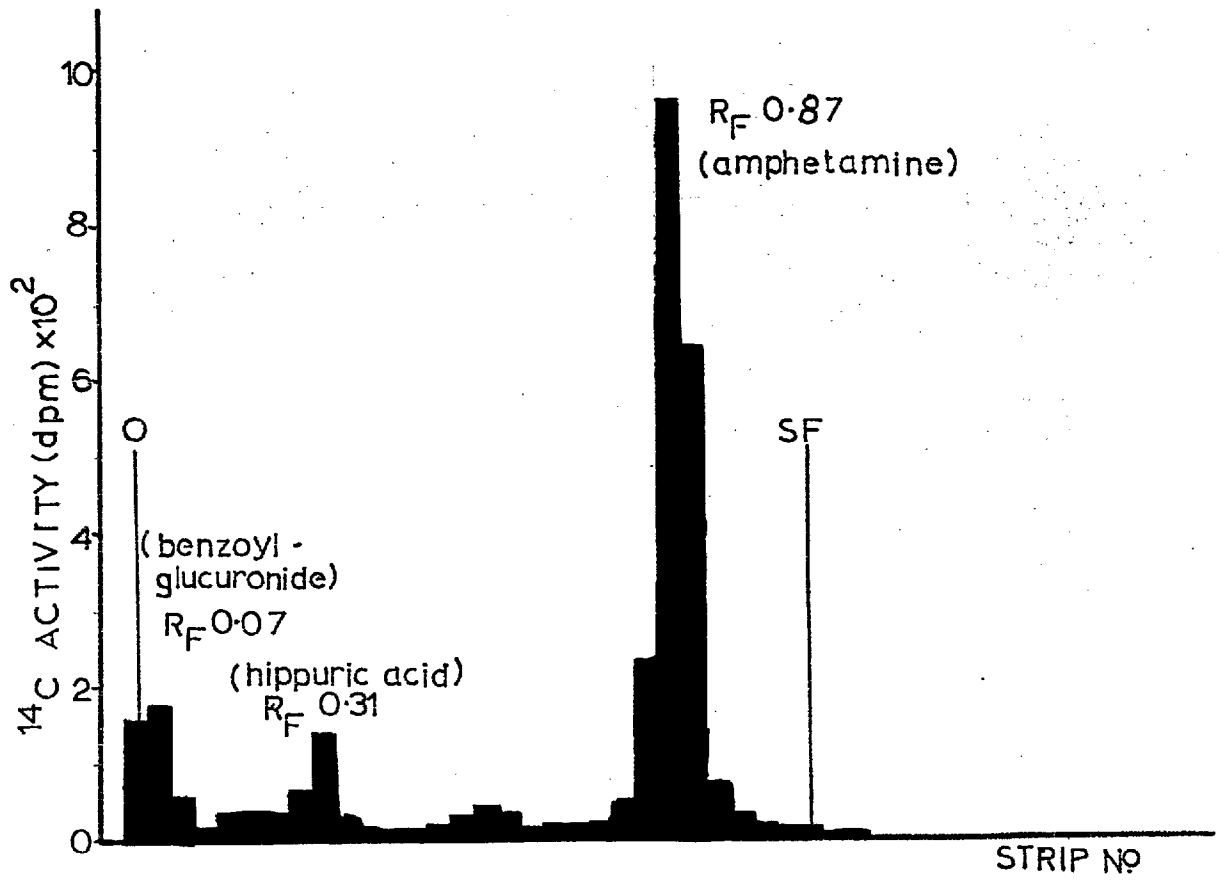
a = benzoyl glucuronide, b = hippuric acid, c = 4-hydroxyamphetamine

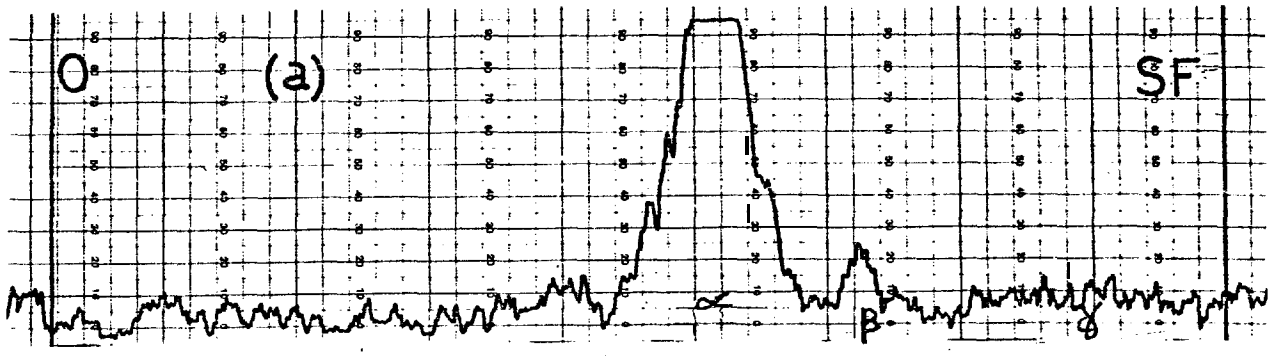
d = amphetamine e = benzoic acid

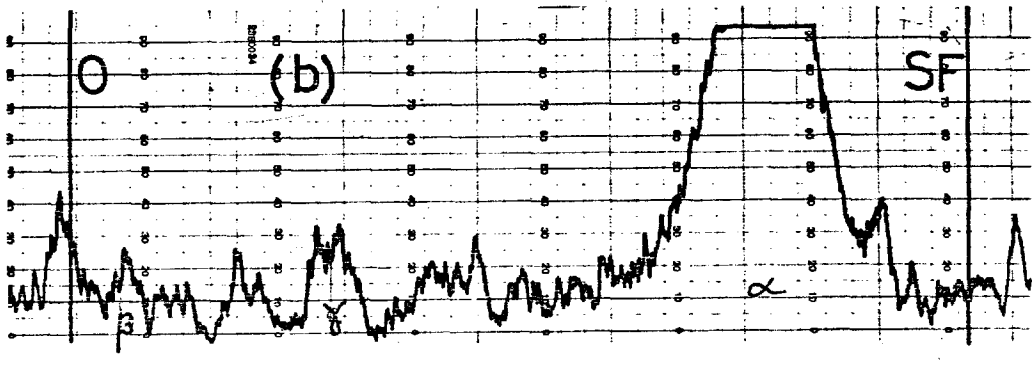


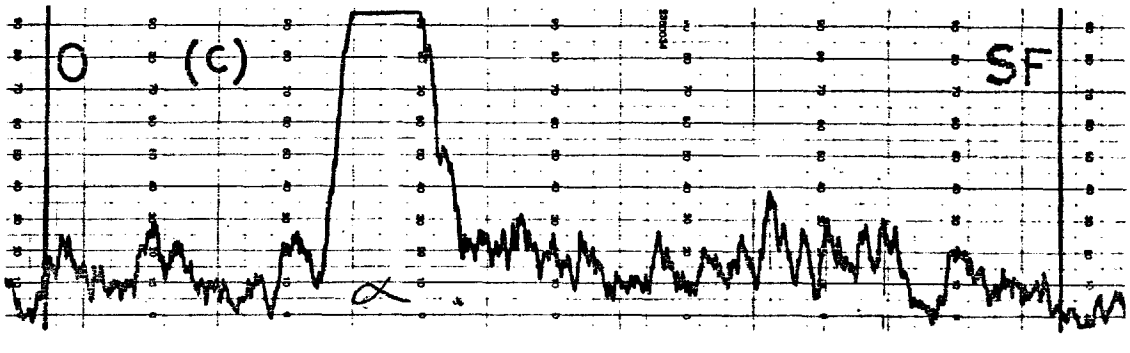
Histograms from chromatograms of 24 hour urine from Tamarin monkey dosed intramuscularly with [^{14}C]-(±)-amphetamine at 0.3 mg/kg (1.9 μCi) and chromatographed on Whatman No. 1 paper in Solvent systems, II (upper histogram) and I (lower histogram) (see Chapter 3, Materials and Methods).

O = Origin. SF = Solvent front.









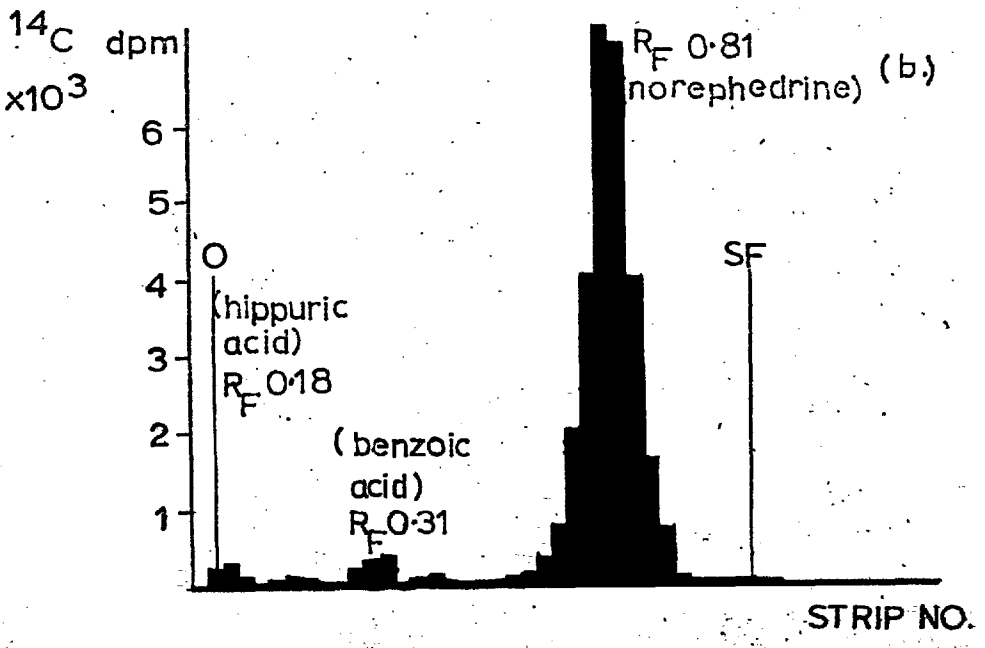
Radiochromatogram scans of 24 hour urine from Tamarin monkey dosed intramuscularly with [^{14}C]-(±)-norephedrine (0.3 mg/kg) and chromatographed on Whatman No. 1 paper.

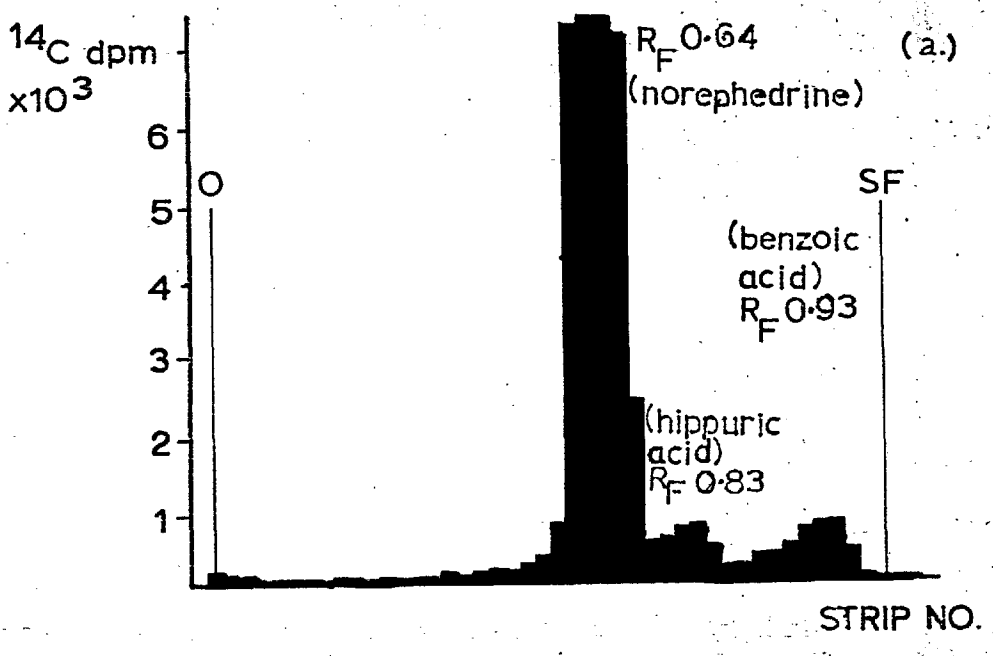
(a) Solvent system III
(b) Solvent system IV see Chapter 4, Materials and Methods
(c) Solvent system V

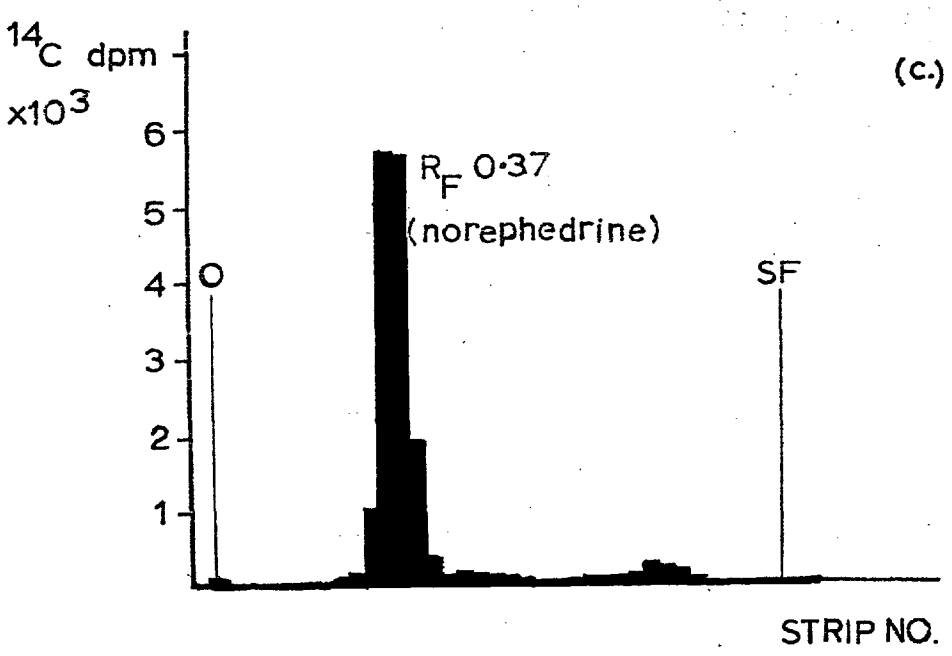
α = norephedrine. β = hippuric acid. γ = benzoic acid.

The respective histograms of (a), (b) and (c) may be found on the following page.

O = Origin. SF = Solvent Front.


^{14}C dpm
x10^3
6
5
4
3
2
1
O
(hippuric acid)
R_F 0·18
(benzoic acid)
R_F 0·31
R_F 0·81
(norephedrine)
(b.)
SF
STRIP NO.


^{14}C dpm
x10^3
6
5
4
3
2
1
O
R_F 0·64
(norephedrine)
(a.)
SF
(benzoic acid)
R_F 0·93
(hippuric acid)
R_F 0·83
STRIP NO.


^{14}C dpm
x10^3
6
5
4
3
2
1
O
R_F 0·37
(norephedrine)
(c.)
SF
STRIP NO.

