α -CARBON HYDROXYLATION IN THE METABOLISM OF TOBACCO ALKALOIDS

by

Nguyen thi Trong-Lang
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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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in the

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[redacted]

To Larry

A Lily grows

A life is born

And the world is

a better place

and

To my father

Công cha như núi Thái-Sơn

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ABSTRACT OF THE DISSERTATION

α-Carbon Hydroxylation in the

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Nguyen thi Trong Lang

Doctor of Philosophy in Pharmaceutical Chemistry

University of California San Francisco

Many of the biotransformation processes observed in the metabolism of xenobiotics are oxidative in nature. Generally oxidative metabolism is mediated by the microsomal mixed function oxidases with cytochrome P_{450} as the terminal oxidase. Many carbon-heteroatom oxidative transformations are believed to involve an initial hydroxylation at carbon α to the heteroatom. These α-hydroxylated intermediates are generally chemically unstable and are either further metabolized or break down into other products. In order to extend our knowledge of the α-carbon hydroxylation pathway in drug metabolism, the metabolism of nicotine has been examined.

The chemical structure of the major tobacco alkaloid (S)-nicotine contains three unique carbon atoms α to the nitrogen atom of the pyrrolidine moiety. Nicotine is known to undergo metabolism at two of the three α carbon atoms to form nornicotine and cotinine. Earlier work has shown that the α-hydroxylated intermediate in the metabolic transforma-

tion of nicotine to cotinine can be trapped via the nicotine-$\Delta^{1'(5')}$-iminium ion to form 5'-cyanonicotine.

The work described in this dissertation is concerned with the further elucidation of the structures and the modes of formation of metabolites of the major nicotine alkaloids. For these studies a synthetic scheme was designed which provides for the introduction of deuterium at all sites of the five membered ring of nicotine and of cotinine as well as the synthesis of nornicotine deuterium labeled at the $C_{2'}$ and $C_{3'}$ positions of the pyrrolidinyl moiety. The use of these specifically deuterium labeled substrates in conjunction with mass spectrometry allowed for the detection and structure elucidation of the metabolites.

In a separate study on the structure of 5-hydroxycotinine, a major metabolite of cotinine, it was shown by spectral studies on a synthetic sample that this compound exists in slow equilibrium with its open ring form, γ-(3-pyridyl)-γ-oxo-N-methylbutyramide. The position of this equilibrium is influenced by the presence of base and the nature of the solvent. The structure of this α-C-hydroxylated metabolite may serve as a model for the less stable α-C-hydroxylated metabolic intermediates formed in the metabolism of nicotine and nornicotine.

Studies on the metabolism of nornicotine by 10,000 x g rabbit liver supernatant fractions led to the discovery of two new metabolites,

myosmine and 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline. These structures were confirmed by the use of the deuterium labeled substrates, nornicotine-3',3'-d_2, nornicotine-2'-d_1 and nicotine-5',5'-d_2 (as an indirect source of nornicotine-5',5'-d_2). These metabolites may arise from the parent compound by elimination of H_2O from the initial α-hydroxylated intermediate.

Studies on the metabolism of nicotine by 10,000 x g rabbit liver supernatant fractions and the 100,000 x g microsomal fractions in the presence of 0.01 M sodium cyanide led to the gc-ei mass spectral characterization of N-cyanomethylnornicotine. The location of the cyano group was established by gc-ei mass spectral analysis of the deuterium labeled products obtained from the specifically deuterium labeled substrates nicotine-5',5'-d_2, nicotine-2',5',5'-d_3 and nicotine-N-methyl-d_3 as well as by synthesis. Formation of N-cyanomethylnornicotine appears to occur at least in part without prior nitrogen-carbon bond cleavage implicating the <u>in situ</u> generation of the N-methyleniminium species during the course of metabolic oxidative N-demethylation of nicotine. These same studies also confirmed the formation of 5-cyanonicotine. By co-incubating nicotine-d_0 and nicotine-5',5'-d_2 or nicotine-2',5',5'-d_3 it was shown that there is no deuterium isotope effect (k_H/k_D = 1.0 ± .1) in the metabolic formation of 5'-cyanonicotine.

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INTRODUCTION

All lipid soluble compounds which are foreign to the biological organism should be regarded as potential toxic agents. If metabolism did not occur to convert these substances into the more readily excretable polar metabolites they could accumulate in the organism and continue to produce toxic effects. This is a major problem in premature infants where the metabolizing enzyme systems are not yet fully developed. Thus the metabolism of xenobiotics is necessary for the survival of the organism. Metabolic alterations usually occur at reactive positions of the molecule and in general result in the formation of more polar transformation products or in primary metabolites which may then undergo conjugation with a highly water soluble moiety.

In biological terms drugs are also foreign compounds which undergo metabolism as other xenobiotics. Although drug metabolism generally leads to metabolites which are pharmacologically less active than the parent compound, in some cases the reverse has been found to be true. In certain cases transformation products may damage the organism by interactions with macromolecules. For these reasons the study of drug metabolism is important to our understanding of the effects of drugs on the biological system.

Metabolic conversions may be classified into four types of reactions: oxidation, reduction, hydrolysis and synthesis (conjugation). In terms of primary or first phase metabolic events, oxidative metabolism is by far the most important pathway since the majority of xenobiotics undergo this type of biotransformation. Even though only a few simple conversions are involved, the catalytic actions of several enzyme systems are generally required.

Drug metabolizing enzymes are associated mainly with the endoplasmic reticulum of liver cells. For *in vitro* studies the liver is homogenized. This results in a fragmentation of the endoplasmic reticulum to form small vesicles called microsomes.[1] The microsomes may be separated from other cell constituents by differential centrifugation. The resuspended microsomes in the presence of the appropriate cofactors (NADPH or NADPH generating system, O_2 and magnesium ion) are capable of metabolizing xenobiotics. By studying the metabolic activity of such *in vitro* preparations it is hoped that an understanding of similar processes in the whole animal can be obtained.

Early workers[2] were able to separate the microsomal fraction into three basic components necessary for drug metabolizing activity: cytochrome P_{450}, NADPH cytochrome P_{450} reductase and a phospholipid containing fraction. More recent work has shown that many other enzymes

are present as well. One of these is the flavoprotein enzyme which is known to convert primary and secondary amines to the corresponding N-hydroxy compounds and tertiary amines into their N-oxides.[3] However the cytochrome P_{450} system accounts for much of the bulk of the microsomes. Cytochrome P_{450} was so named because it forms a reduced carbon monoxide complex having an absorption band at 450 nm. The structure of this enzyme is known to include an iron-containing porphyrin moiety.

Cytochrome P_{450} is a mixed function oxidase which requires NADPH or NADH as the source of reducing equivalents and inserts one of the two atoms of molecular oxygen into the substrate. This process is illustrated for cytochrome P_{450} in Figure I. It involves the transfer of one electron from NADPH to the substrate-enzyme complex via an electron transport system which consists of the flavoprotein cytochrome c reductase (Fp) and a non-heme protein (NHI). The source of the second electron is still not clear but it is hypothesized to be from cytochrome b_5.[4]

Recent work has added to the complexity of this simple picture. It is now known that multiple forms of cytochrome P_{450} exist in liver.[5] Liver microsomes isolated from animals pretreated with various enzyme inducers show different abilities to metabolize various substrates, different patterns of reactivity towards chelating reagents and different absorption spectra. That these differences are due to different forms

of cytochrome P_{450} has been confirmed by the separation of the protein fractions of liver microsomes from untreated animals and from animals treated with inducers.[13]

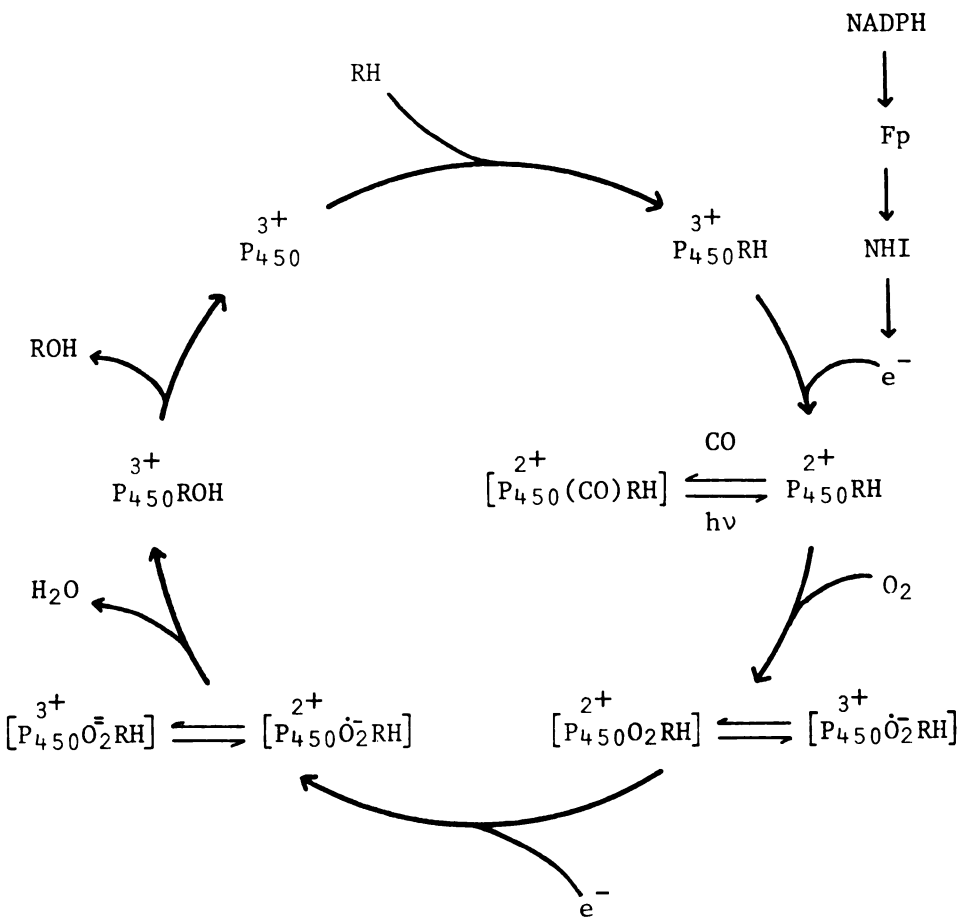
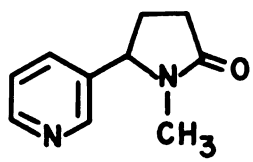