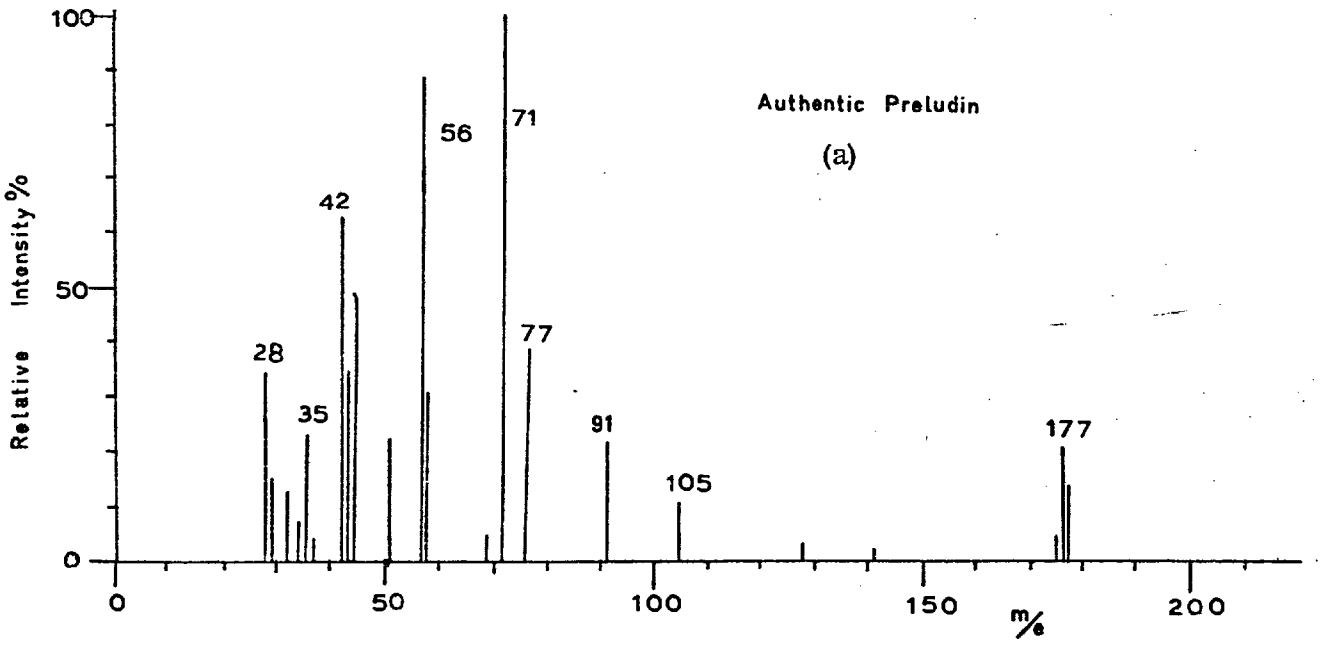
APPENDIX 2

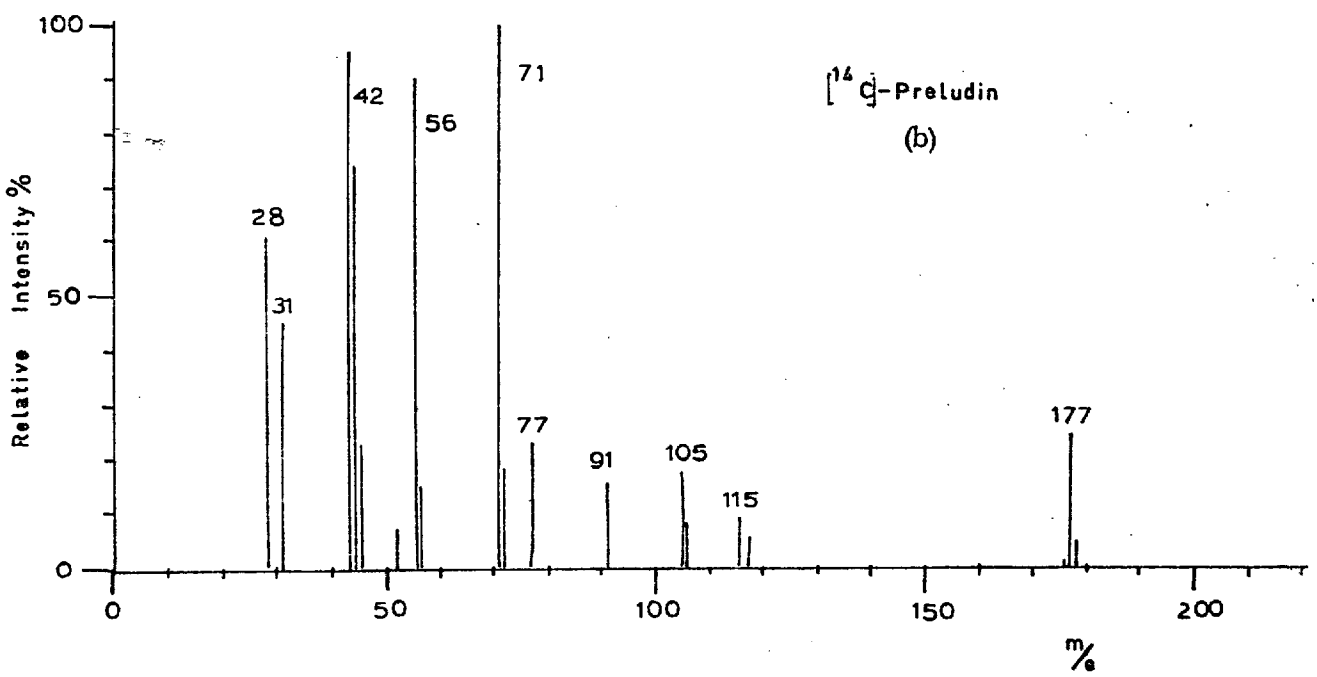
MASS SPECTRA AND POSSIBLE FRAGMENTATION PATTERNS

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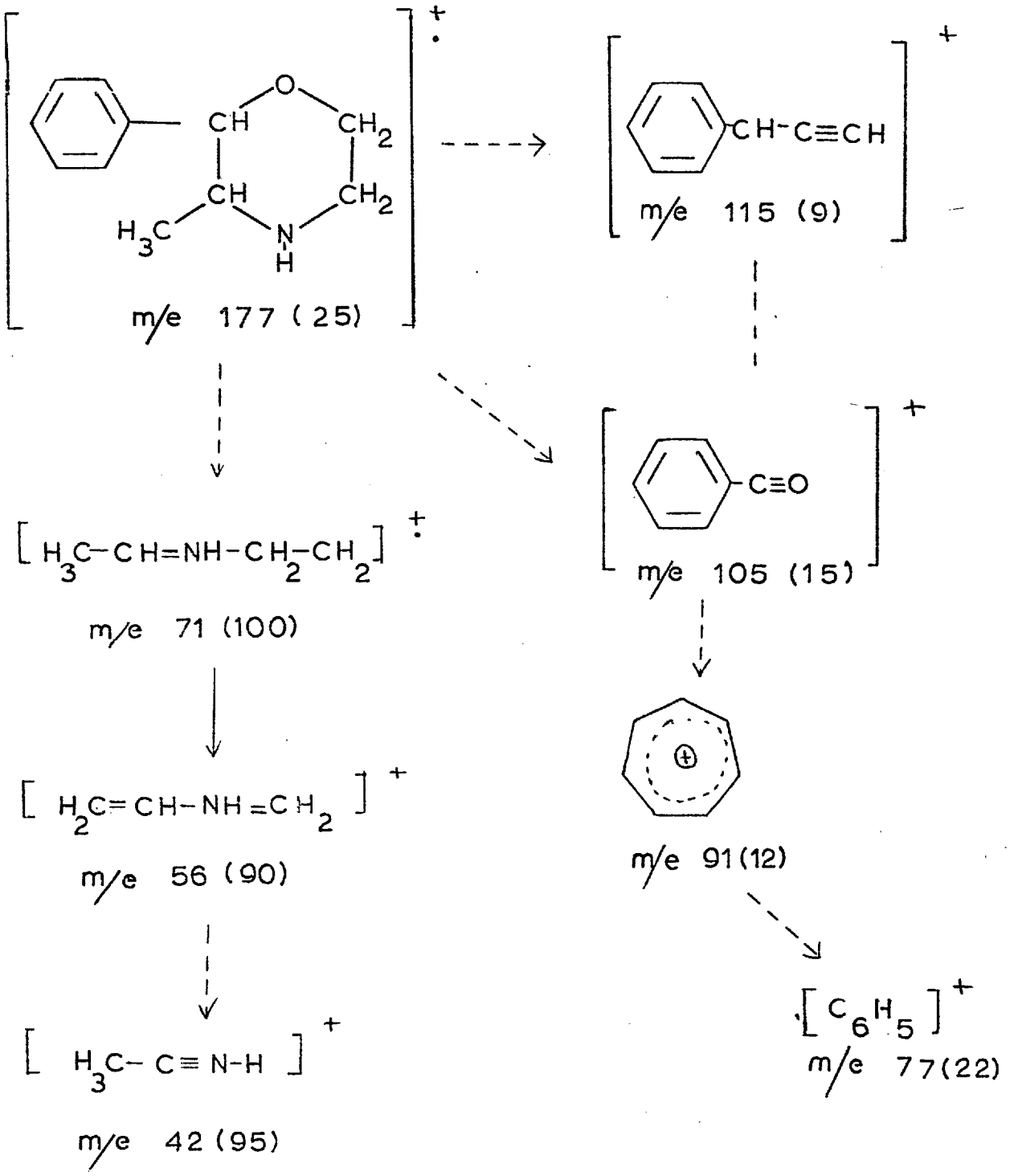
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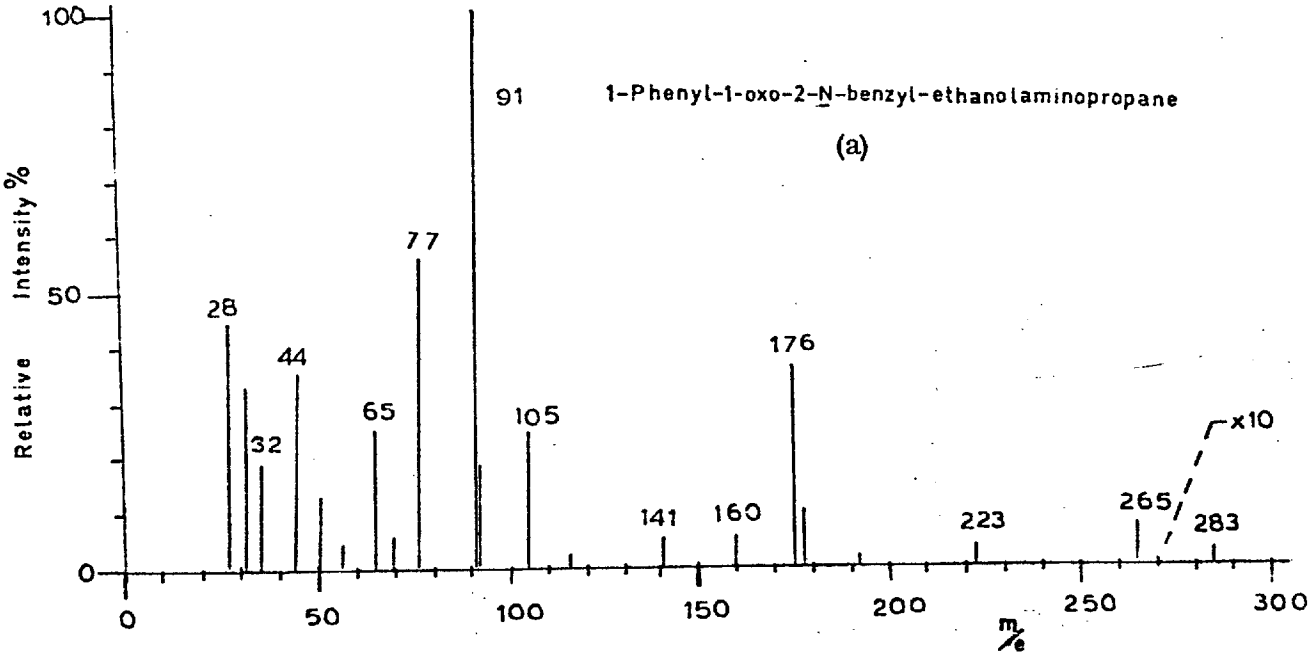


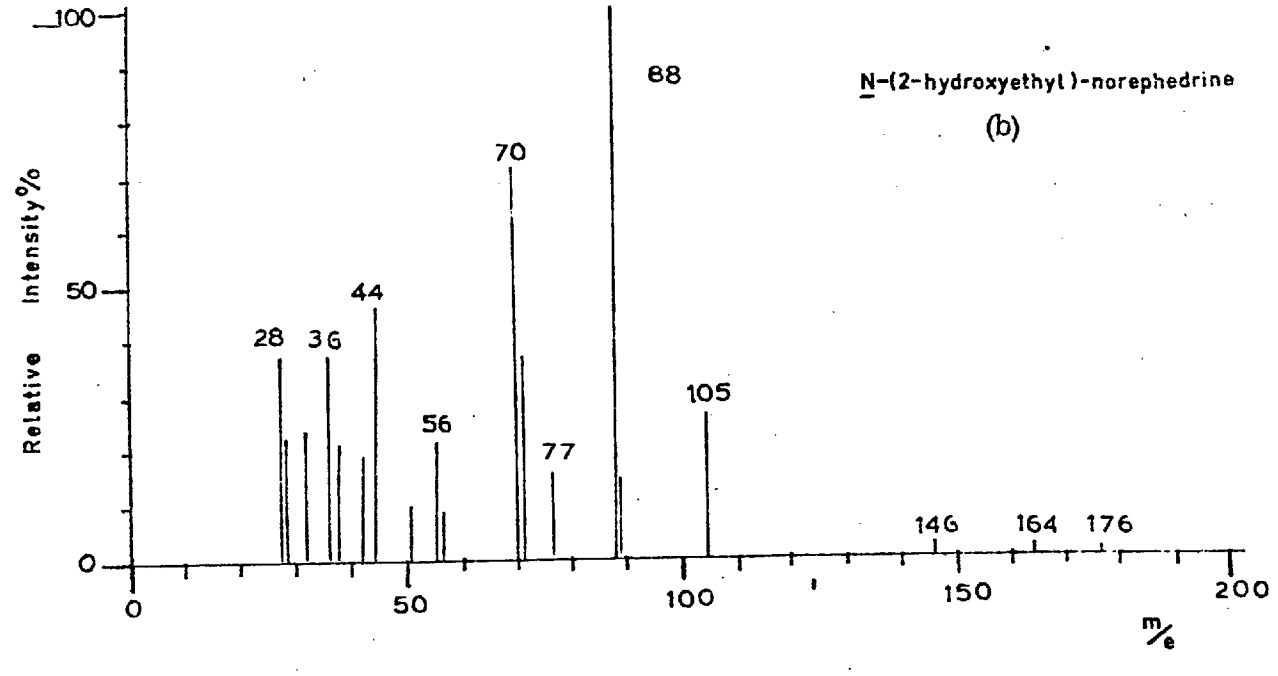
Graphical Representation of Mass Spectra of (a) Authentic Preludin and (b) Synthesised [^{14}C]-Preludin



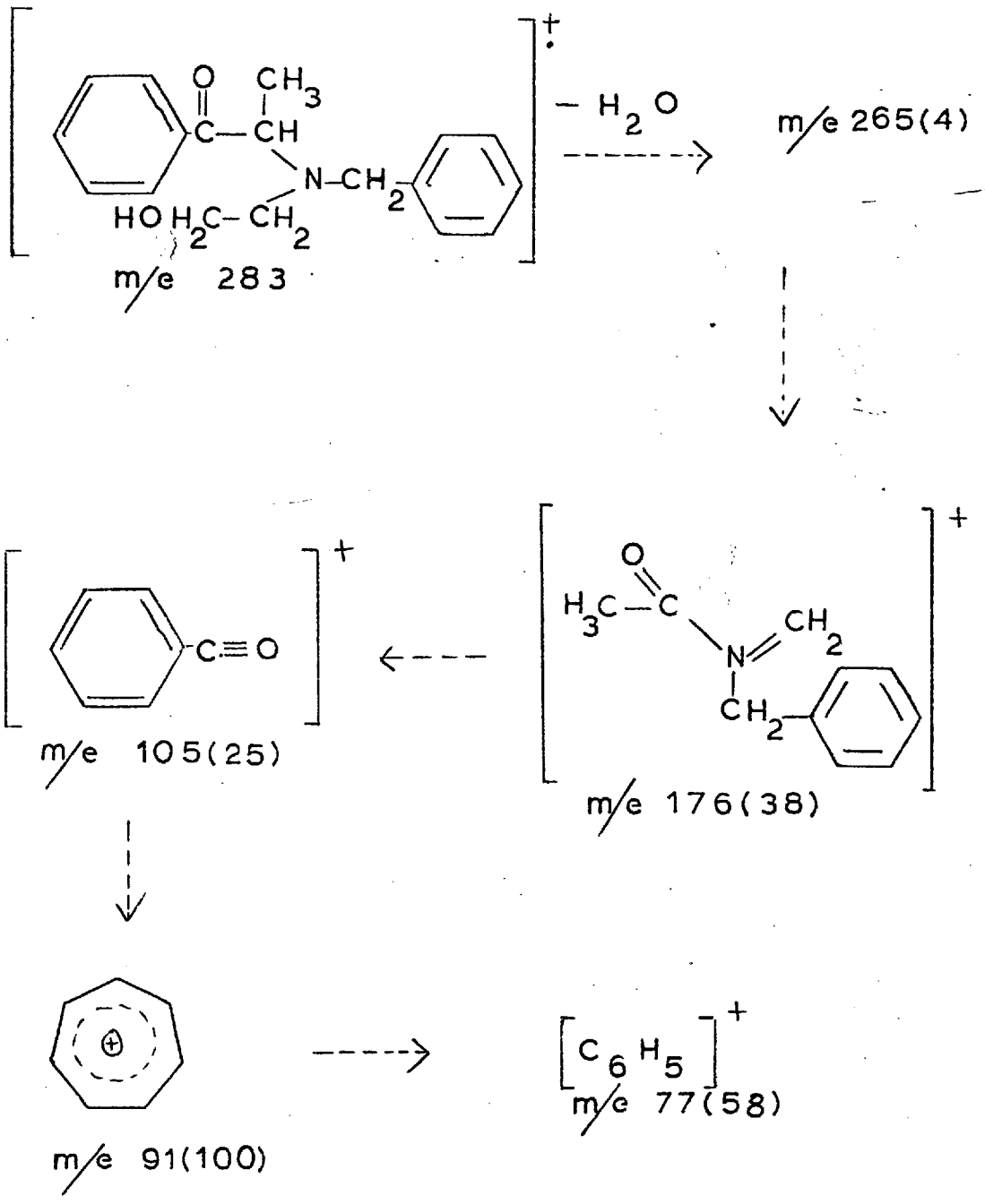
Possible fragmentation pattern for Preludin

Relative intensities are shown in parentheses. (Arrows depict a possible fragmentation pattern).



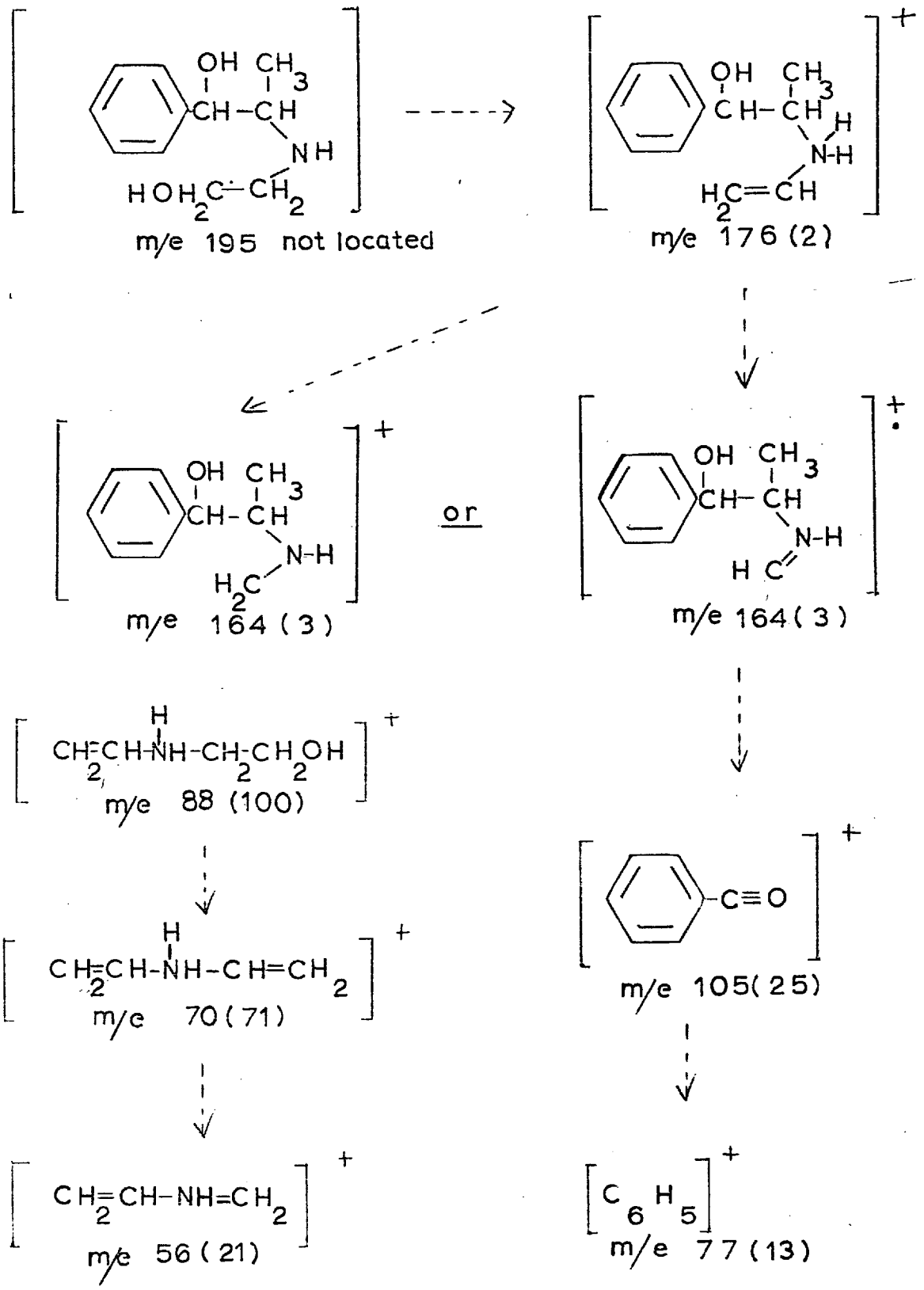


Graphical Representation of Mass Spectra of (a) 1-Phenyl-1-oxo-N-benzyl-ethanolaminopropane and (b) N-(2-Hydroxyethyl)-norephedrine or 1-Phenyl-2--ethanolaminopropan-1-ol



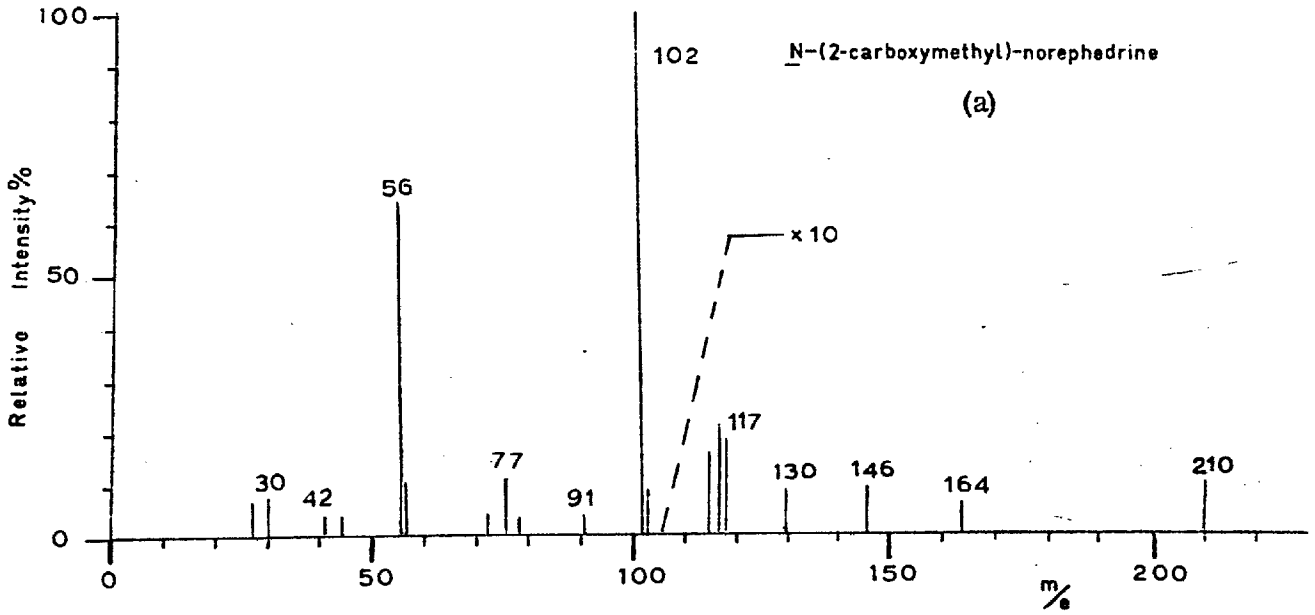
Possible fragmentation pattern of 1-phenyl-1-oxo-2-N-benzylethanolaminopropane

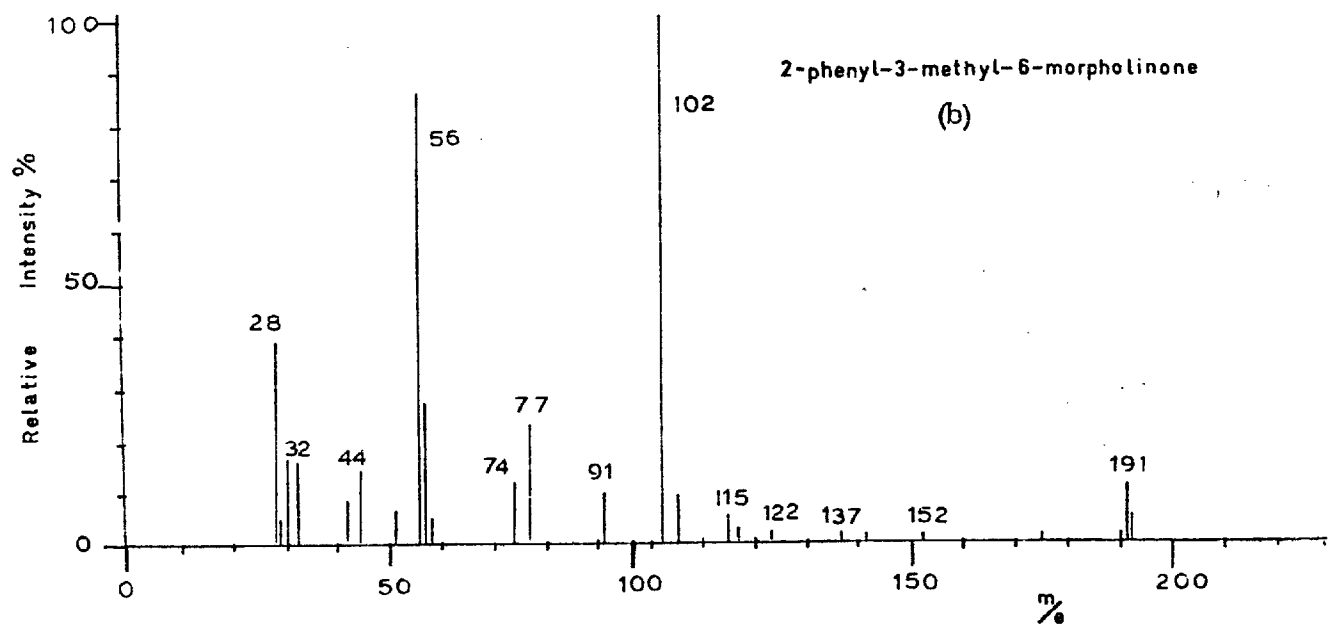
Relative intensities are shown in parentheses. Arrows depict a possible fragmentation pattern.



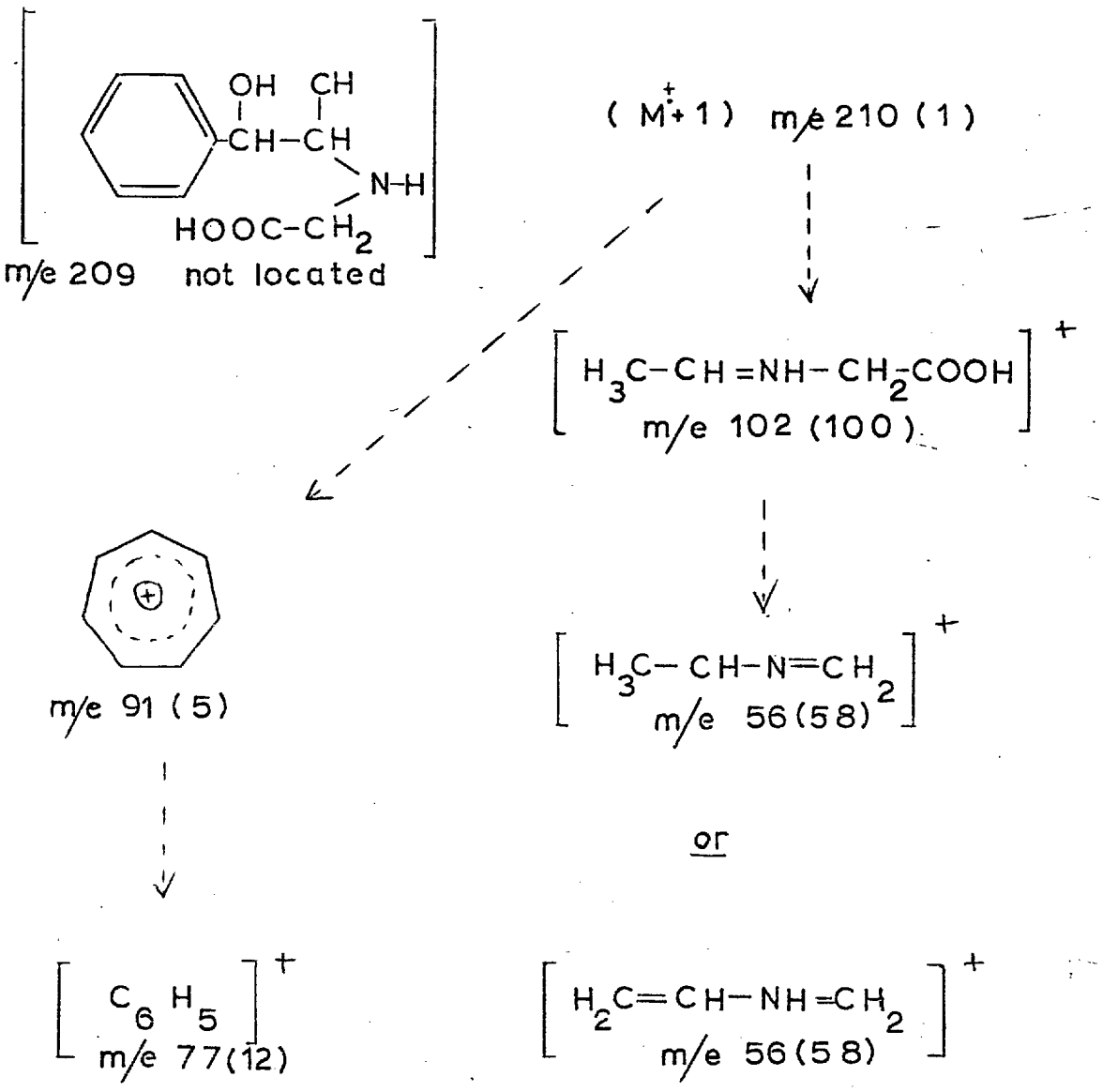
Possible fragmentation pattern of N-(2-hydroxyethyl)-norephedrine

Relative intensities are shown in parentheses. Arrows depict a possible fragmentation pattern.



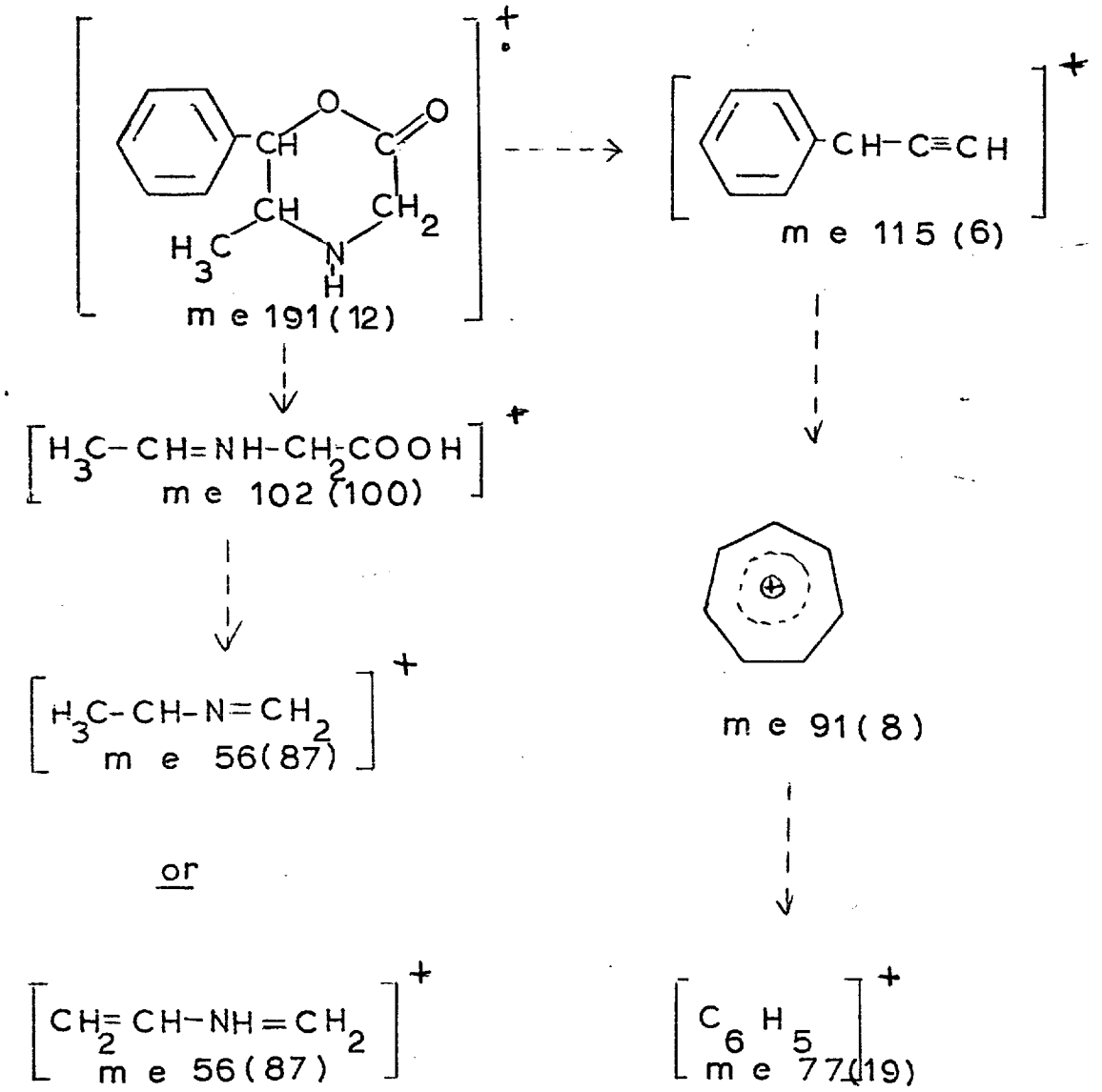


Graphical Representation of Mass Spectra of (a) N-(2-Carboxymethyl)-norephedrine and (b) 2-Phenyl-3-methyl-6-morpholinone



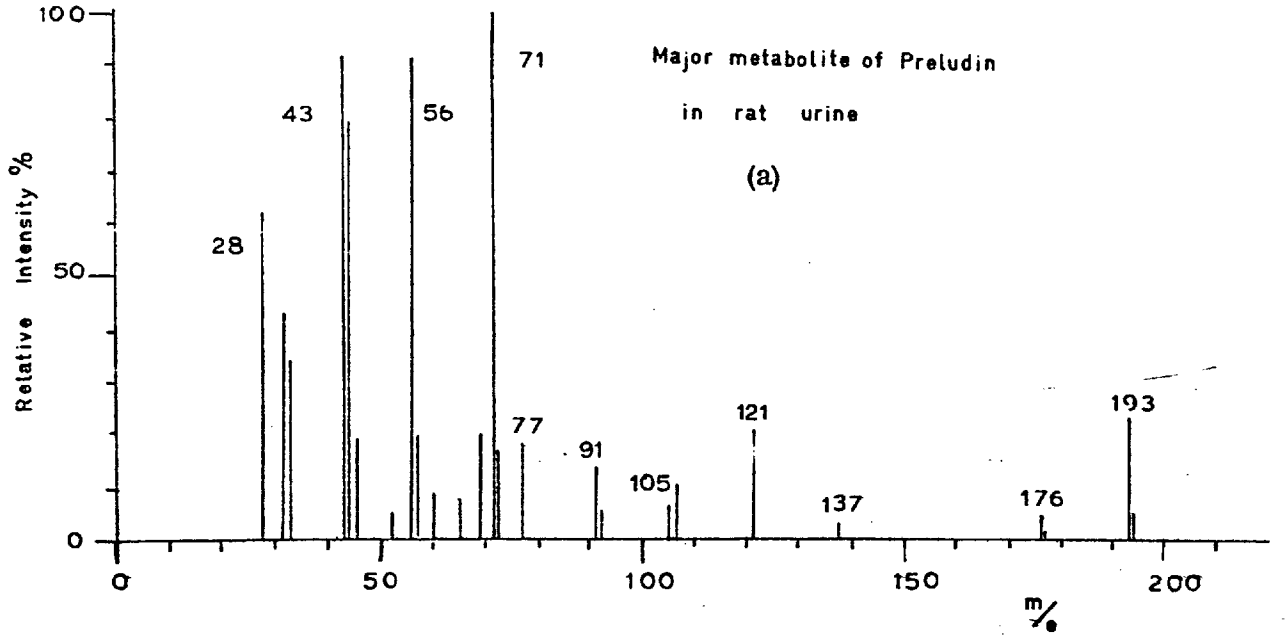
Possible fragmentation pattern of N-(2-carboxymethyl)-norephedrine

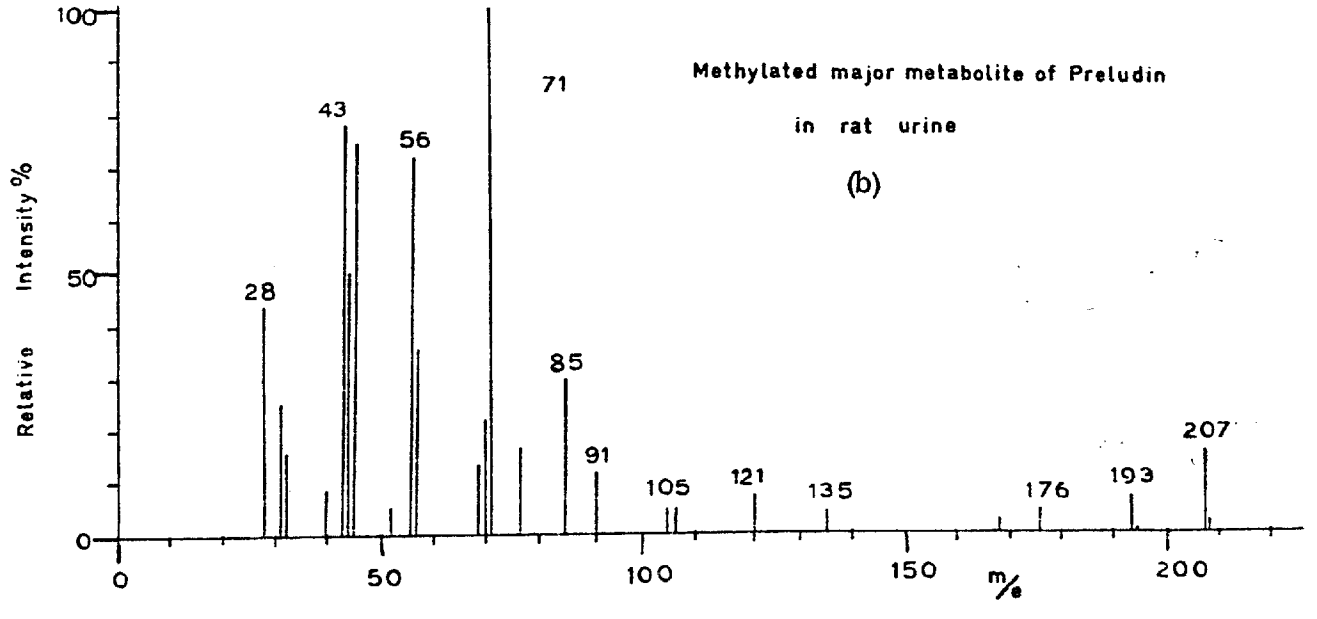
Relative intensities are shown in parentheses.