Figure I. The accepted scheme of electron transport associated with oxidative reactions mediated by cytochrome P_{450}. The substrate is designated RH.

It is the purpose of the work presented in this dissertation to give some insight into the mechanism of metabolic oxidation of xenobiotics by the microsomal cytochrome P_{450} enzyme system. More specifically, this thesis is concerned with the study of carbon hydroxylation of three important tobacco alkaloids nicotine (1), nornicotine (2) and cotinine (3).



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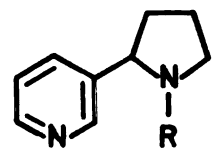


1: R = CH_3

2: R = H

Pharmacologically, nicotine is the most important of the tobacco alkaloids. It is very toxic and has found widespread use as a pharmacological tool for investigating the peripheral cholinergic nervous system. Additionally the nicotine molecule is an excellent candidate for metabolic studies. Next to ethanol, it is probably the most extensively used drug in our society and based on this use alone, studies of its metabolic fate and the metabolic fate of its two principle metabolites nornicotine and cotinine are well justified. Fortunately these three molecules are structurally relatively simple and hence are quite amenable to the necessary synthetic and analytical manipulations

required for in depth metabolic work. Most importantly in terms of the focus of my research, the metabolic hydroxylation of the three unique carbon atoms α to the pyrrolidinyl nitrogen atom of nicotine provides an opportunity to investigate in one molecule a variety of pathways all of which are presumably initiated by a cytochrome P_{450} dependent two electron oxidation at carbon.

In the next two chapters, the important features of enzymatic α-C-hydroxylation and of the metabolism of the tobacco alkaloid nicotine are reviewed. These reviews are not intended to be extensive since many excellent reviews of these topics can be found in the literature;[4,17] it does serve however to introduce the discussion of this dissertation which is presented in Chapter IV.

Chapter II

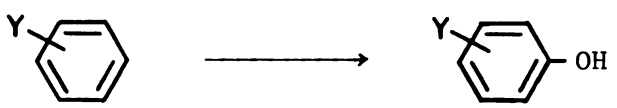
α-CARBON HYDROXYLATION IN DRUG METABOLISM

Metabolic oxidation can be classified into a wide variety of types. Some examples are listed below. Although the mechanisms of drug oxidation by the microsomal enzyme systems are not yet clearly understood, these oxidative conversions can be regarded formally as having been initiated by a carbon or heteroatom hydroxylation.[6] The hydroxylated products may be detected as such or may undergo further enzymatic or spontaneous conversions into other forms depending upon their stability.

Aliphatic hydroxylation

$$R\text{-}CH_3 \longrightarrow R\text{-}CH_2OH$$

Aromatic hydroxylation (via arene oxide)

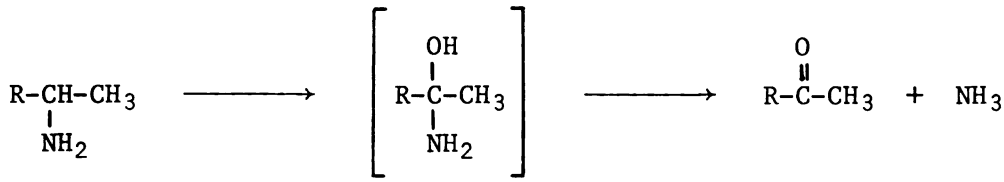


N-, O- or S-dealkylation

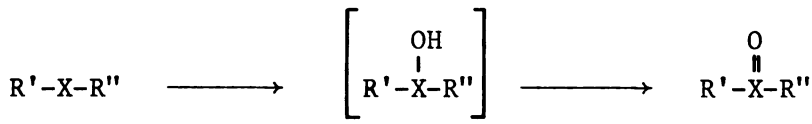
$$R'\text{-}X\text{-}CH_2R'' \longrightarrow \left[R'\text{-}X\text{-}\underset{\displaystyle OH}{\underset{|}{C}}H\text{-}R'' \right] \longrightarrow R'\text{-}XH + R''CHO$$

X = NH, NR, O or S

Deamination

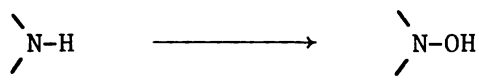


Sulfoxide and N-oxide formation



X = S or NR

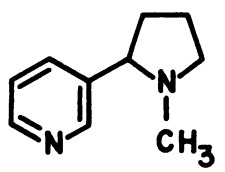
N—hydroxylation



An important subclass of metabolic hydroxylations is composed of those reactions which occur by the formal insertion of oxygen into the C—H bond α to a nitrogen atom or other heteroatom. According to this generalization, oxidative dealkylation and oxidative deamination are essentially the same process involving initial hydroxylation at the carbon atom followed by dissociation of the hydroxylated intermediate into a dealkylated metabolite and a carbonyl compound.

The oxidation of cyclic amines to lactams can also be classified as a metabolic oxidation which involves an initial α-carbon hydroxylation. However in this case a second enzyme system converts the α-hydroxylated intermediate to the carbonyl product. This pathway has been confirmed

for the conversion of nicotine (1) to cotinine (3)[7] and is believed to occur generally for the conversion of cyclic amines and cyclic amine derivatives to the corresponding α carbonyl metabolites. Some typical examples of drugs which undergo this type of metabolic oxidation are Tremorine (4),[8] Mazindol (5),[9] Nitrimidazine (6),[10] Medazepam (7)[11] and Cyclophosphamide (8).[12]



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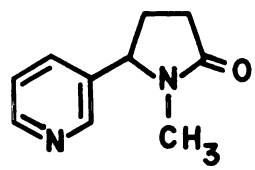

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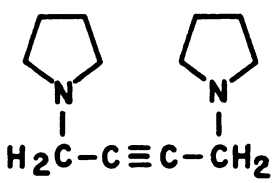

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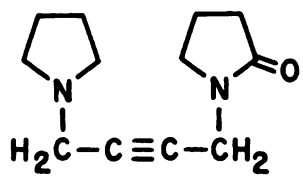

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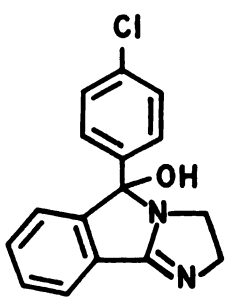

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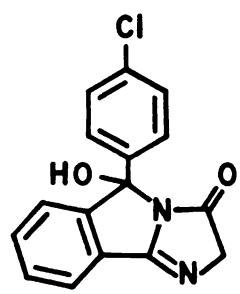

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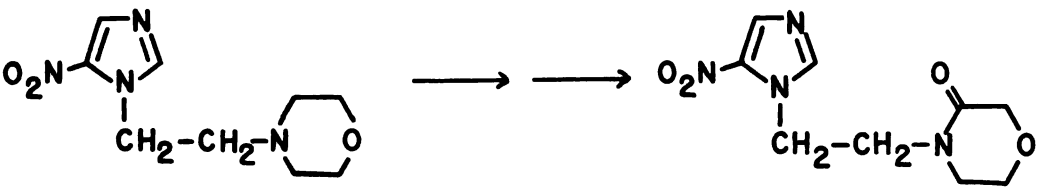

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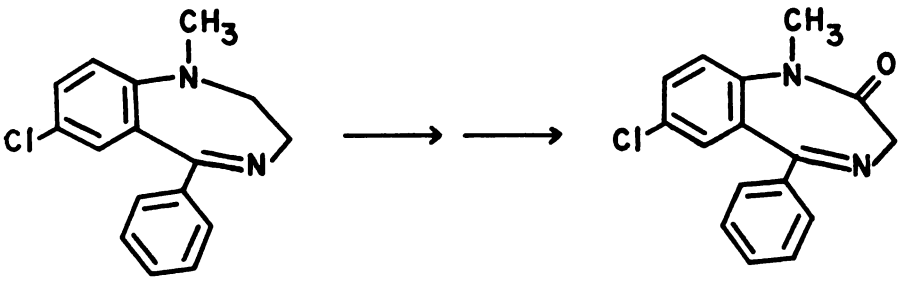