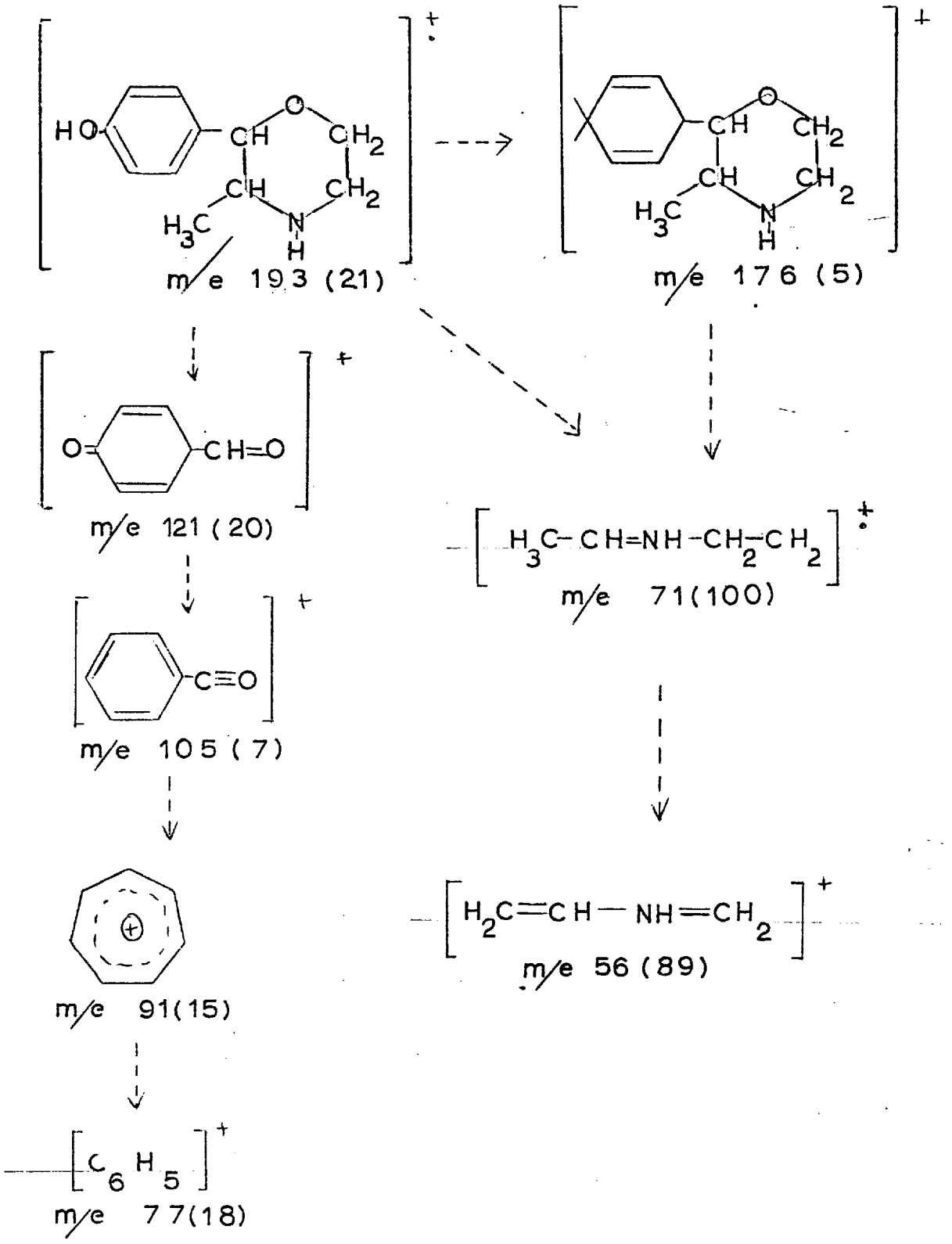
Possible fragmentation pattern of 2-phenyl-3-methyl-6-morpholinone

Relative intensities are shown in parentheses.



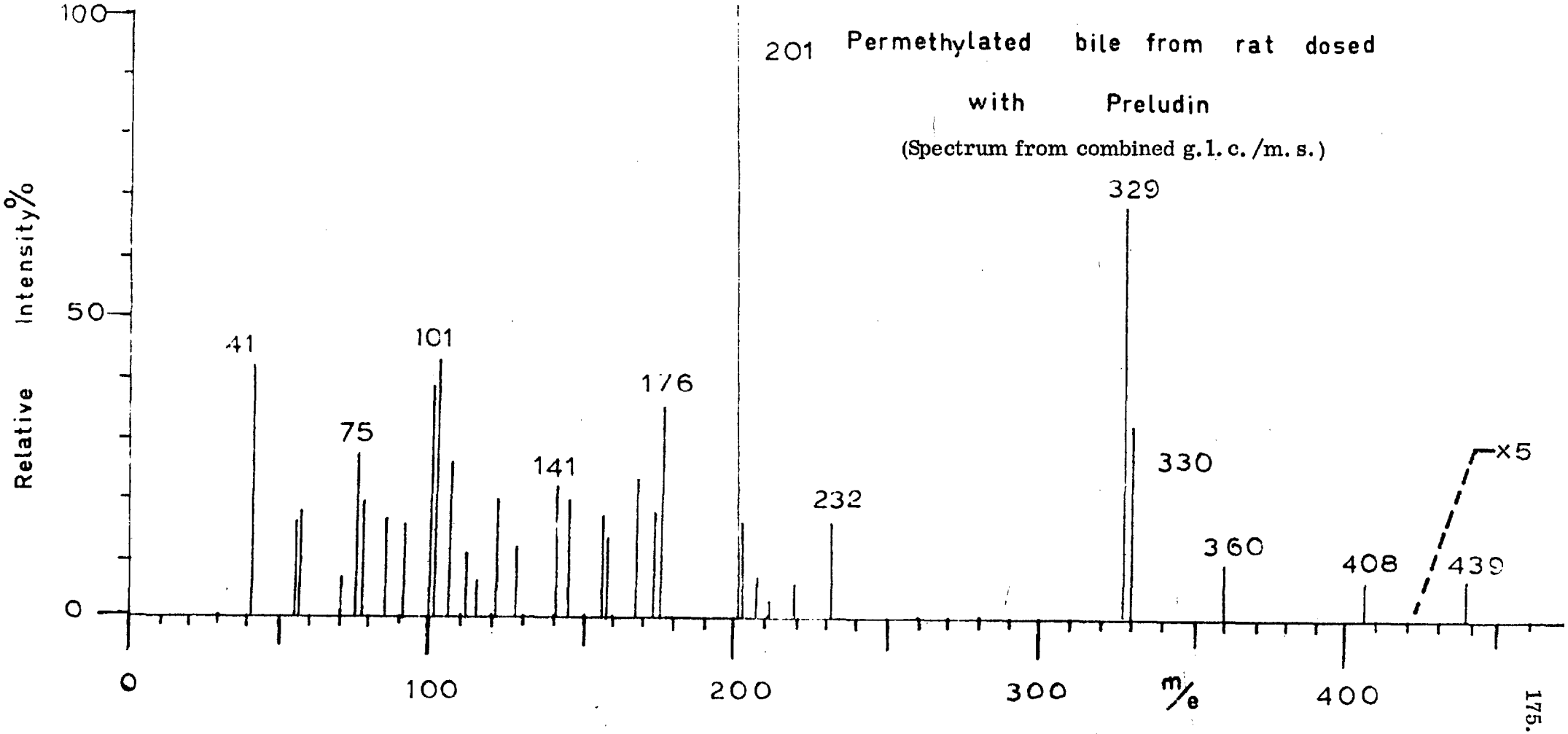


Graphical Representation of Mass Spectra of (a) the Major Metabolite of Preludin in 24 hour Rat Urine Namely the Phenolic Compound and (b) the Methylated Major Metabolite in Rat Urine

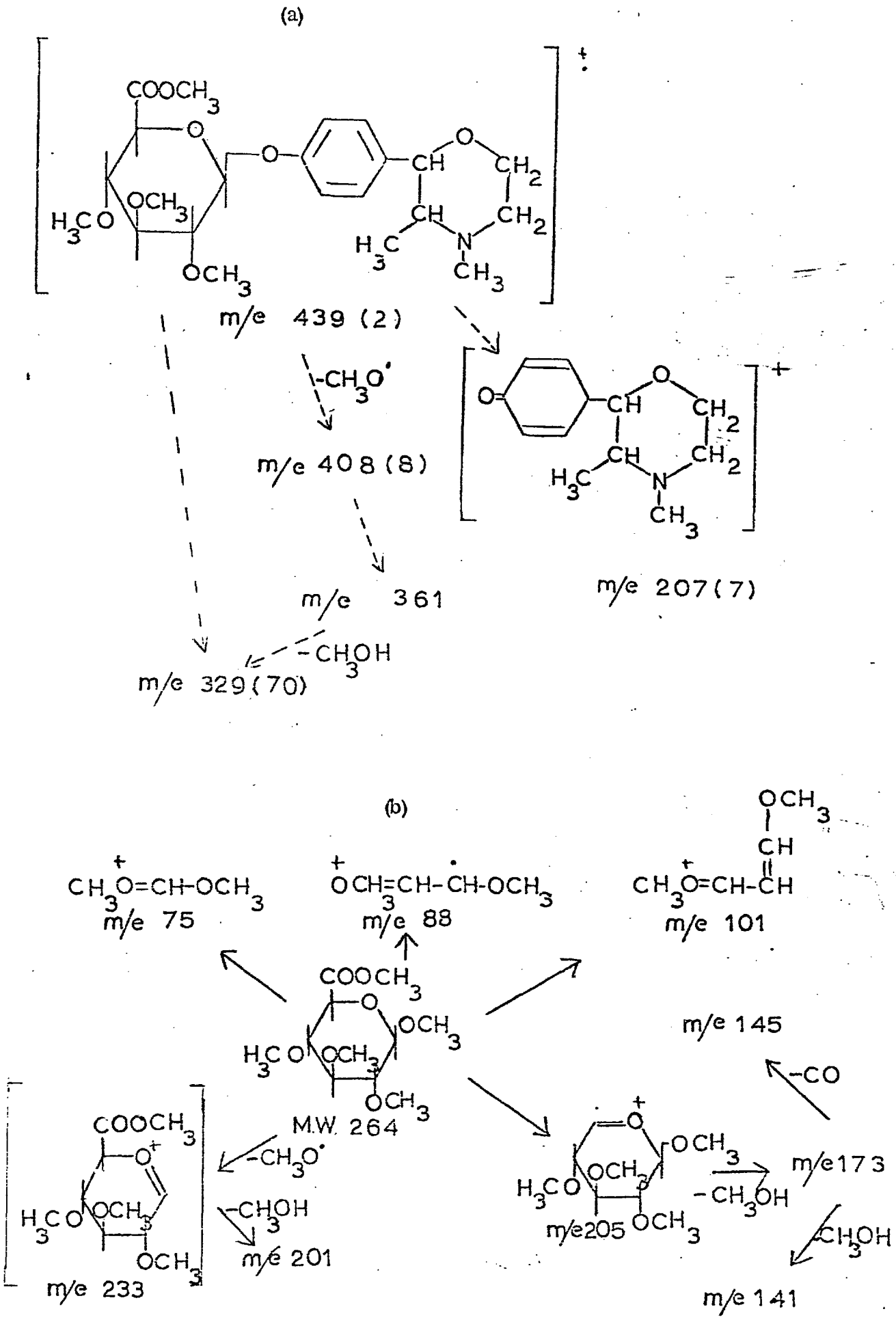


Possible fragmentation pattern for the phenolic compound, 2-(4'-hydroxyphenyl)-3-methylmorpholine.

Relative intensities are shown in parentheses.

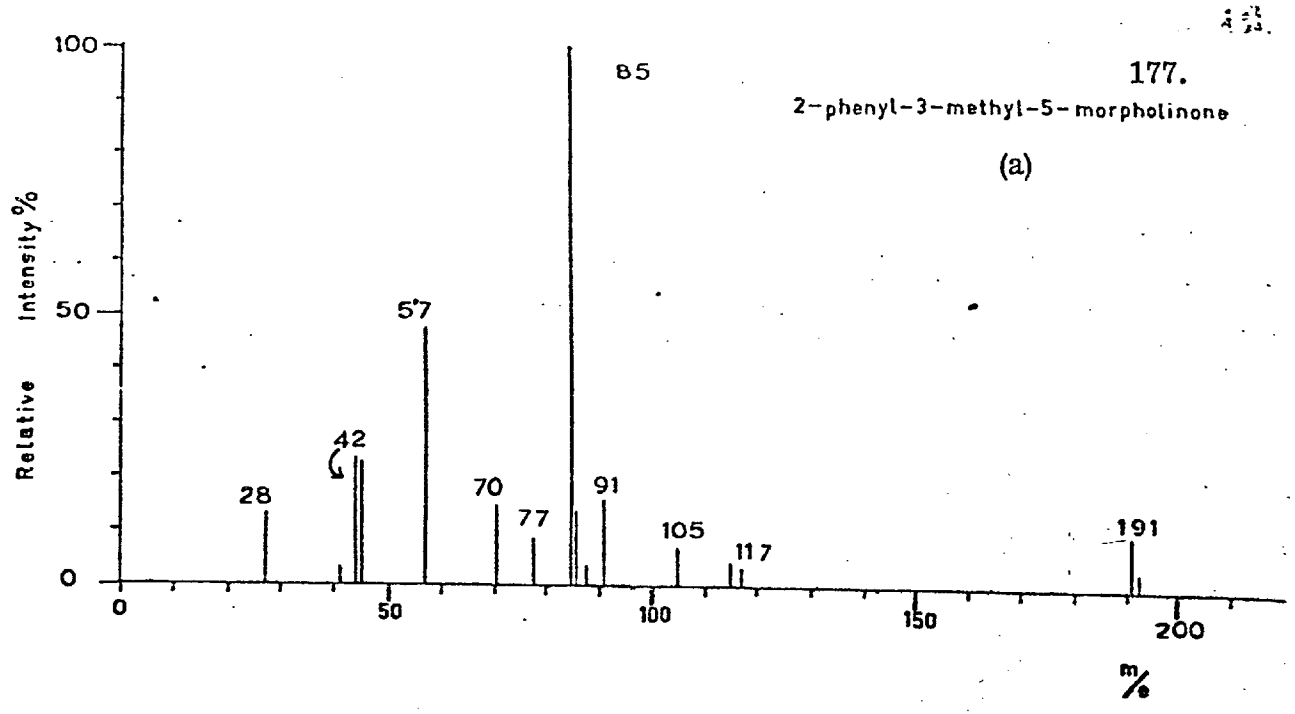


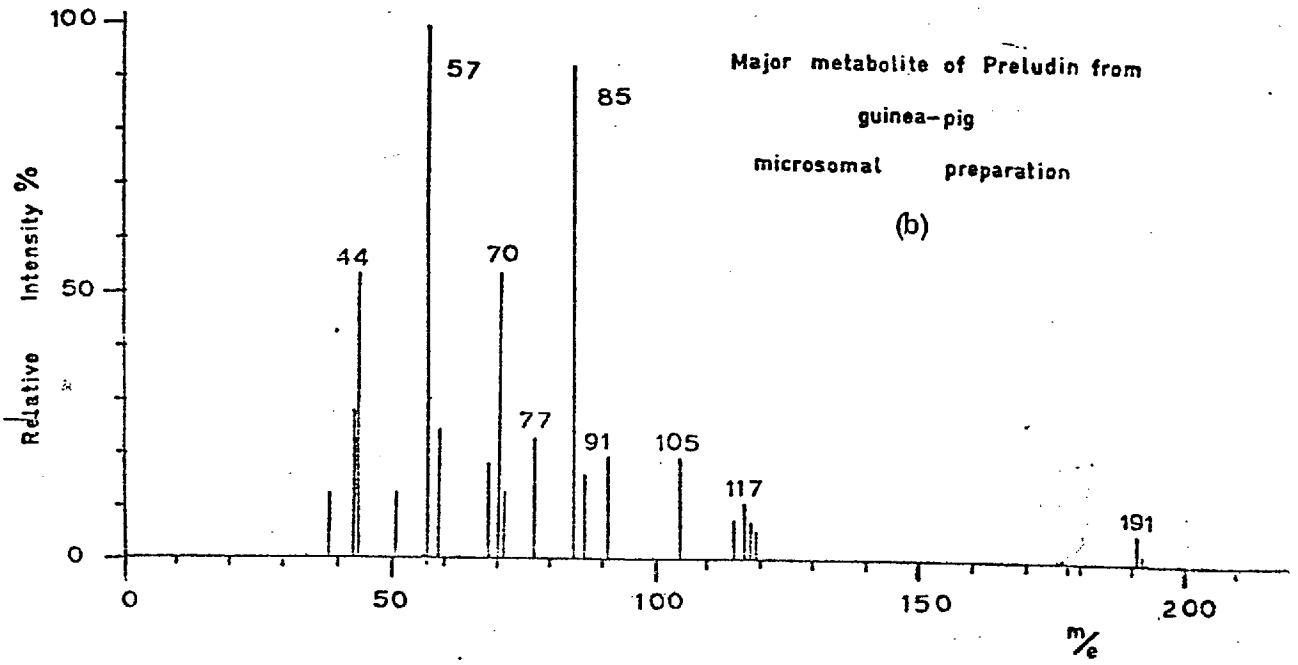
Graphical Representation of Mass Spectrum of Permethylated Bile from Rat Dosed with Preludin at 20 mg/kg Intraperitoneally.

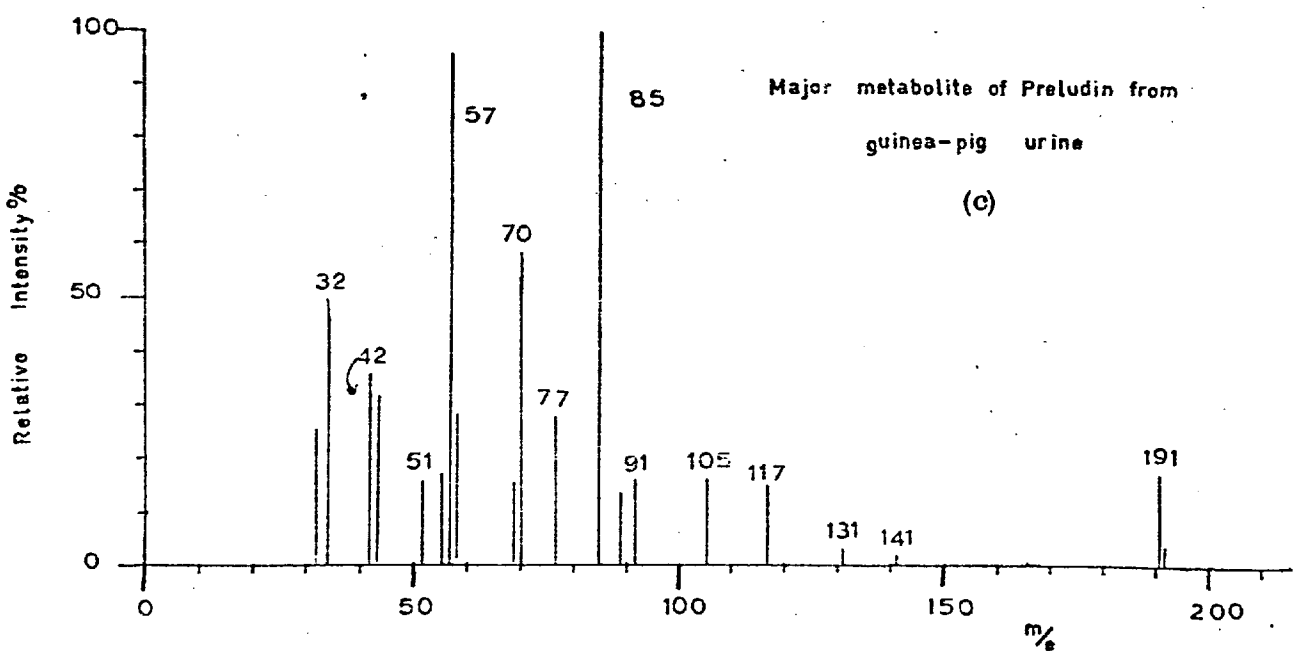


Possible fragmentation patterns of (a) the permethylated glucuronide metabolite in rat bile and (b) the fragmentation pattern of glucuronic acid (after Thompson et al., 1973).

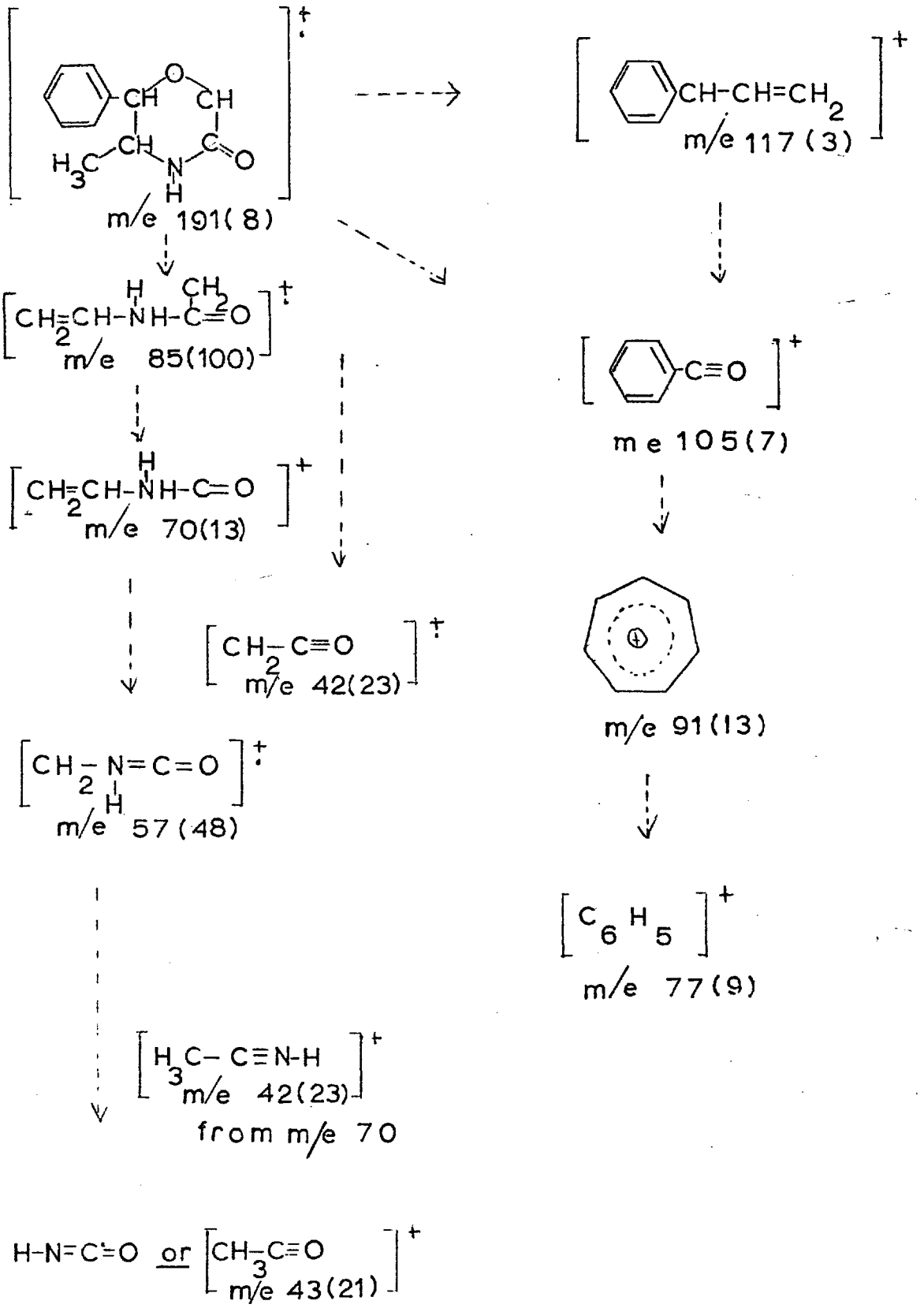
Relative intensities are shown in parentheses





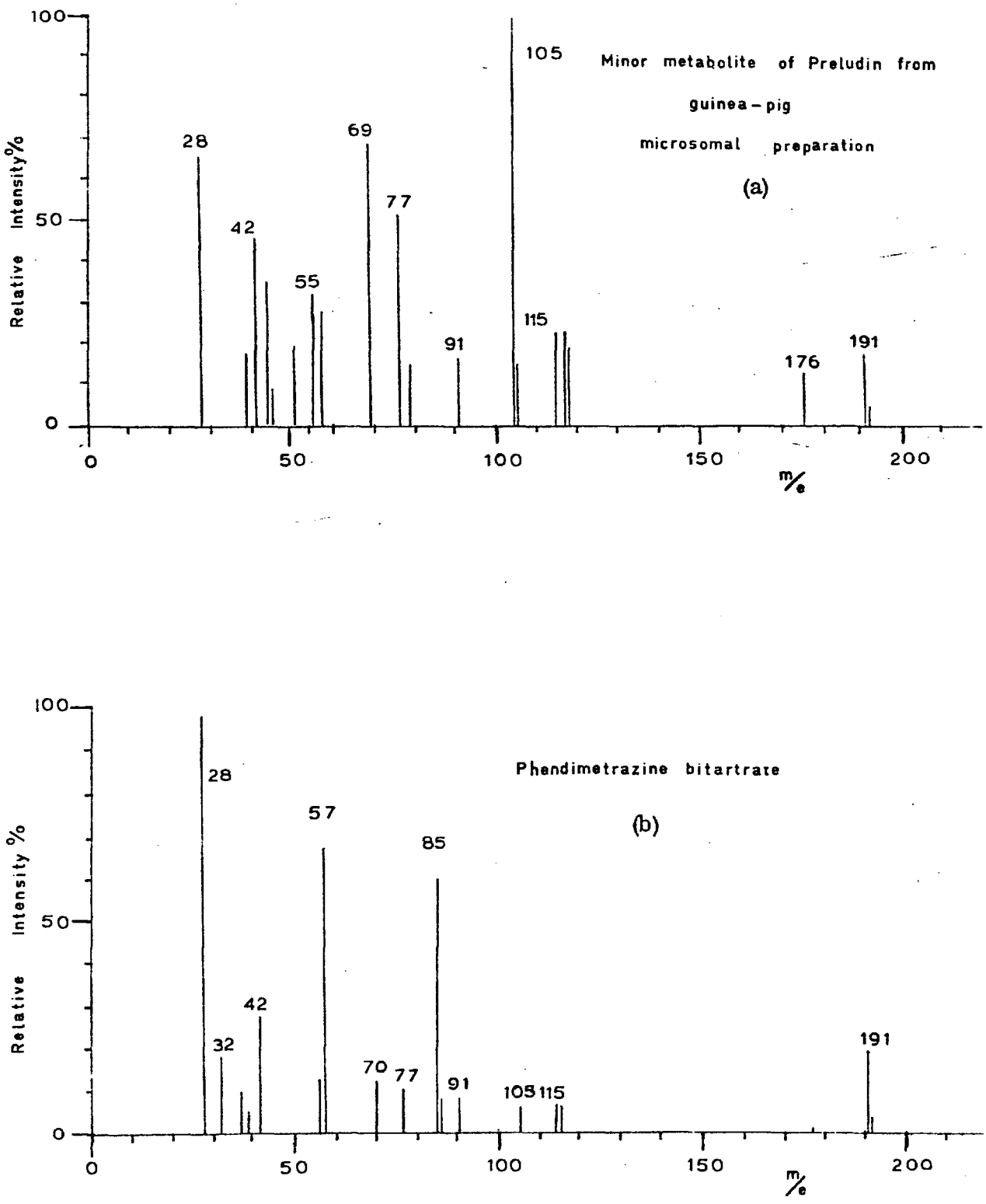


Graphical Representation of Mass Spectra of (a) 2-Phenyl-3-methyl-5-morpholinone, (b) the Major Metabolite of Preludin from the Guinea Pig Microsomal Preparation and (c) the Major Metabolite of Preludin from 24 hour Urine of Guinea Pig

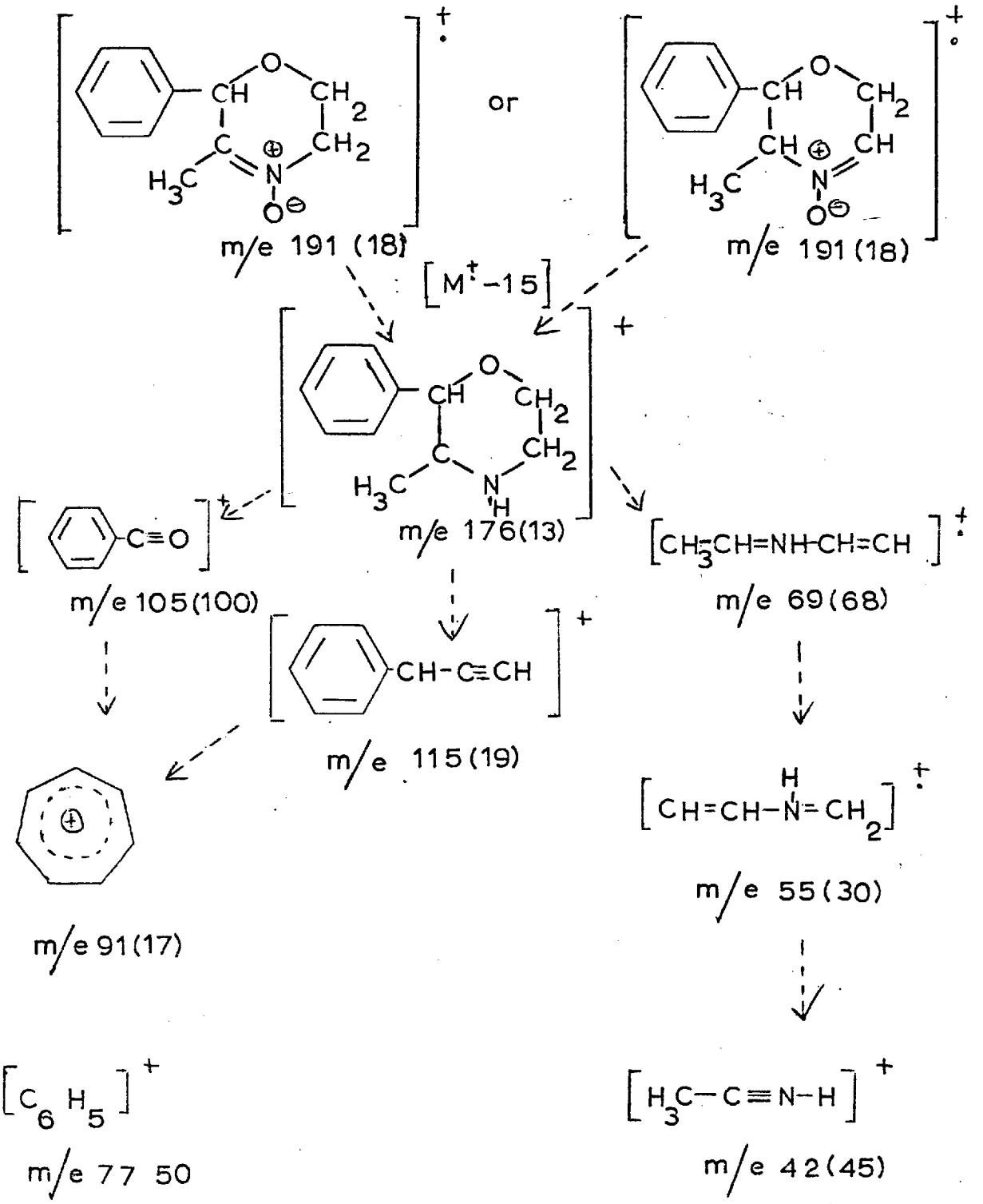


Possible fragmentation pattern of 2-phenyl-3-methyl-5-morpholinone

Relative intensities are shown in parentheses. Arrows depict a possible fragmentation pattern.

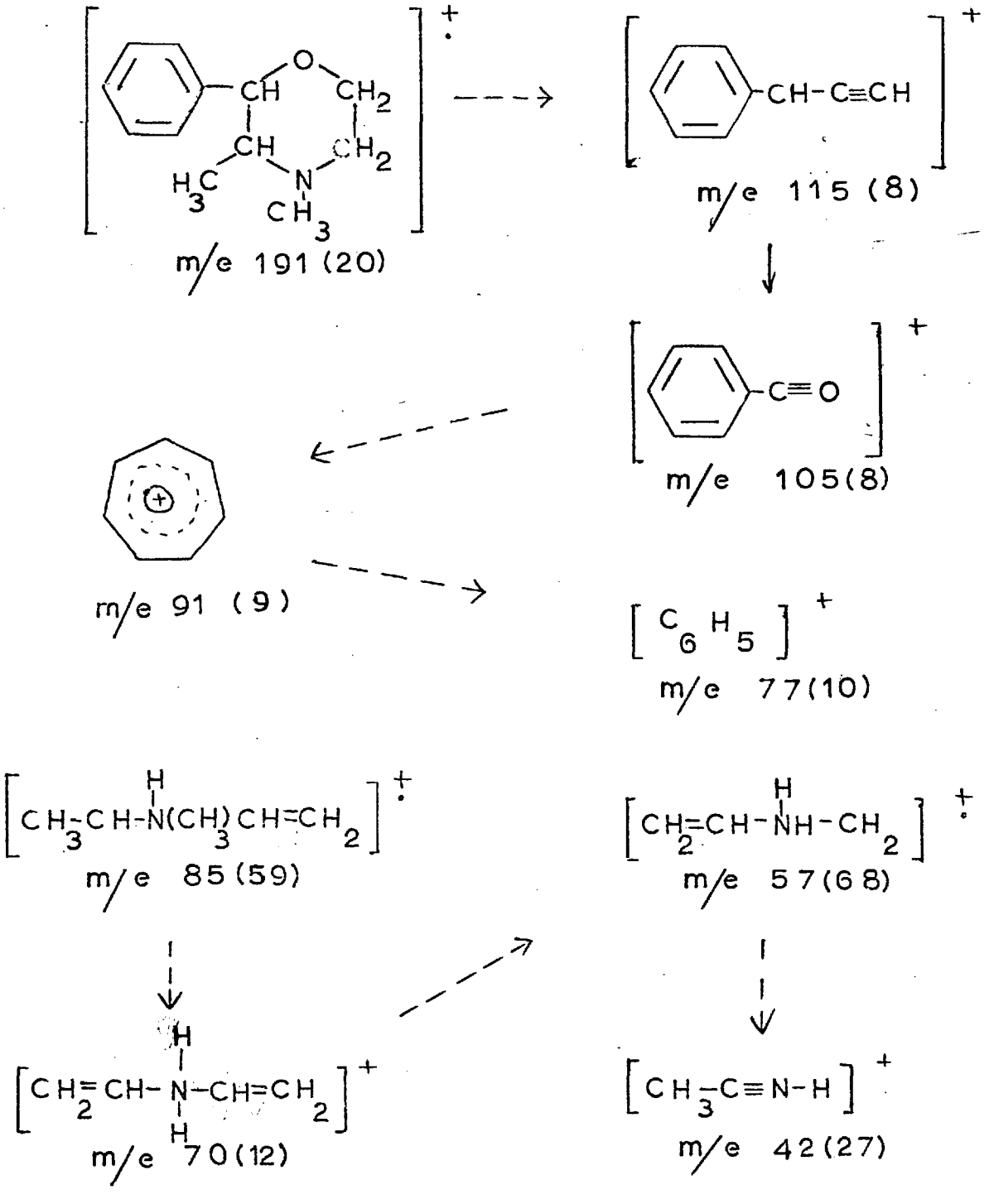


Graphical Representation of the Mass Spectra of (a) the Minor Metabolite (Unidentified) of Preludin from Guinea Pig Microsomal Preparation and (b) Phendimetrazine Bitartrate



Unidentified Metabolite (designated a nitrone)

Relative intensities are shown in parentheses. Arrows depict a theoretical fragmentation pattern.