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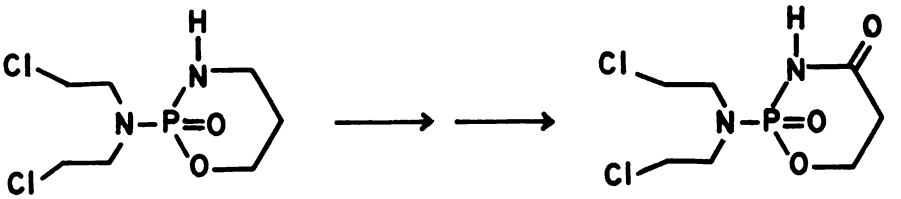
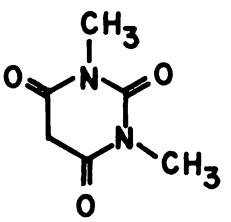


8 8a

In the following discussion only the topic of metabolic N-dealkylation will be reviewed although many of the conclusions reached can also be applied to O- and S-dealkylation.

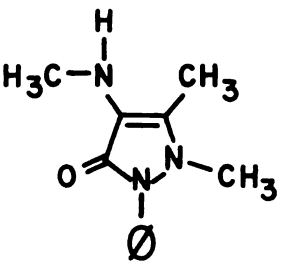
The first report which appeared in 1939[14] of the metabolic oxidative N-dealkylation of a foreign compound described the demethylation of N,N'-dimethylbarbituric acid (9) by dog. Since then this metabolic route has been recognized as a common pathway in the metabolism of

amine drugs. The location of the responsible enzymes was determined in 1953 to be in the hepatic microsomal fraction.[15]



9

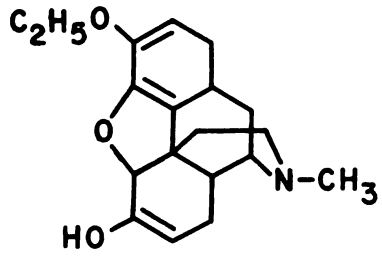
Ø = Phenyl



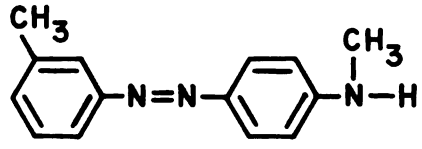
10b

The bulk of the evidence suggests that the N-dealkylase system requires NADPH, molecular oxygen and magnesium ion. Cooper, et al.[16] demonstrated that the N-demethylation of N-monomethyl-4-aminoantipyrine (10b) was inhibited by carbon monoxide; this inhibition could be partially reversed by monochromatic light and the maximal reversal was obtained by light of 450 nm wavelength. Studies using animals pretreated with enzyme inducers showed that the N-dealkylation of xenobiotics is affected differently depending upon the substrate and inducer investigated.[18] In in vitro experiments using rats pretreated with phenobarbital, the N-demethylation of ethylmorphine (11a) was increased several fold but was unaffected when animals pretreated with 3-methylcholanthrene were used. This is in contrast to the N-demethylation of 3'-methyl-4-methylaminoazobenzene (12) which is enhanced by pretreatment

with either phenobarbital or 3-methylcholanthrene.



11a

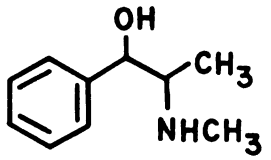


12

It was found that phenobarbital induces the synthesis of the cytochrome P_{450} system while the polycyclic hydrocarbons 3,4-benzpyrene and 3-methylcholanthrene induce the synthesis of a new system which was later called cytochrome P_{448}.[19] In further studies[20] on the *in vitro* N-demethylation of 11a and 12 by rat microsomes, it was observed that SKF 525-A inhibited the metabolism of both substrates when untreated or phenobarbital treated rats were used, but inhibited only the metabolism of ethylmorphine (11a) when 3-methylcholanthrene treated rats were used. Evidence from kinetic studies was presented which suggests that a single enzyme (cytochrome P_{450}) is responsible for the N-demethylation of the two substrates 11a and 12 in preparations from untreated and phenobarbital treated rats and that a second enzyme (cytochrome P_{448}) which metabolizes 12 but not 11a, is present in preparations from 3-methylcholanthrene treated rats. This was confirmed

by spectrographic studies which showed the same ratio of the 455 nm to 430 nm peaks in the uv spectrum of the isocyanide-microsomal enzyme complex prepared from untreated and phenobarbital treated rats but a remarkedly different ratio for the same complex prepared from 3-methylcholanthrene treated rats. The evidence does not rule out the presence of a small amount of cytochrome P_{448} in untreated rats nor does it rule out the possibility that cytochrome P_{448} may be a more significant portion of the natural microsomal enzymes in some other animal species.

The N-dealkylase system is one of several enzyme systems which make up the microsomal mixed function oxidases. As expected from the lack of substrate specificity of the mixed function oxidases in general, a wide spectrum of substrates are reported to undergo enzymatic N-dealkylation. Some examples are: secondary amines (eg. ephedrine, 13[23]), tertiary amines (eg. imipramine, 14[23]), N-alkylamides (eg. diphenamide, 15[24]), N-alkylimides (eg. N-methyl-α-phenyl-α-ethylglutarimide, 16[25]), N-alkylcarbamates (eg. carbaryl, 17[26]), N-alkylbarbitutic acids (eg. N-methylphenobarbital, 18[27]), N-alkylsulfonamides (eg. N-propyl-4-bromobenzenesulfonamide, 19[28]), N-alkylpurine bases (eg. caffein, 20[29]) and N-alkylphosphoramides (eg. hexamethylphosphoramide, 21[30]). Lists of a number of drugs which are known to undergo biological N-dealkylation have been compiled according to the alkyl group cleaved.[21,22]



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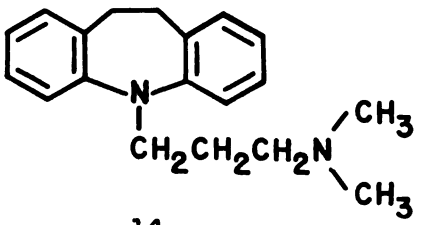

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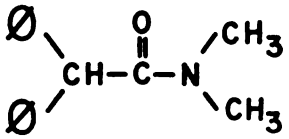

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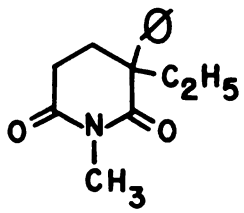

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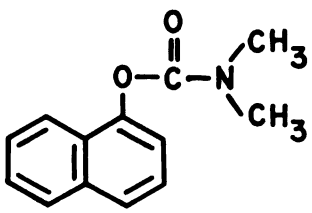

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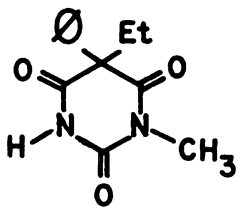

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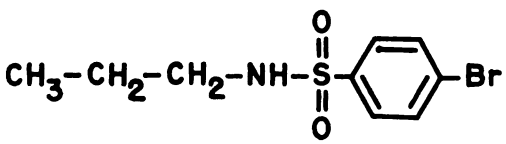

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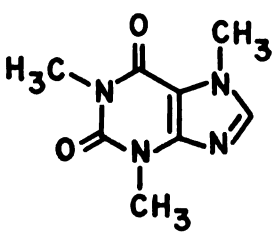

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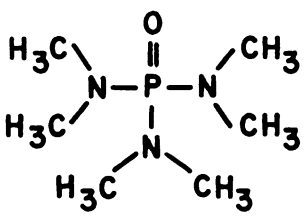
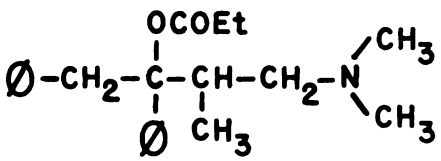


21

Ø = Phenyl

Although these compounds include diverse functionalities, all are lipid soluble. This is in contrast to the N-methylamino acids which are polar and which do not undergo N-demethylation by the microsomal mixed function oxidases; the N-demethylation of these polar amino acids is however catalyzed by hepatic enzymes located in the mitochondria.[21]

The relationship between the lipid solubility of substrates and the rate of N-demethylation has been the subject of several studies. McMahon[31] studied three pairs of benzyldimethylamines (compounds 22-27) in rats, mice and guinea pigs.



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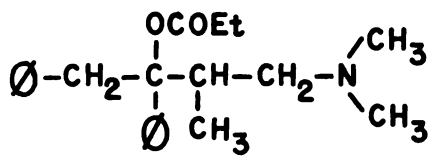

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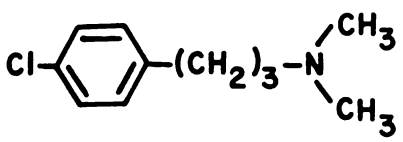

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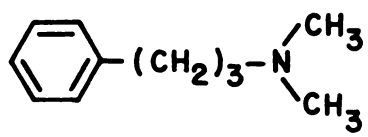

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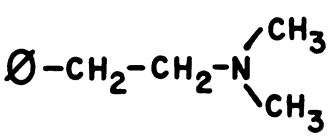

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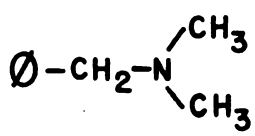
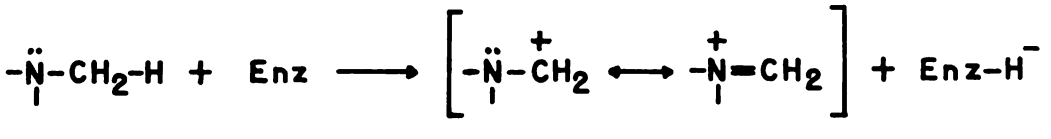


27

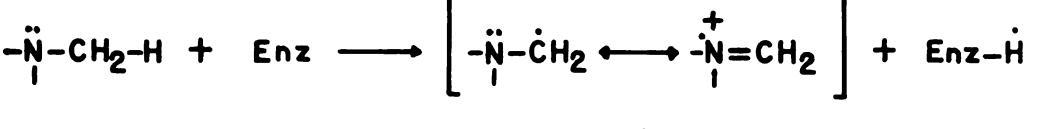
Although the extent of demethylation for each amine is different for each species, a consistent correlation between lipid solubility of the drug with the rate of N-demethylation was observed using either isolated enzyme preparations or whole animals. Hansch, et al.[32] derived a mathematical equation which correlates the pKa and the lipophilicity of 18 disubstituted methylamines as well as the six amines (22-27) studied by McMahon with their rates of N-demethylation by rat microsomes. The equation includes both first and second power terms with negative coefficients for pKa which indicates a non-linear dependence of the N-demethylation rate on the electron density of the nitrogen atom.

The rates of N-demethylation of methylamines with bulky substituents do not differ much from those of simpler methylamines. This fact led Hansch to propose a mechanism which involves the lone pair of electrons while minimizing the steric influence of highly branched N-alkyl substituents.



28a

or



28b

In this mechanism a hydride or a hydrogen radical is removed by the enzyme to form the intermediate methyleniminium ion 28a or radical ion 28b. Thus the lone pair electrons may have a dual role in determining the rate of N-demethylation. Amines with high pKa's will be highly protonated at pH 7.4 and thus will not readily cross the lipoidal barrier of the endoplasmic reticulum. Amines with low pKa's will not have much capability to stabilize intermediate 28a or 28b if formed.

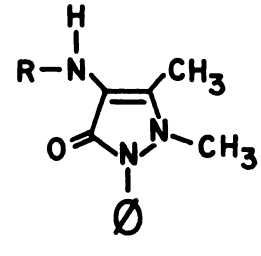
Although the bulkiness of the nitrogen substituents does not appear to influence the rate of N-demethylation, the size of the alkyl group undergoing cleavage is certainly of importance. La Du, et al.[33] studied the N-dealkylation of a series of aminopyrine homologues (10). Under the same conditions, the amount of 4-aminoantipyrine (10a) derived from the N-dealkylation of 4-methylaminoantipyrine (10b) was about three times that from the dealkylation of 4-ethylaminoantipyrine (10c) and four times that from the dealkylation of 4-tert-butylaminoantipyrine (10d). The same trend was also observed in the N-dealkylation of N-alkyl-4-bromobenzenesulfonamide homologues (29).[28]

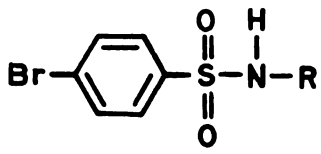
10a : R = H

10b : R = CH_3

10c : R = C_2H_5

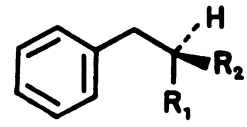
10d : R = t-C_4H_9





R = H, CH_3, Et, n-Pr

29



30a : $R_1 = CH_3$, $R_2 = NH_2$

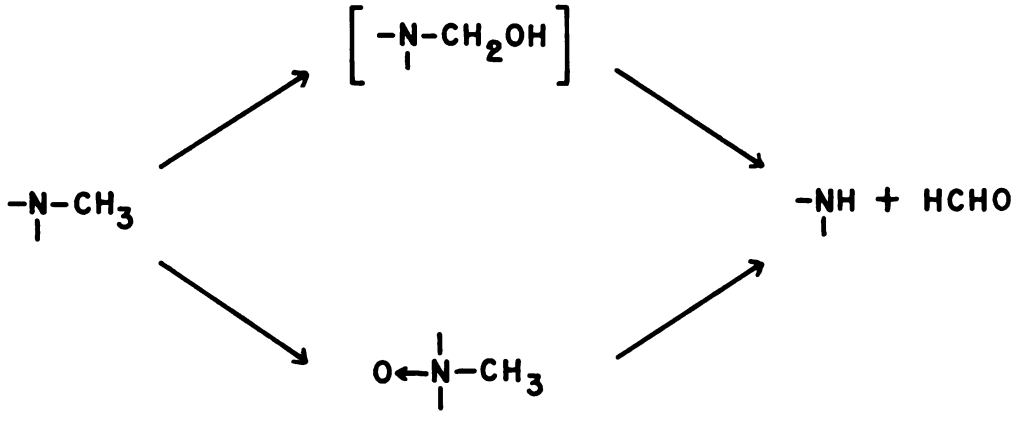
30b : $R_1 = NH_2$, $R_2 = CH_3$

In both in vivo and in vitro studies on dimethylalkylamines it was found that generally the removal of the first methyl group is faster than the removal of the second one, as a result the secondary amines accumulate in the tissues.[22] Many authors have attributed this difference in the N-demethylation rate to product inhibition.[34,22]

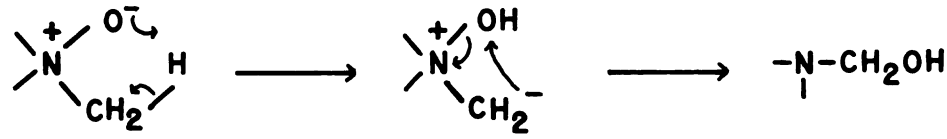
Even though lacking in general substrate specificity the mixed function oxidases do show stereospecificity in the N-demethylation process. Reports of substrate specificity during this oxidative biotransformation are numerous. However these reports are often contradictory. Which isomer is preferentially metabolized seems to depend not only on the class of compound being investigated but also on the source of the enzymes. For example in man[35] S(+)-amphetamine (30a) is preferentially deaminated while in rabbits[36] R(-)-amphetamine (30b) is metabolized faster than its enantiomer. A number of other examples can be found in a recent review on stereochemical factors in drug

metabolism.[37]

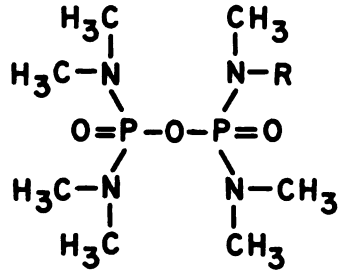
A number of studies have attempted to reveal some of the mechanistic features of the enzymatic N-demethylation of tertiary amines.[38-40] Two major mechanisms have been put forth. One mechanism proposes a direct introduction of oxygen on the methyl carbon[14, 40, 41] resulting in a carbinolamine which subsequently breaks down into the dealkylated amine and formaldehyde. The second mechanism proposes a direct attack on the nitrogen itself[42] producing a tertiary amine N-oxide which then undergoes dealkylation by an undefined pathway.



Chemical model studies[38, 43] suggested that if N-oxides are involved as intermediates in oxidative N-demethylation, product formation might proceed by rearrangement of the N-oxide to the N-hydroxymethyl intermediate via an intramolecular migration[44] of the oxygen on the nitrogen to the α-carbon atom.



Thus whether the initial step involves N-oxidation or direct C-oxidation, both mechanisms involve the carbinolamine as an intermediate. In fact there is considerable evidence for the intermediacy of carbinolamines in the N-demethylation of tertiary amines. Stable amide metabolites such as N-hydroxymethylheptamethylpyrophosphoramide (31b) and N-hydroxymethyl-N-methyl-p-nitrophenylcarbamate (32b) have been found as metabolites of octamethylpyrophosphoramide (31a)[45] and of N,N-dimethyl-p-nitrophenylcarbamate (32a),[46] respectively.