Possible fragmentation pattern of phendimetrazine

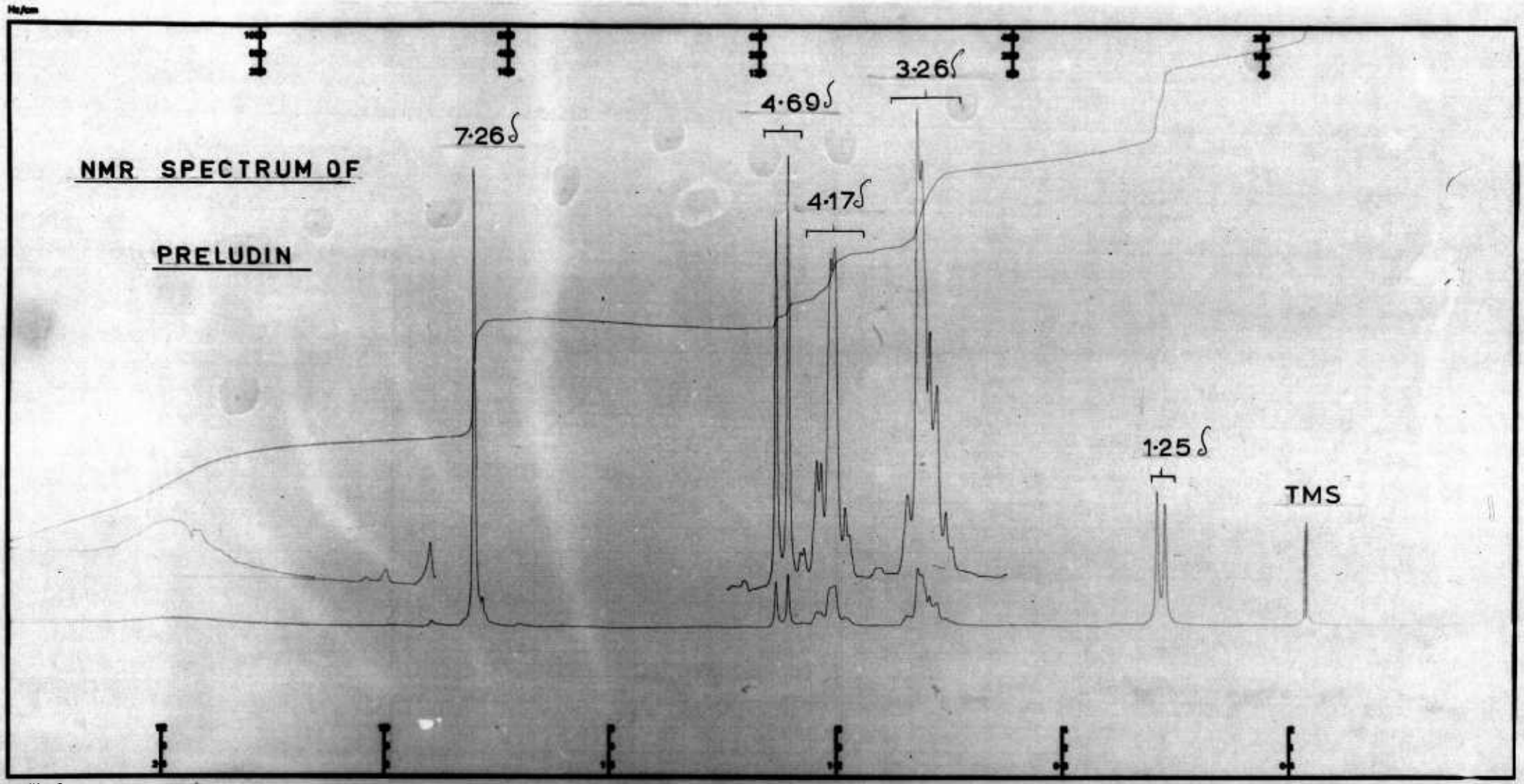
Relative intensities are shown in parentheses. Arrows depict a possible fragmentation pattern.

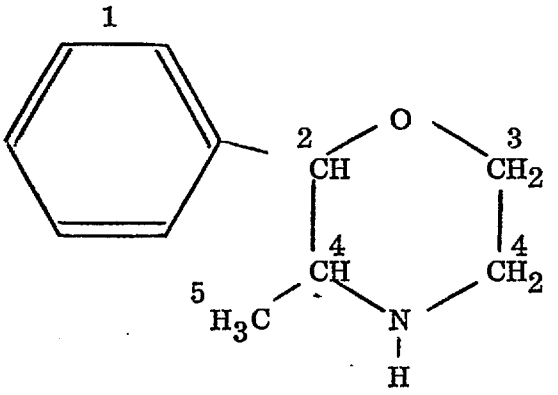
APPENDIX 3

NUCLEAR MAGENTIC RESONANCE (n. m. r.) SPECTRA AND PEAK ASSIGNMENT

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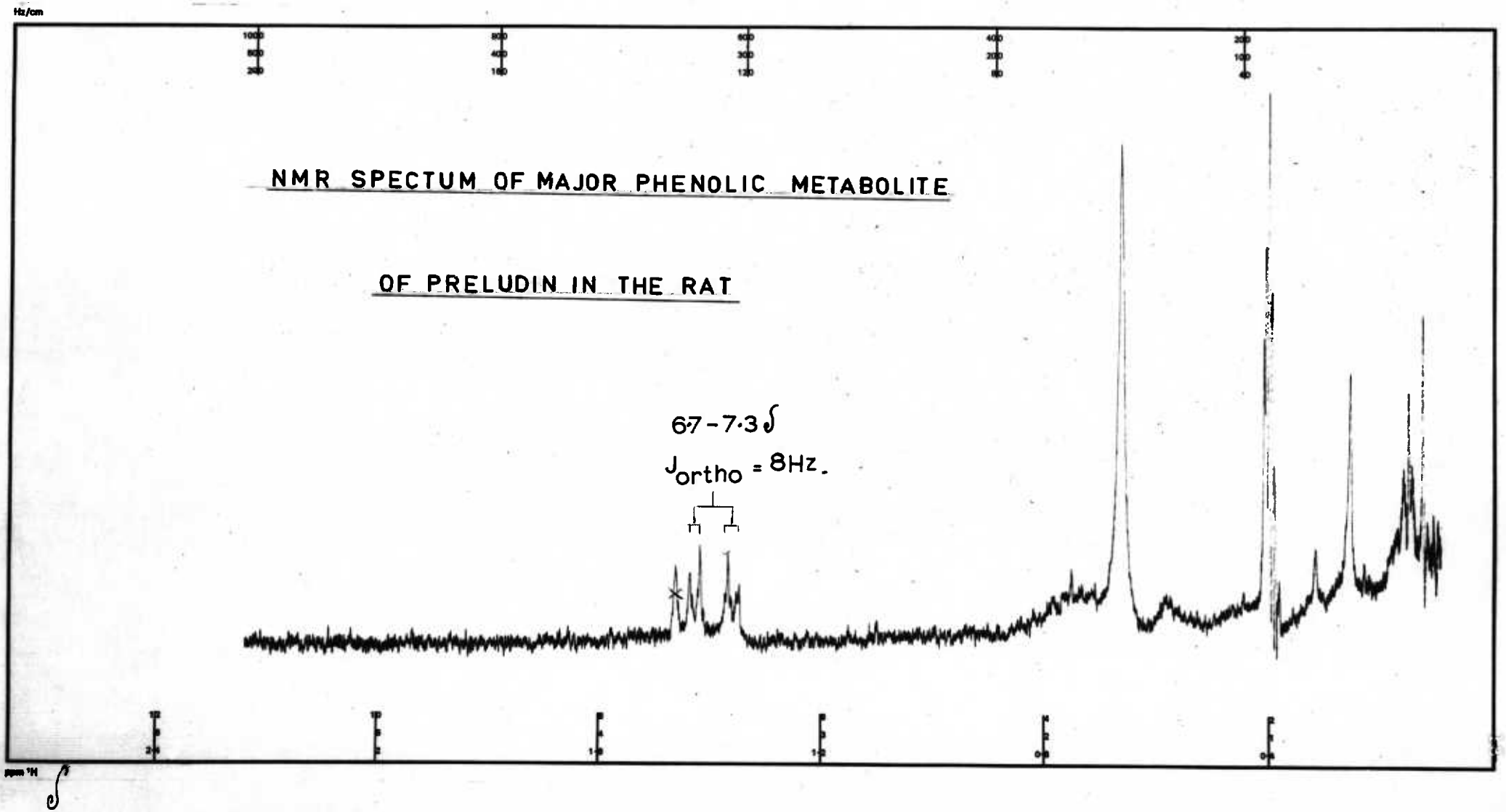

NMR SPECTRUM OF
PRELUDIN
7·26∫
4·69∫
3·26∫
4·17∫
1·25∫
TMS

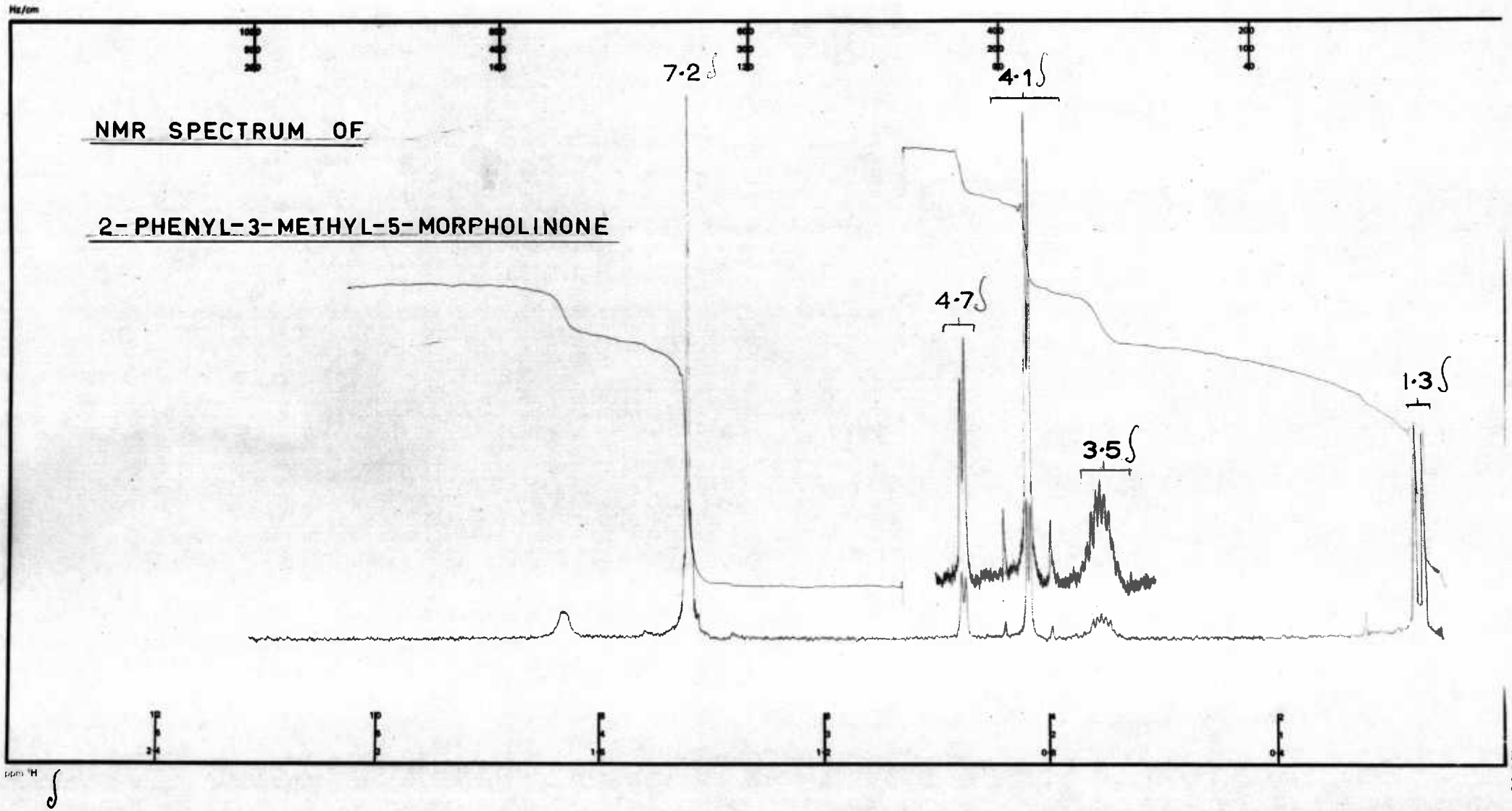


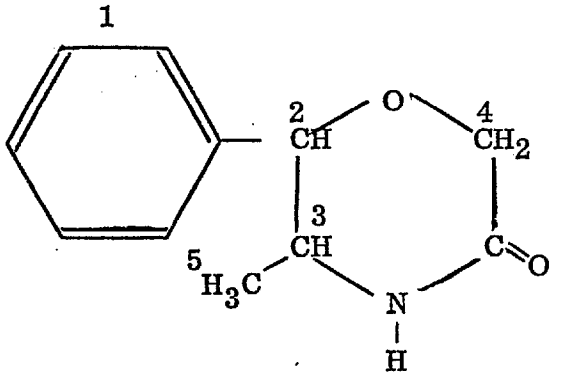
1.	Phenyl -	-	7.2δ	singlet
2.	Benzylic proton	-	4.6δ	doublet
3.	O-C<u>H</u>$_2$-	-	4.17δ	multiplet
4.	C<u>H</u>-N(H)-C<u>H</u>$_2$-	-	3.26δ	multiplet
5.	-C<u>H</u>$_3$	-	1.25δ	doublet

The chemical shifts are shown for the protons underlined.

Peak Assignment for Preludin


Hz/cm
NMR SPECTUM OF MAJOR PHENOLIC METABOLITE
OF PRELUDIN IN THE RAT
6·7 - 7·3 δ
J ortho = 8Hz.
ppm τH
δ


NMR SPECTRUM OF
2-PHENYL-3-METHYL-5-MORPHOLINONE
7·2
4·1
4·7
3·5
1·3

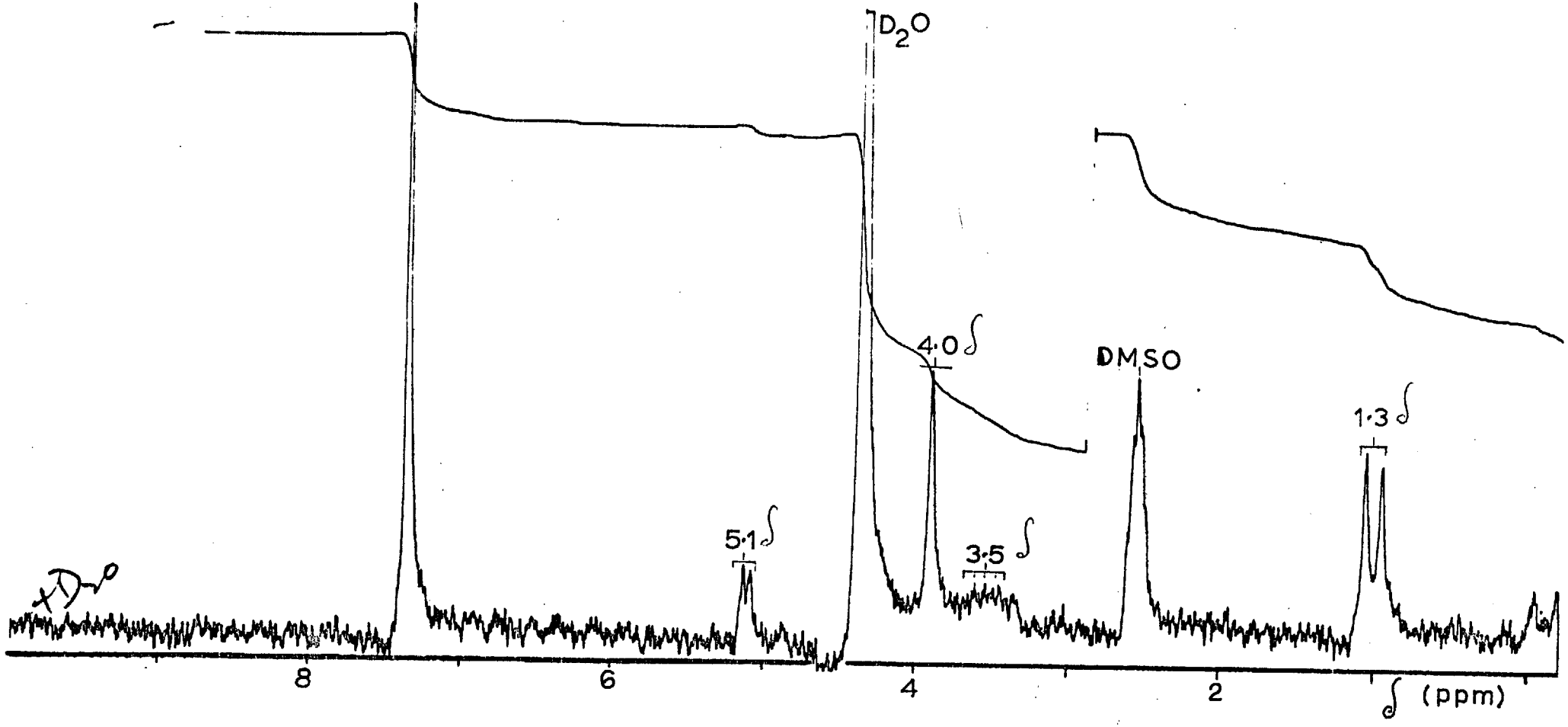


1.	Phenyl-	- 7.2δ	singlet
2.	Benzylic proton	- 4.7δ	doublet
3.	CH_3-C<u>H</u>-NH-	- 3.5δ	multiplet
4.	O-C<u>H</u>$_2$ -	- 4.1δ	split quadruplet
5.	-C<u>H</u>$_3$ -	- 1.3δ	doublet

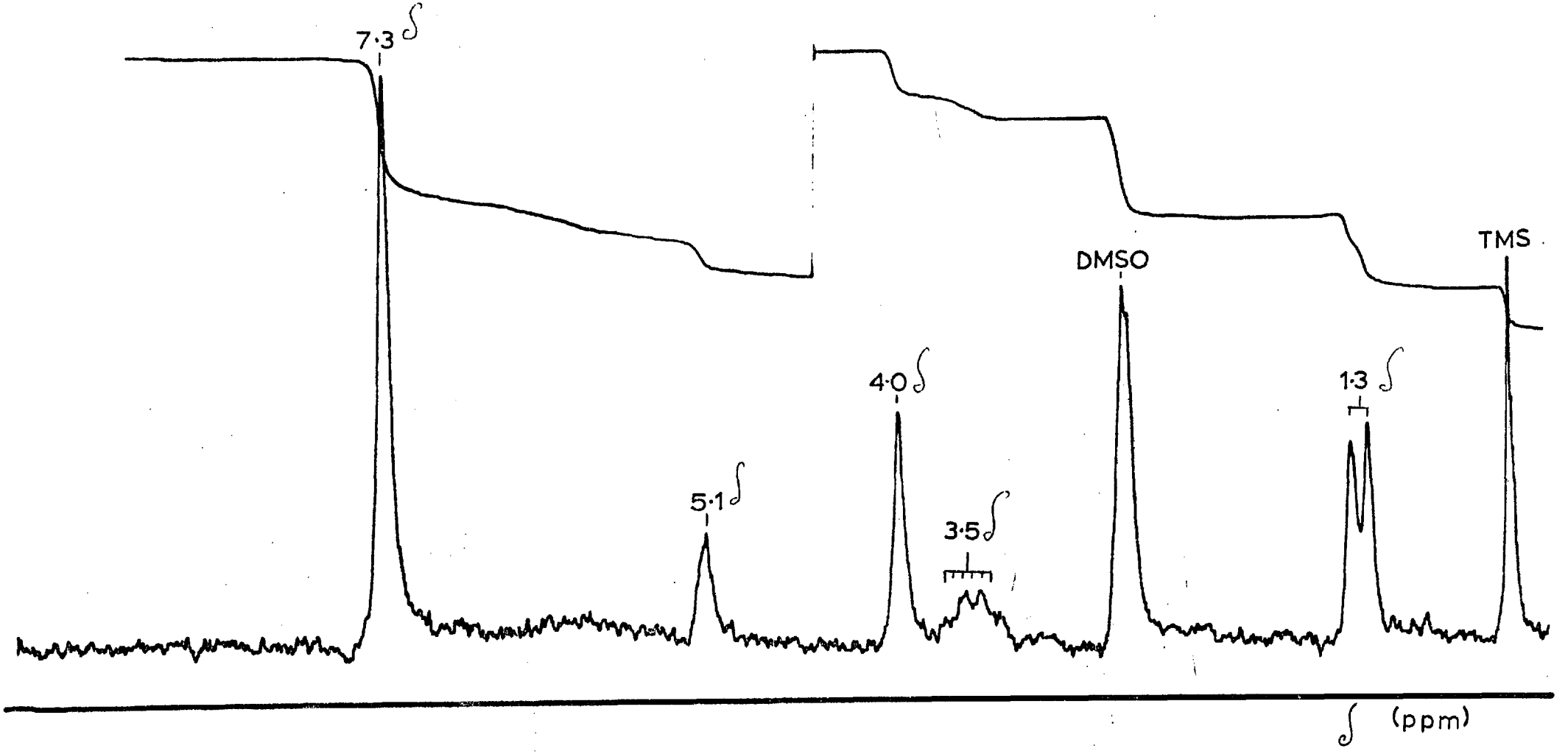
The chemical shifts are shown for the protons underlined.