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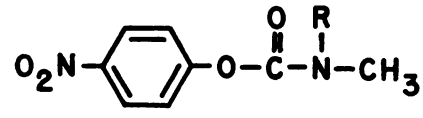


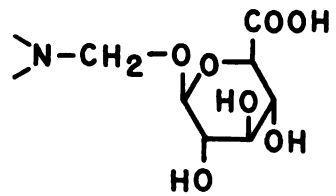
32

a: R = CH_3

b: R = CH_2OH

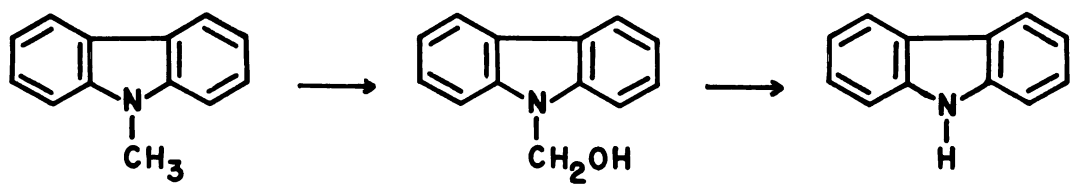
Most other N-hydroxymethyl intermediates have been detected as glucuronide conjugates (33). Examples include the glucuronides of diphenamide (14),[24] of N-methyl-α-phenyl-α-ethylglutarimide (17),[27]

of carbaryl (16)[26] and of several others.[47,48]



33

The corresponding carbinolamines are inherently unstable and have been almost impossible to detect. However, direct evidence of an N-hydroxymethyl species as an intermediate in the N-demethylation of an aromatic amine has been reported.[48] N-Hydroxymethylcarbazole (35) and carbazole (36) were among the metabolites obtained from the *in vivo* and *in vitro* metabolism of N-methylcarbazole (34).



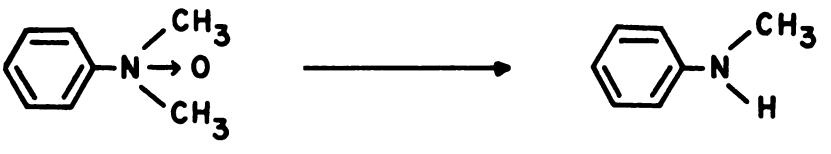
34 35 36

The stability of this N-hydroxymethyl intermediate 35 was explained by the presence of the two aromatic systems attached to the nitrogen. It was shown that more formaldehyde was formed from the enzymatic incubation of 35 than from the incubation of 34. The corresponding N-oxide was not detected as a metabolite. However, attempts to synthesize the

N-oxide from N-methylcarbazole with hydrogen peroxide in acetic acid were reported to be without success despite the claim in 1957[49] that the reaction works.

Many N-oxides have been identified as metabolites of tertiary amines which are known to undergo N-demethylation readily. Evidence suggesting that the N-oxide might be an intermediate in N-demethylation has been reviewed.[50] One example is the conversion by the microsomal enzyme system isolated from pig liver of N,N-dimethylaniline N-oxide (37) to N-methylaniline (38) and formaldehyde at a rate which is compatible with the overall rate of N-demethylation of the corresponding tertiary amine itself.[42]

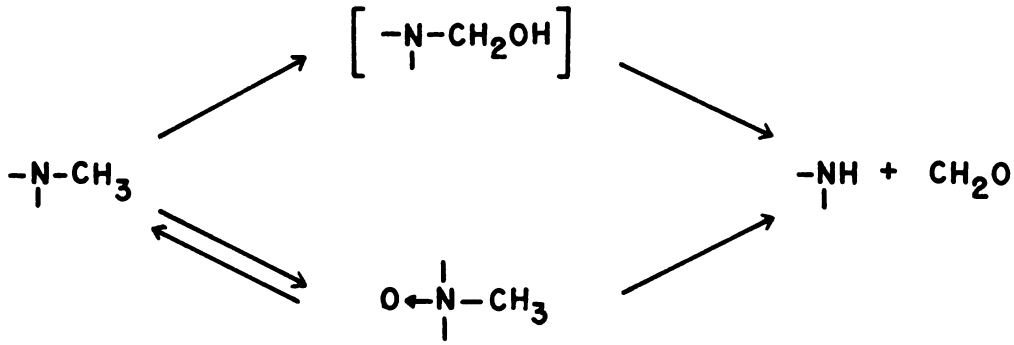
37 38



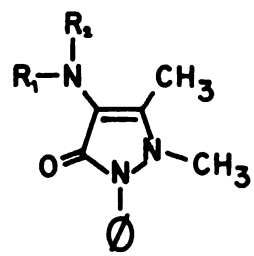
A few other N-oxides have also been reported to undergo similar dealkylation but at a much slower rate.[58]

The discovery that tertiary amine N-oxides are enzymatically[52-55] reduced to the corresponding tertiary amines raised the question as to whether the N-oxides undergo dealkylation directly or via the tertiary

amines.



The involvement of initial N-oxidation in the demethylation process was strongly held in several cases where the substrates had no α-C-H bonds. A few examples are the debutylation of N-tert-butyl and N,N-di-tert-butyl-4-aminoantipyrine (10d and 10e)[33] and of N-tert-butylnorchlorcyclizine (39).[56]



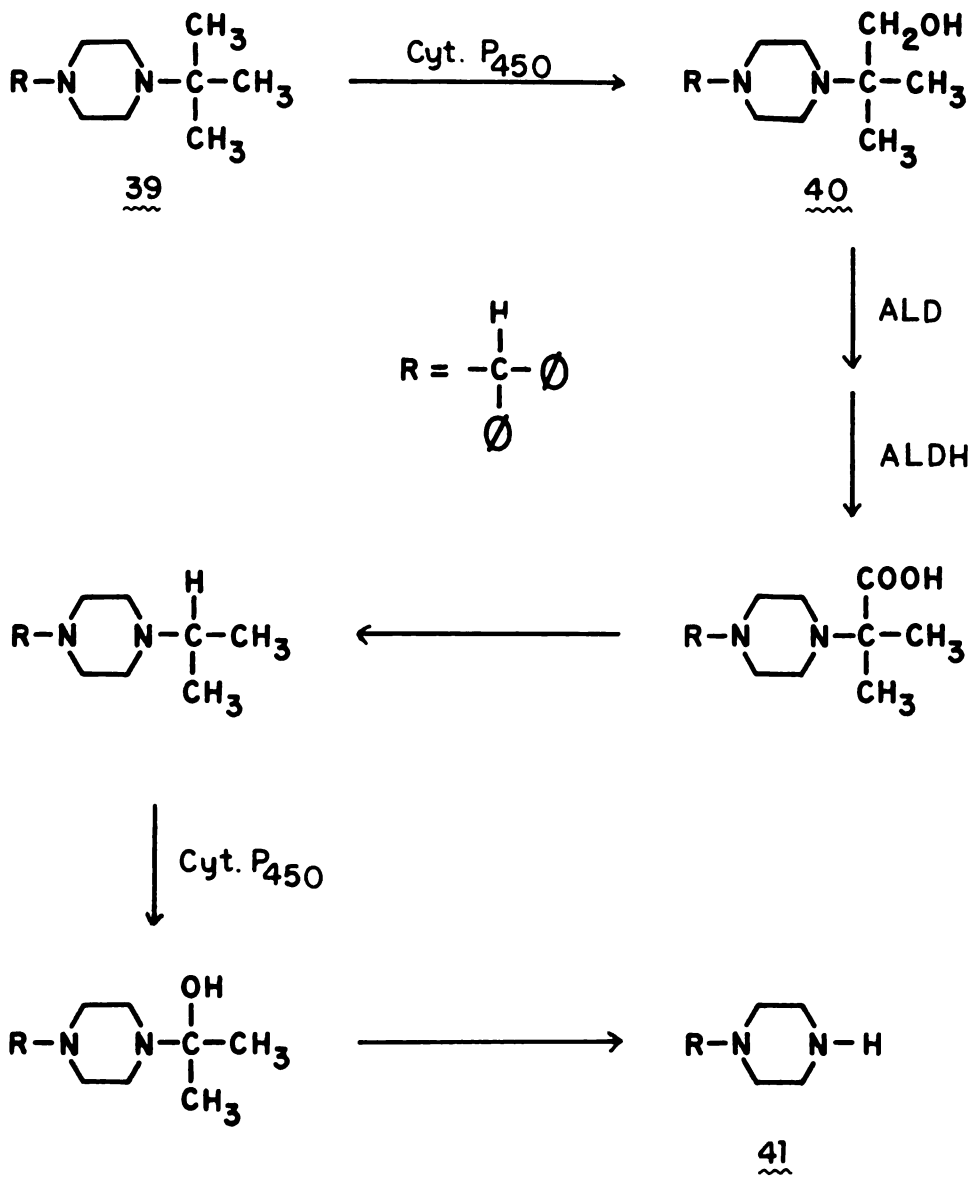
10

d: $R_1 = H$ $R_2 = t\text{-}C_4H_9$

e: $R_1 = R_2 = t\text{-}C_4H_9$

However, more recent evidence makes the intermediacy of the N-oxide in the dealkylation of N-tert-butylnorchlorcyclizine (39) seem doubtful. Kamm[57] isolated N-tert-(β-hydroxy)-butylnorchlorcyclizine (40) as a metabolite of the corresponding tertiary amine 39. The enzymatic

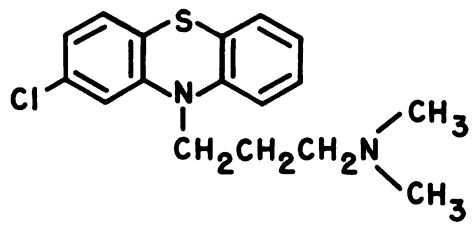
formation of norchlorcyclizine (41) from 39 was suggested to occur via a "modified α-carbon hydroxylation." The author also reported that studies with inhibitors of alcohol dehydrogenase (ALH) and aldehyde dehydrogenase (ALDH) confirmed the participation of these two enzyme systems in the N-dealkylation of N-tert-butylnorchlorcyclizine.



39 40 41

It should also be noted that many tertiary amides, imides and carbamates are known to undergo N-demethylation readily and yet their N-oxides are unknown. Presumably these types of N-oxides are not chemically stable due to the low electron density on the nitrogen. In spite of these considerations, the question of whether N-oxidation or α-C-hydroxylation is the initial step in metabolic dealkylation has been the subject of continued research and debate for many years. However a careful analysis of the recent evidence suggests that N-oxidation and α-C-hydroxylation represent two alternative pathways in drug metabolism.[51,58-62] Beckett[51] and Bickel[58] have reviewed the evidence which suggests that the two oxidative pathways are not related. Although both pathways are NADPH dependent, N-oxidation is usually mediated by a microsomal flavoprotein enzyme system (N-oxidation of non-basic nitrogen containing compounds may be an exception[63a]) and α-C-hydroxylation by a microsomal cytochrome P_{450} dependent enzyme system. Furthermore the rate of tertiary amine dealkylation generally exceeds the combined rate of N-oxide formation and rate of N-oxide dealkylation. However, the most convincing evidence has resulted from studies based on the utilization of flavoprotein enzyme inhibitors. Cysteamine and dithiothreitol inhibited the N-oxidation route of nicotine (1)[62] and chlorpromazine (42)[51] but the α-C-hydroxylation routes were unaffected. Thus it is

considered settled that N-oxidation and α-C-hydroxylation are usually two independent pathways in drug metabolism.



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Chapter III

A REVIEW OF NICOTINE METABOLISM

The major tobacco alkaloid nicotine (1) occurs naturally in tobacco plants exclusively in the S-(-) form.[63] The literature on the pharmacology of nicotine is extensive and dates back to the last century. Early metabolic studies were attempted when it was found that not all of nicotine absorbed from tobacco smoke or from nicotine given to experimental animals was excreted. The details of the metabolic fate of nicotine has received much attention since the late 1940's. The peak publication period was in the 1960's. Studies on the metabolism of nicotine have been carried out with almost all the common experimental mammals:[17] cats, dogs, rats, mice, rabbits, hamsters, monkeys, guinea pigs and humans. Comparative studies with insects have also been reported in the literature.[64]

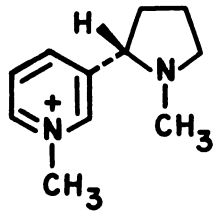
The mammalian metabolism of nicotine is found to take place mainly in the liver. Early investigators[65] have shown that animals with liver damage or hepatectomized animals are more sensitive to nicotine than are normal animals. *In vitro* experiments[66] with liver slices and homogenates have also confirmed the *in vivo* experiments. Other organs such as the lungs and kidneys[66-69] have also been found to be capable of metabolizing nicotine, although to a much lesser extent.

The metabolism of nicotine is NADPH and molecular oxygen dependent.[69] Nicotine is known to be metabolized by two distinct major pathways: α-C-oxidation and N-oxidation. When SKF-525A and carbon monoxide were used as inhibitors of cytochrome P_{450} dependent mixed function oxidases, the products of α-C-oxidation were not seen. However nicotine metabolism was not completely blocked and in fact, the amount of metabolism by N-oxidation was increased.[70] The inhibition of N-oxidation with cysteamine and dithiothreitol was mentioned at the end of the previous chapter. Thus the primary metabolism of nicotine in mammals appears to be mediated by at least two different microsomal enzyme systems: the cytochrome P_{450} dependent and the FMN dependent enzyme systems.

The in vitro metabolism of nicotine was found to be maximal with homogenized liver supernatant fractions obtained at 9000xg to 18,000xg The microsomal fractions (100,000xg to 140,000xg pellets) give only 60% as much metabolic activity.[70, 71]

Most of the known urinary metabolites of nicotine have been observed in in vitro experiments as well. Aside from the N-methylpyridinium ion 43 and the products of N-oxidation, (1R,2S)-nicotine N'-oxide (44a) and (1S,2S)-nicotine N'-oxide (44b), the other nicotine metabolites are believed to be products of enzymatic hydroxylation at the α carbon atoms of the five membered ring moiety.

Figure II shows the current suggested pathways leading to the metabolites resulting from N-oxidation and α-C-hydroxylation.



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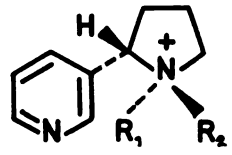


44a: $R_1 = CH_3$, $R_2 = O^-$

44b: $R_1 = O^-$, $R_2 = CH_3$

Nicotine N-oxide (44) was first identified as a nicotine metabolite by Papadopoulos in 1964[72] and it was shown later that both (1R,2S) and (1S,2S) nicotine N-oxide (44a and 44b)[73] were formed from S(-)-nicotine. It was found that S-nicotine yields more (1R,2S) nicotine N'-oxide than (1S,2S) nicotine N'-oxide in all species studied.[74] It has also been reported that these N-oxides can be enzymatically reduced to nicotine both *in vivo* and *in vitro*.[52-54]

Cotinine (3) was the first nicotine metabolite identified[75] and is the major metabolite formed in all species studied both *in vivo* and in *vitro*.[17] The metabolic conversion of nicotine to cotinine was proposed to be mediated by at least two different enzyme systems: the cytochrome P_{450} enzyme and the aldehyde oxidases via the intermediate 5'-hydroxy-

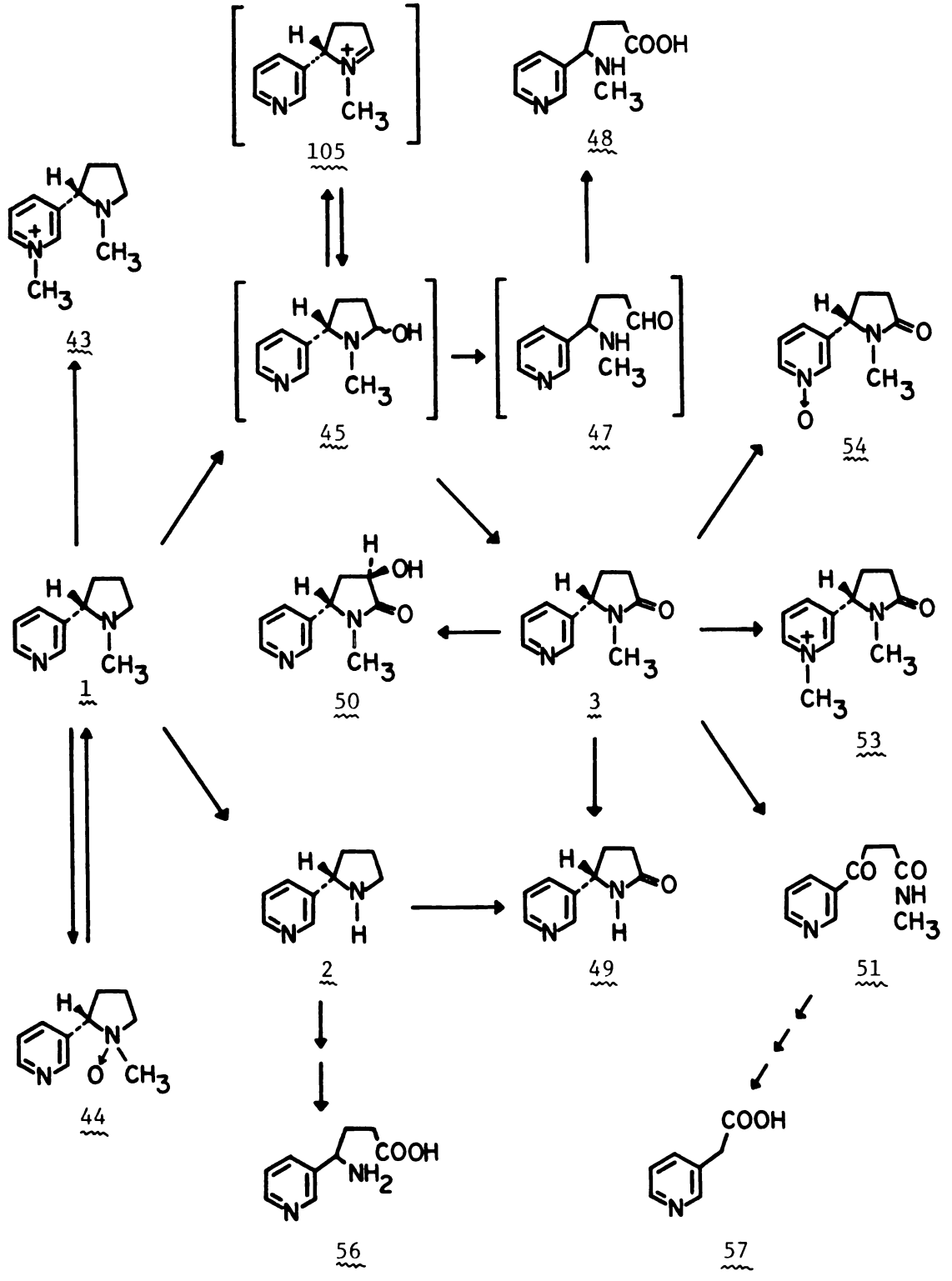


Figure II. Current suggested pathways in the metabolism of nicotine.