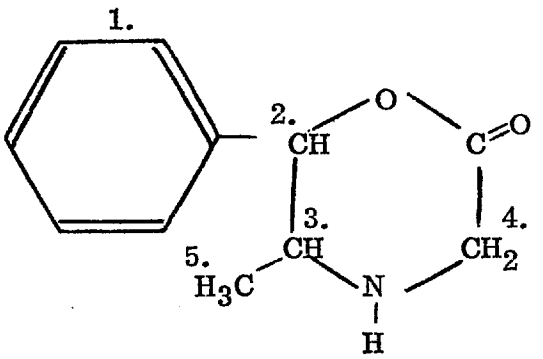
<u>Peak Assignment for 2-Phenyl-3-methyl-5-morpholinone</u>

NMR SPECTRUM OF 2-PHENYL- 3-METHYL- 6 - MORPHOLINONE + D_2O



NMR SPECTRUM OF 2-PHENYL-3-METHYL-6-MORPHOLINONE





1.	Phenyl-	-	7.2δ	singlet
2.	Benzylic proton	-	5.1δ	doublet
3.	CH_3-C<u>H</u>-NH-	-	3.5δ	multiplet
4.	CO-C<u>H_2</u>-NH-	-	4.0δ	singlet
5.	-C<u>H_3</u>	-	1.3δ	doublet

The chemical shifts are shown for the protons underlined

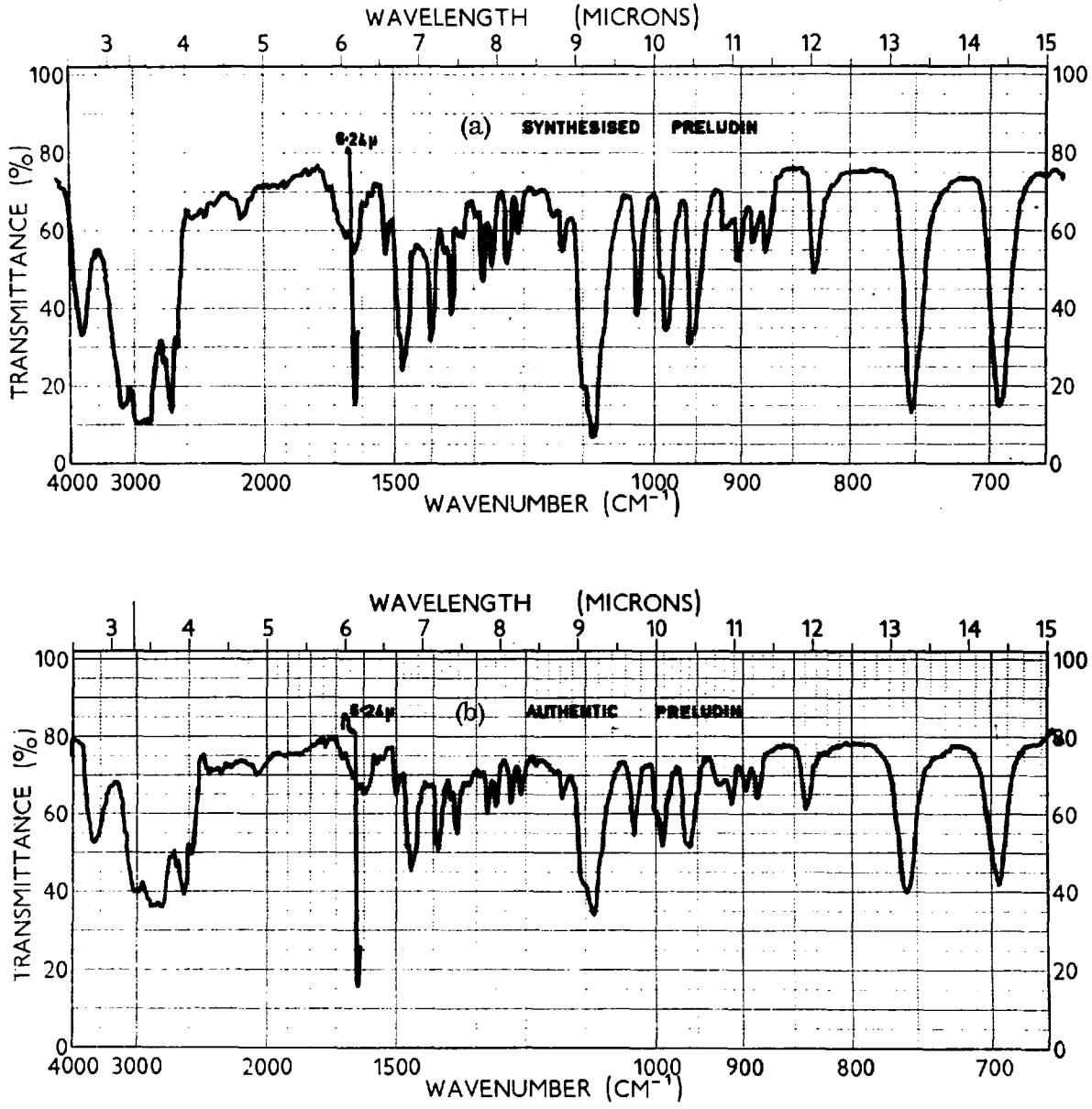
Peak Assignment for 2-Phenyl-3-methyl-6-morpholinone

APPENDIX 4.

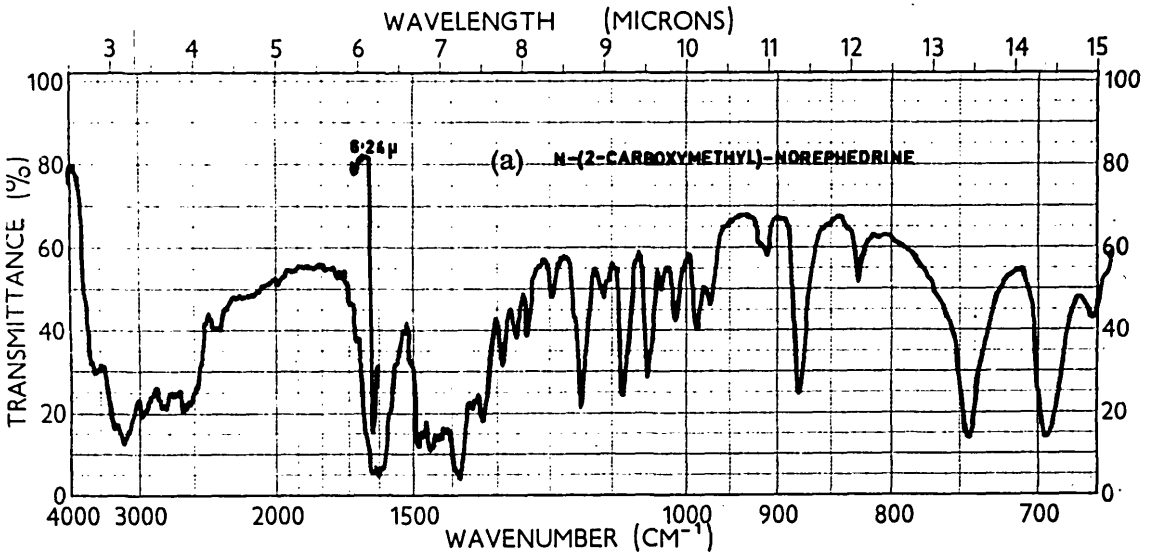
INFRA-RED (i. r.) SPECTRA

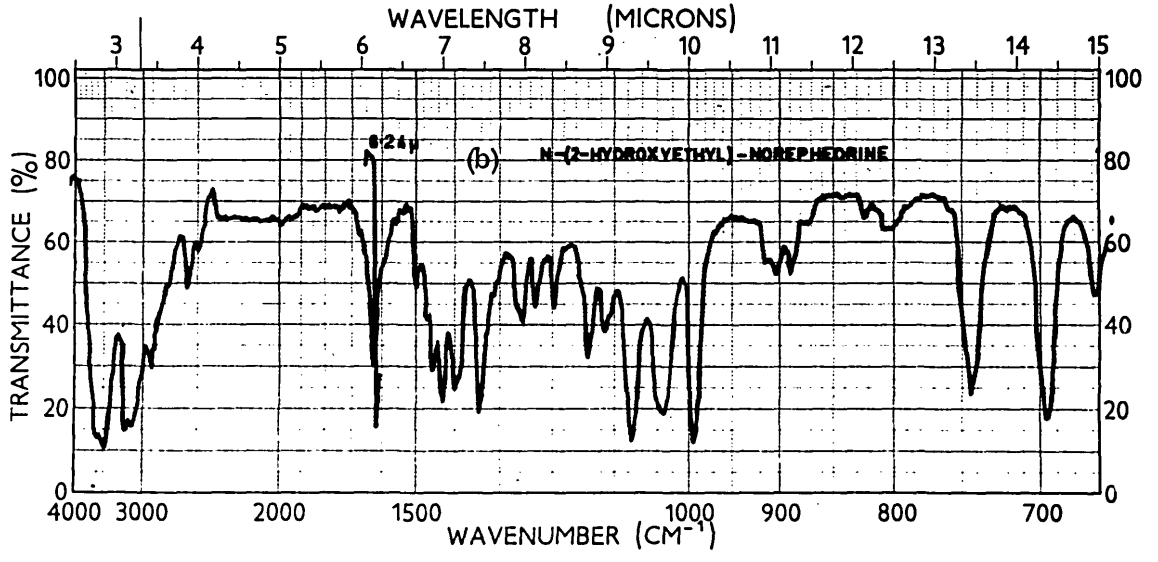
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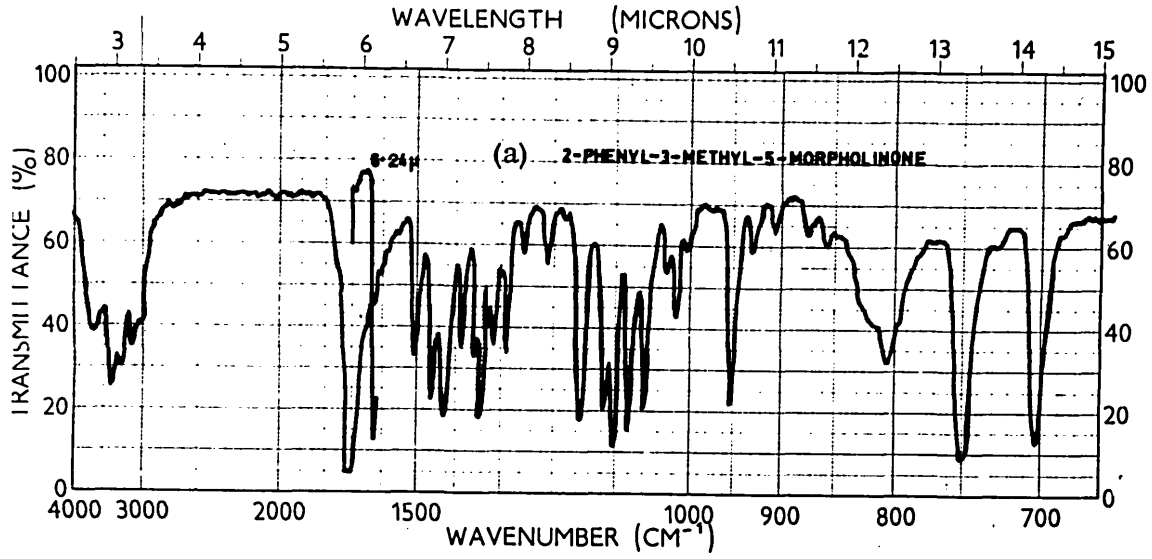


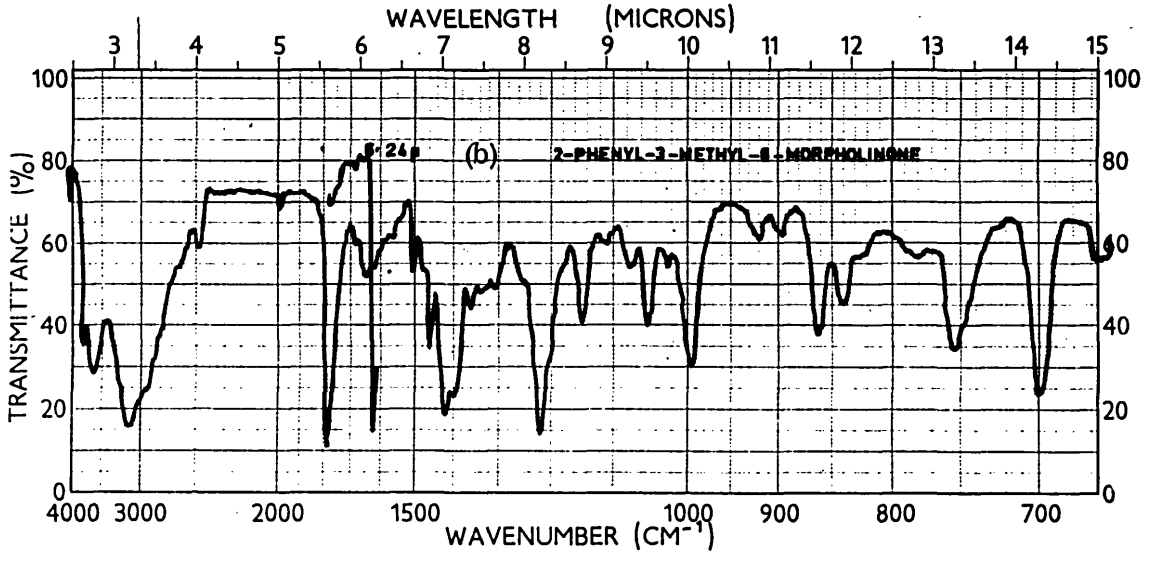
Infra-red Spectra of (a) Synthesised Preludin and (b) Authentic Preludin





Infra-red Spectra of (a) N-(2-Carboxymethyl)-norephedrine and (b) N-(2-Hydroxyethyl)-norephedrine or 1-Phenyl-2-ethanolaminopropan-1-ol





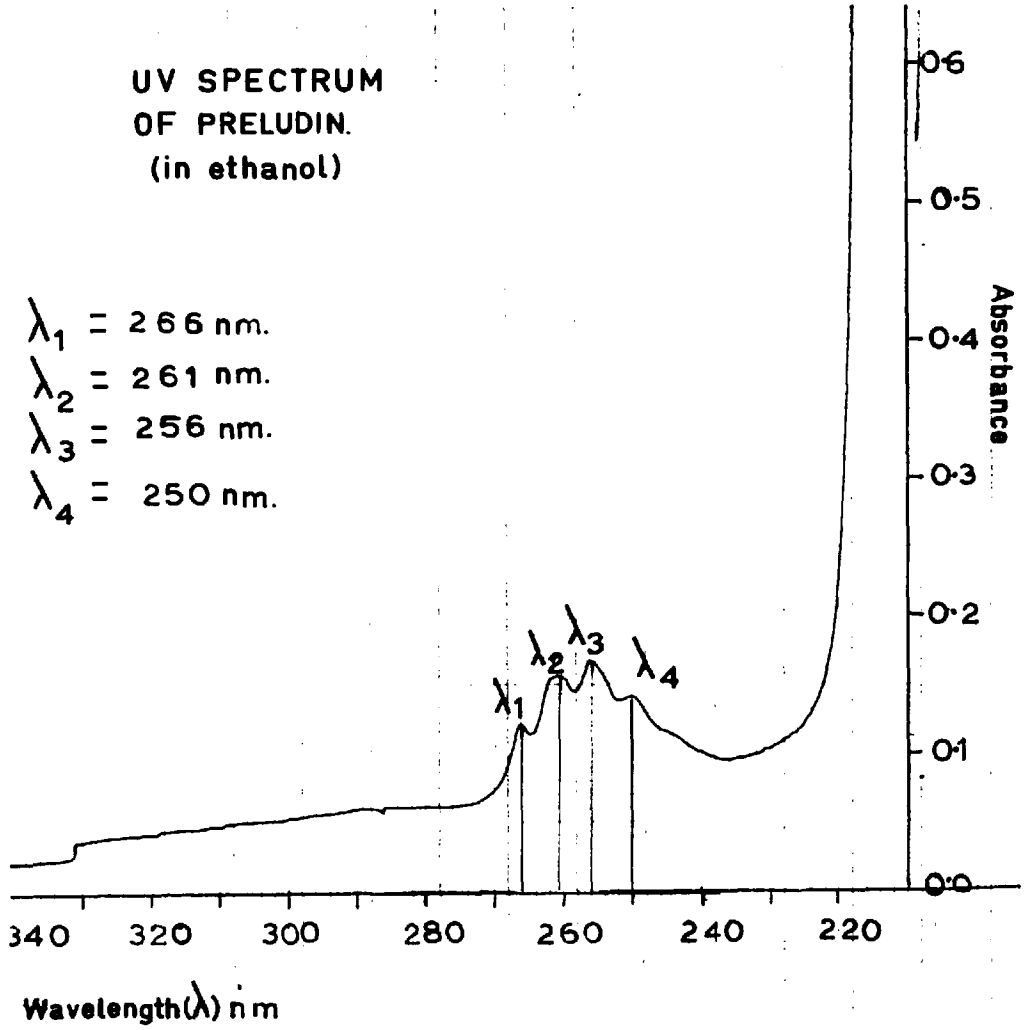
Infra-red Spectra of (a) 2-Phenyl-3-methyl-5-morpholinone and (b) the lactone, 2-phenyl-3-methyl-6-morpholinone