nicotine (45)[7] which is in equilibrium with nicotine $\Delta^{1'(5')}$-iminium ion (105).[76] The hydrolysis product of cotinine, γ-(3-pyridyl)-γ-methylaminobutyric acid (48), is found in the urine of dogs[77, 78] given nicotine and is also found in the urine of smokers.[79] Whether this acid is the precursor of or the enzymatic hydrolysis product of cotinine *in vivo* has been the subject of some debate.[17] However at least in *in vitro* rabbit liver preparations it has been shown that the rate of cotinine formation when the acid 48 is incubated is much too slow to account for the rate of cotinine formation when nicotine is incubated under the same conditions.[7] Another acidic metabolite of nicotine which has been found in both *in vivo*[78, 84] and *in vitro*[71] studies is 3-pyridylacetic acid. This acid is believed to be the end metabolite of nicotine.

Cotinine is further metabolized at the carbon atoms α to the nitrogen atom and to the carbonyl group of the pyrrolidinone moiety to give demethylcotinine (49),[80-84] *trans*-3-hydroxycotinine (50)[68, 85, 86] and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide (51).[88] Cotinine is also reported to undergo N-methylation and N-oxidation at the pyridyl nitrogen to produce the N-methylpyridinium ion 53[89] and cotinine N-oxide (54).[90]

The enzymatic N-demethylation of nicotine to nornicotine (2) has not been well investigated. Although the *in vivo* and *in vitro*[84, 91-93] formation of nornicotine from nicotine has been reported, there is still

some controversy in the literature as to whether nornicotine is truly a hepatic metabolite of nicotine. Gorrod and Jenner failed to detect nornicotine in their studies and stated in a recent extensive review[17] of the metabolism of tobacco alkaloids that if the N-demethylation of nicotine to nornicotine occurred at all it must occur in the kidneys not liver. This subject will be discussed in detail in the next chapter.

Nornicotine is further metabolized[92,94] to demethylcotinine (49), and γ-(3-pyridyl)-γ-aminobutyric acid (56). The pathway leading to these products have not been established. However due to the similarity of its structure with that of nicotine, nornicotine is assumed to be metabolized by similar pathways.

The in vitro metabolism of nicotine is linear with time up to 60 minutes and is linear with substrate concentrations up to 0.3 mM.[69,70] The presence of more substrate does not inhibit the metabolism.[69,70]

The extent of in vitro metabolism of nicotine was found to vary greatly with the animal species used.[74] In a series of experiments under otherwise comparable conditions it was found that guinea pig liver preparations gave at least 40% metabolism of nicotine and an average of 80% metabolism was observed. However of the metabolites observed only 15% is cotinine, most of the rest being nicotine N'-oxide (44). Rat liver preparations do not have as much capacity for metabolizing

nicotine. The extent of metabolism was only 15%, of which 5% was cotinine. The average extent of metabolism with hamster and mouse liver preparations was about 30 to 40%. Cotinine and nicotine-N'-oxide were found in about equal amounts. Rabbit liver preparations gave an average of 50% metabolism, 25% of which was cotinine while the N'-oxide formed was less than 10%. Thus it seems that rabbit liver preparations are the most suitable for studies of α-C-oxidation. In all species studied there was no reported difference in the metabolism of R(+) and S(-) nicotine.

Chapter IV

RESULTS AND DISCUSSION

A very powerful technique for studying drug metabolism utilizes specifically deuterium labeled substrates in conjunction with mass spectrometry. This approach allows for the oftentimes facile detection and structure elucidation of metabolites and also for the investigation of reaction mechanisms via deuterium isotope effects. For this reason, a synthetic scheme was designed (Figure III) for the synthesis of nicotine (1) and its major metabolite cotinine (3) which would provide for the introduction of deuterium at all sites of the five membered ring. The scheme also could be utilized for the synthesis of labeled nornicotine (2) with deuterium located at the $C_{2'}$ and $C_{3'}$ positions of the pyrrolidinyl moiety.

In this synthetic sequence, myosmine (58) is the key intermediate. Myosmine is another important tobacco alkaloid[63] which also is generated by pyrolysis of nicotine during the smoking process.[95] Myosmine has been reported to be a metabolite of nornicotine in tobacco plants.[96]

Synthetically the imino functionality of myosmine provides an opportunity to introduce deuterium atoms at $C_{2'}$ (by reduction) and $C_{3'}$ (by exchange) of the five membered ring. This pathway requires conversion to the chiral nornicotine and hence either an asymmetric reduction

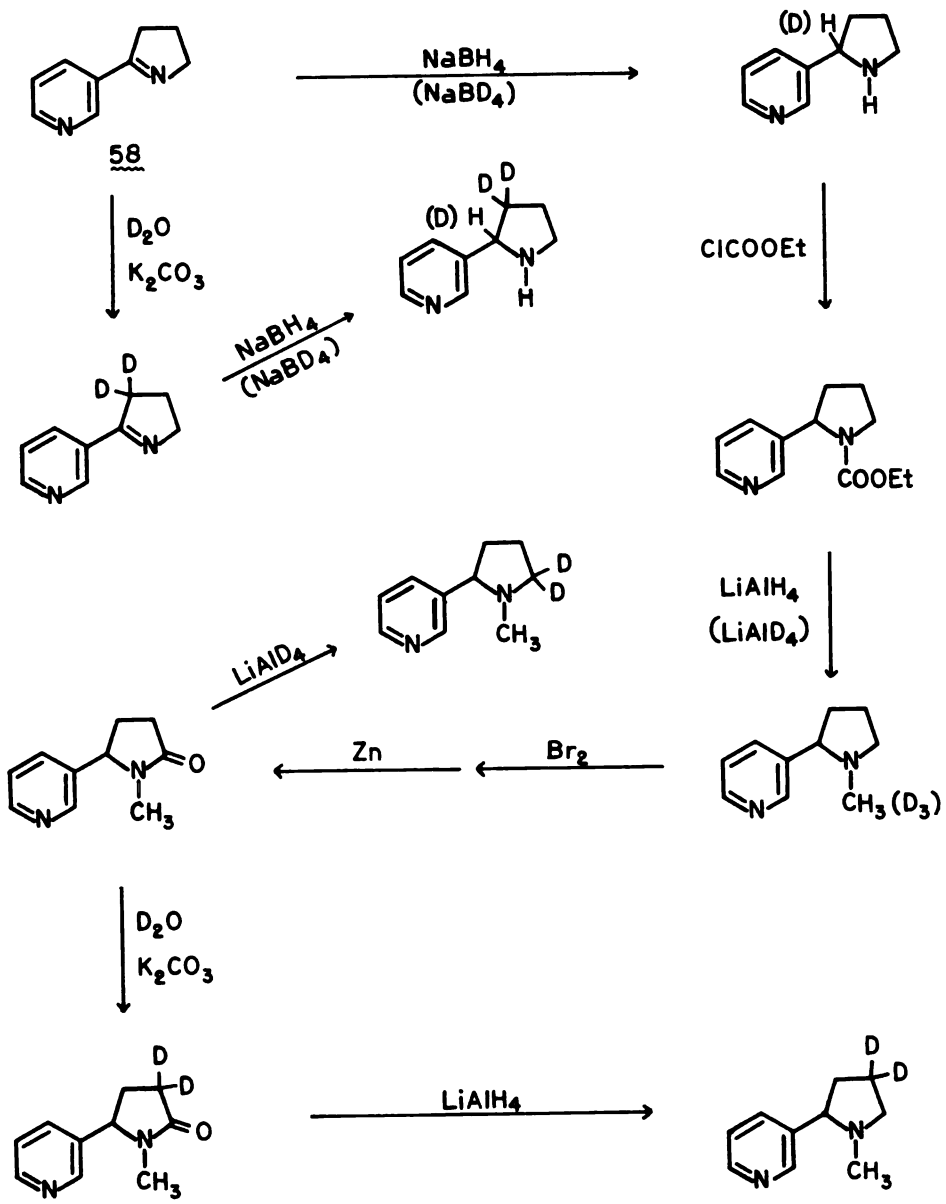
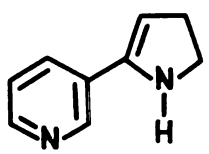


Figure III. Synthetic sequence for the syntheses of deuterated tobacco alkaloids.

or resolution if one is to work with the naturally occurring (S) enantiomer. An alternative to the pathway shown in Figure III which does provide the required stereochemistry would be to synthesize (S)-nornicotine by the N-demethylation of the readily available (S)-nicotine. While this approach could not be used to prepare nicotine alkaloids labeled at the $C_{2'}$ or $C_{3'}$ positions, it would provide an easy synthesis of these compounds labeled at the N-methyl, the $C_{4'}$ and the $C_{5'}$ positions. Unfortunately none of the attempts to N-demethylate nicotine reported in the literature has been successful[113, 114] in obtaining the desired product in a reasonable yield.