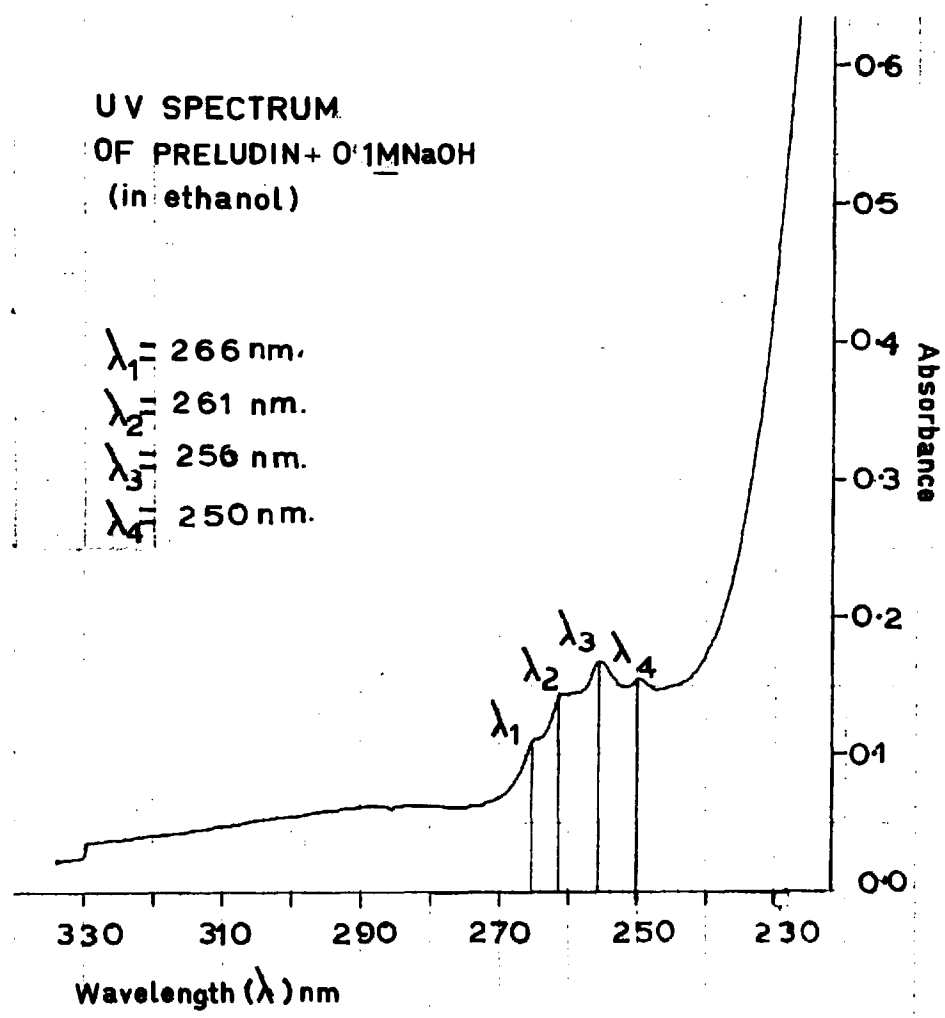
APPENDIX 5.

ULTRA-VIOLET (u. v.) SPECTRA

Contents

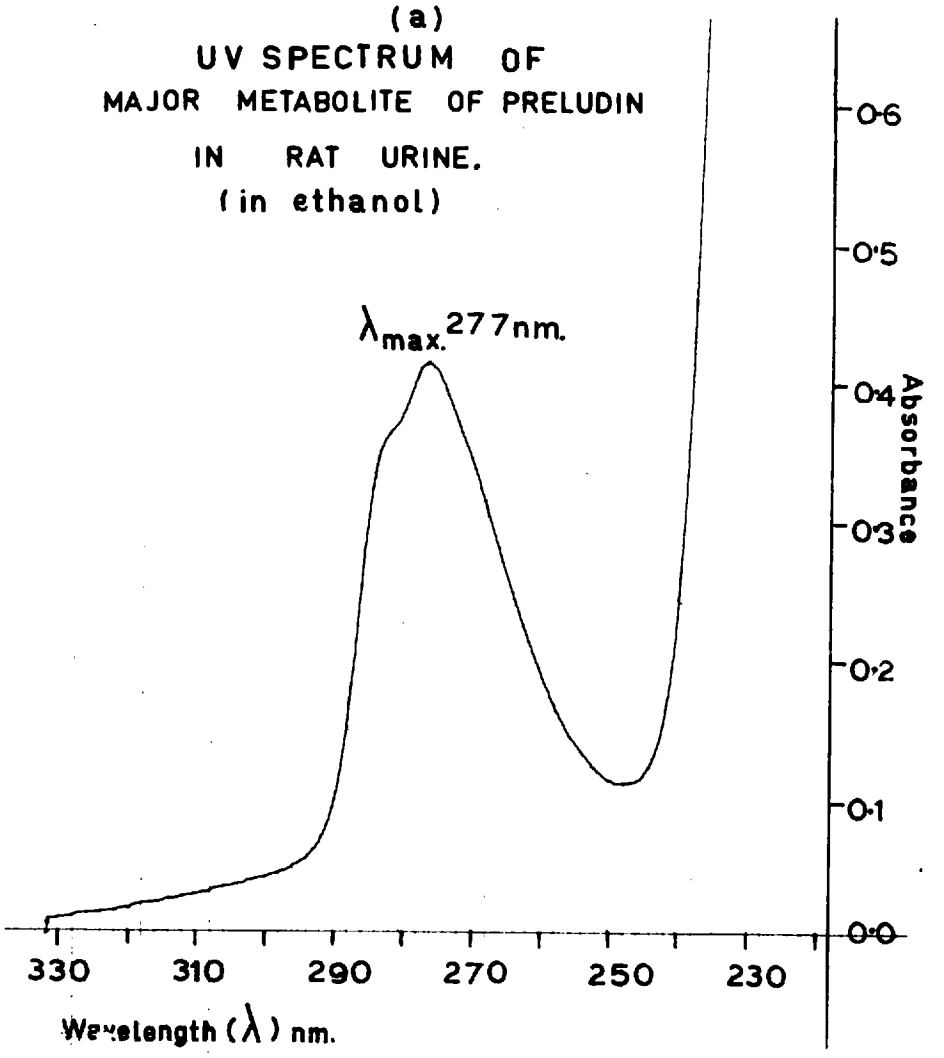
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UV SPECTRUM
OF PRELUDIN.
(in ethanol)
λ1 = 266 nm.
λ2 = 261 nm.
λ3 = 256 nm.
λ4 = 250 nm.
λ1
λ2
λ3
λ4
0·6
0·5
0·4
0·3
0·2
0·1
0·0
Absorbance
340
320
300
280
260
240
220
Wavelength(λ) nm


UV SPECTRUM
OF PRELUDIN+ 0·1MNaOH
(in ethanol)
λ1= 266 nm.
λ2= 261 nm.
λ3= 256 nm.
λ4= 250 nm.
λ1
λ2
λ3
λ4
0·6
0·5
0·4
0·3
0·2
0·1
0·0
Absorbance
330
310
290
270
250
230
Wavelength (λ) nm

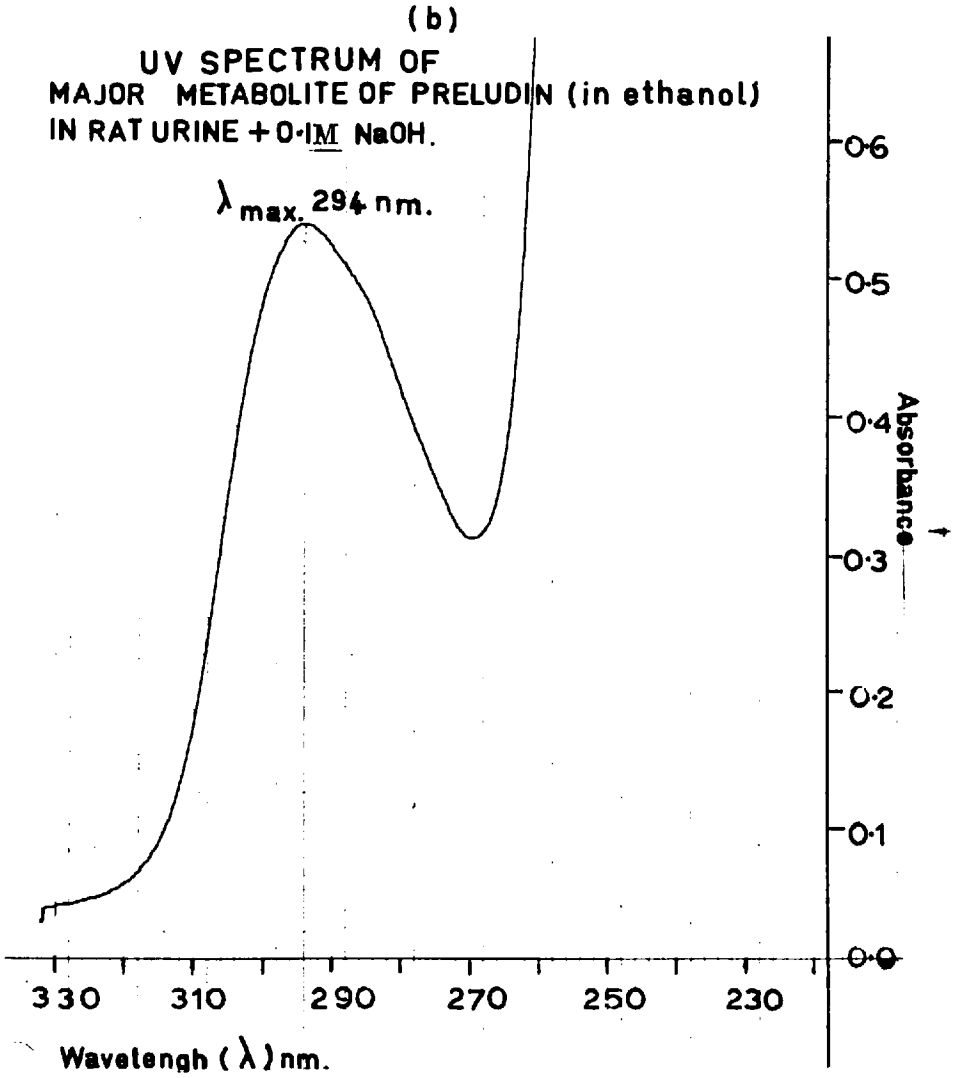
(a)

UV SPECTRUM OF
MAJOR METABOLITE OF PRELUDIN
IN RAT URINE.
(in ethanol)



(b)

UV SPECTRUM OF
MAJOR METABOLITE OF PRELUDIN (in ethanol)
IN RAT URINE + 0·1M NaOH.



APPENDIX 6

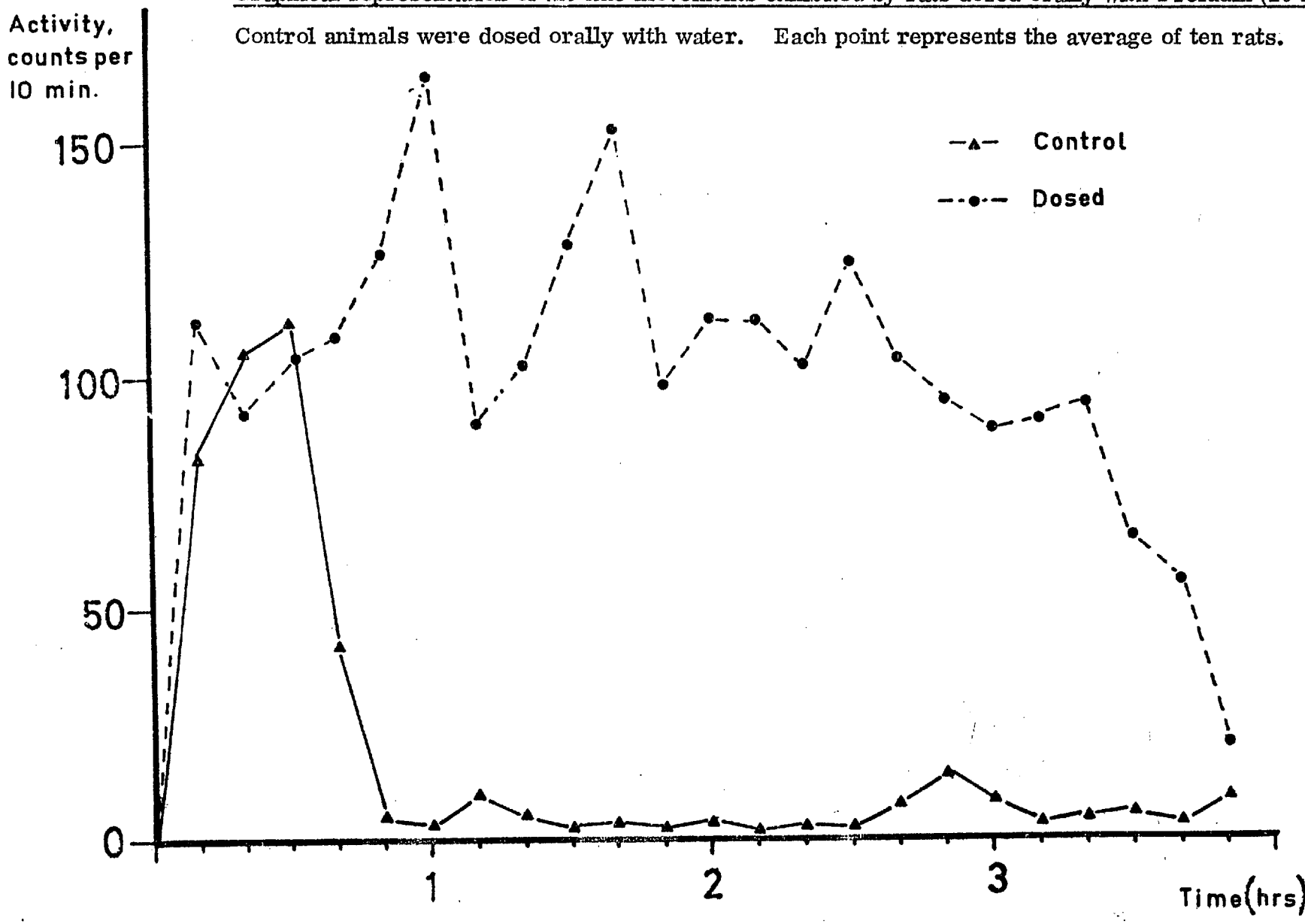
PHARMACOLOGICAL DATA

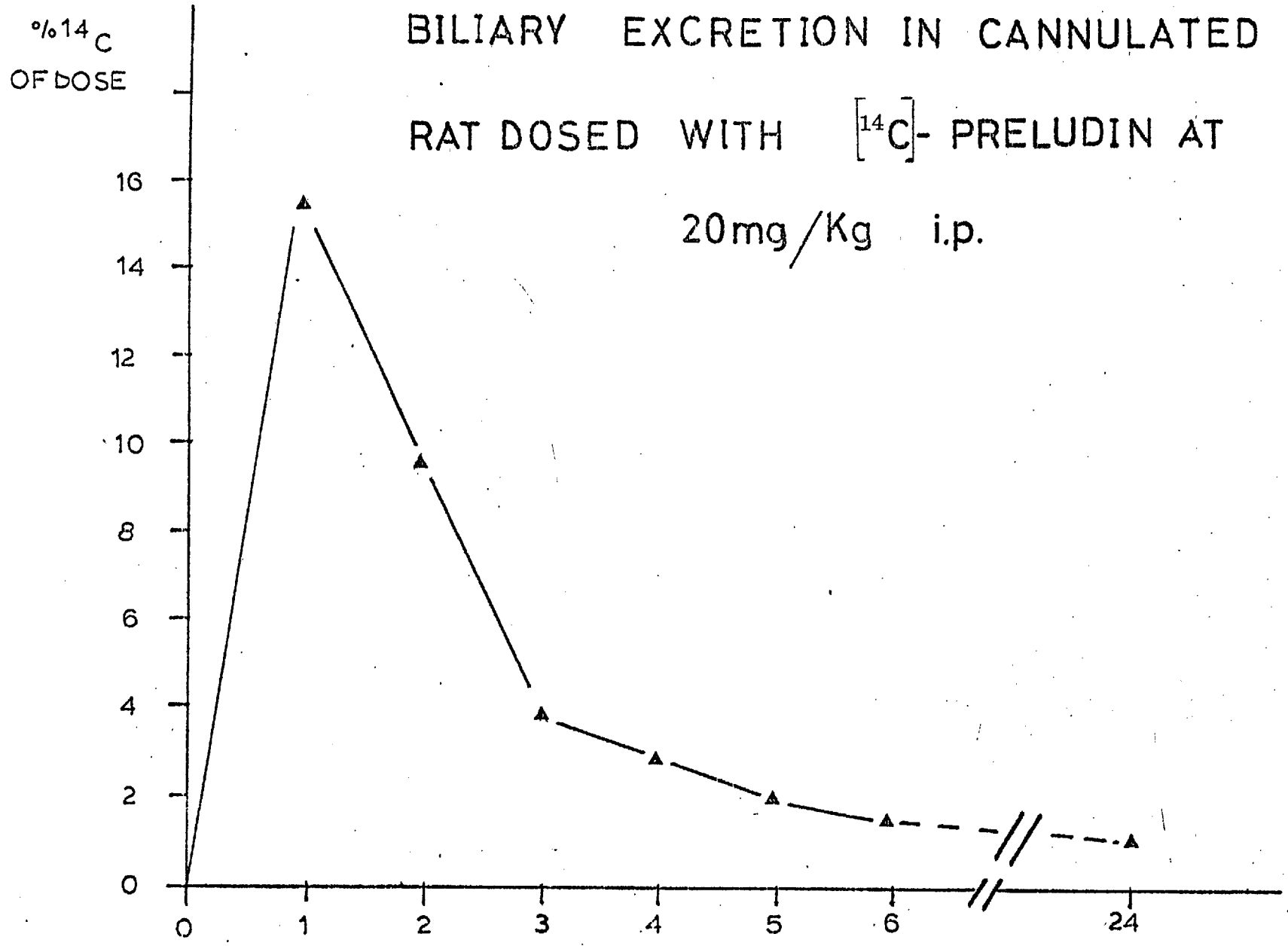
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Graphical representation of the fine movements exhibited by rats dosed orally with Preludin (20 mg/kg).

Control animals were dosed orally with water. Each point represents the average of ten rats.




BILIARY EXCRETION IN CANNULATED
RAT DOSED WITH [14C]- PRELUDIN AT
20mg/Kg i.p.
%14C OF DOSE
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
24

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