A. Synthetic studies.

For our studies on the metabolism of nornicotine we desired substrates labeled at the sites of probable metabolic alteration including the positions α to the nitrogen atom ($C_{2'}$ and $C_{5'}$) and at $C_{3'}$ to study the potential metabolite 59, an isomer of myosmine. For these studies nornicotine-d_0, -2'-d_1, -3',3'-d_2 and -2',3',3'-d_3 were synthesized. Although nornicotine labeled at the 5' position could not be obtained by our synthetic approach, the metabolism of this compound was studied by using nicotine-5',5'-d_2 as the substrate.



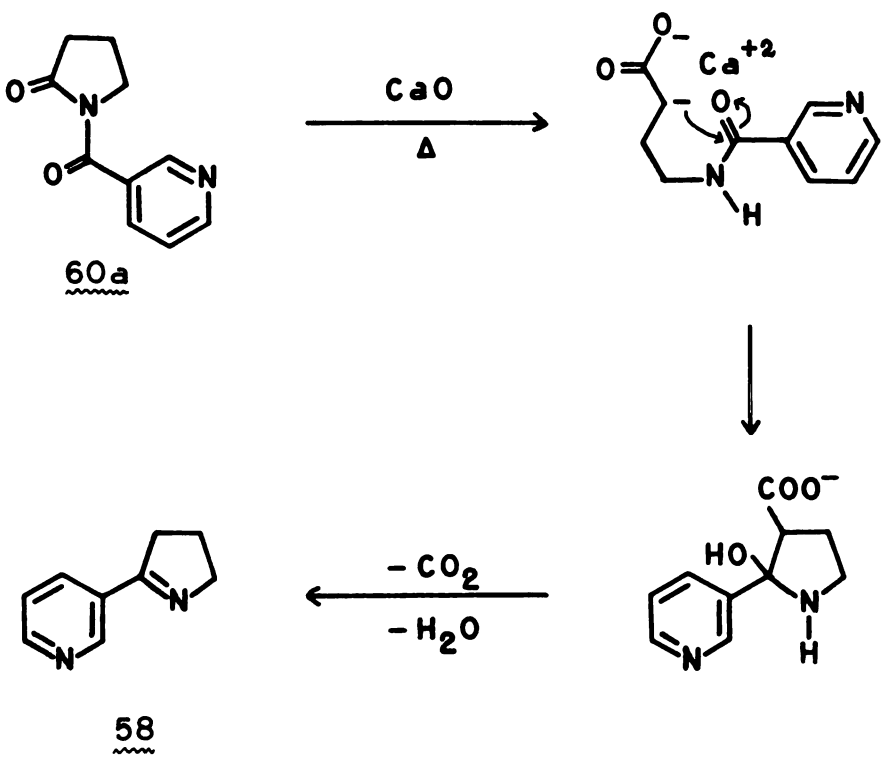
59

For our studies on the metabolism of nicotine the following substrates labeled at all of the sites α to the nitrogen were synthesized: nicotine-2'-d_1, nicotine-5',5'-d_2, nicotine-2',5',5'-d_3 and nicotine-N-methyl-d_3.

All of the above compounds were synthesized as racemic mixtures via myosmine and were used as such in metabolic studies. (S)-nicotine-5',5'-d_2 was synthesized from (S)-nicotine via (S)-cotinine, through a sequence of steps which does not involve the chiral center.[97]

1. Myosmine (58).

Several procedures leading to myosmine have appeared in the literature.[98-102] The simplest is a free flame pyrolysis of a mixture of equal weight of calcium oxide and N-nicotinoyl-2-pyrrolidinone (60a).[100] The proposed mechanism[103] is as follows:

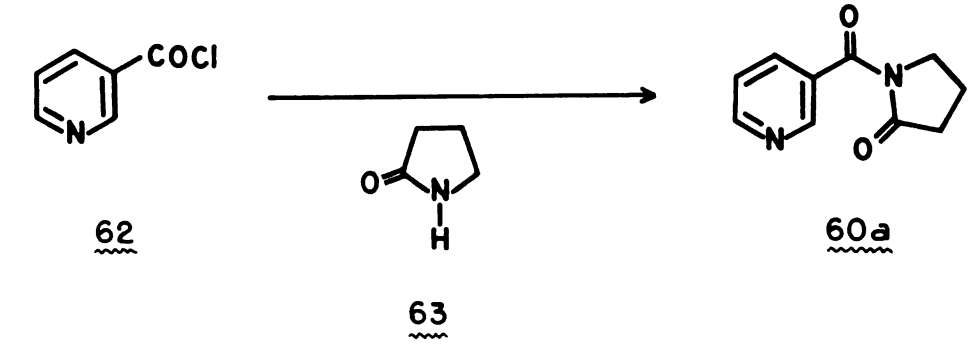


60a

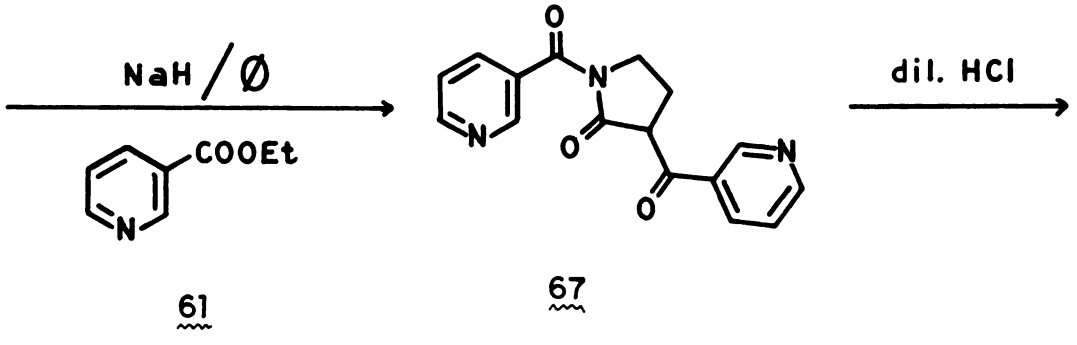
58

The yield of the reaction was reported to be 65%. However, all our attempts to repeat this procedure failed. Even though extensive modifications involving various heating techniques (such as a salt bath instead of a free flame), using specially purified calcium oxide, working entirely in a nitrogen atmosphere and using specially designed glass-

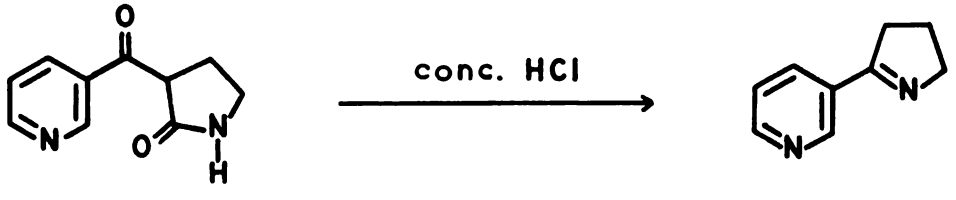
ware (see experimental) were tried, the yield of myosmine as determined by direct gc analysis (3% OV-17, 70° to 235°C at 10° per minute) of the pyrolysate was consistently poor (8-10%). The need for a large amount of myosmine encouraged the use of an older approach[99] involving the condensation of ethyl nicotinate (61) with N-nicotinoyl-2-pyrrolidinone (60a), followed by acid catalyzed decarboxylation.



62 63 60a

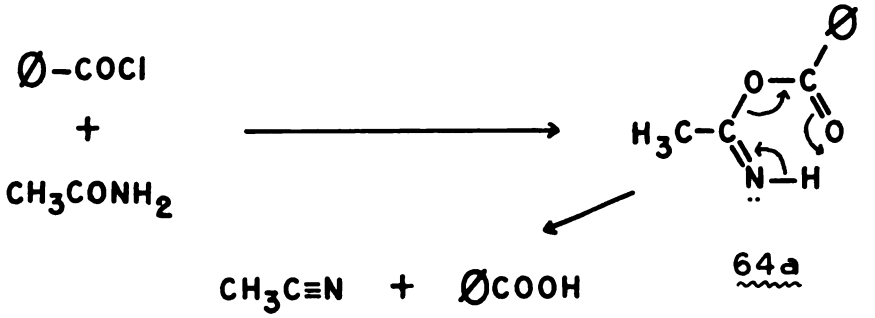


61 67

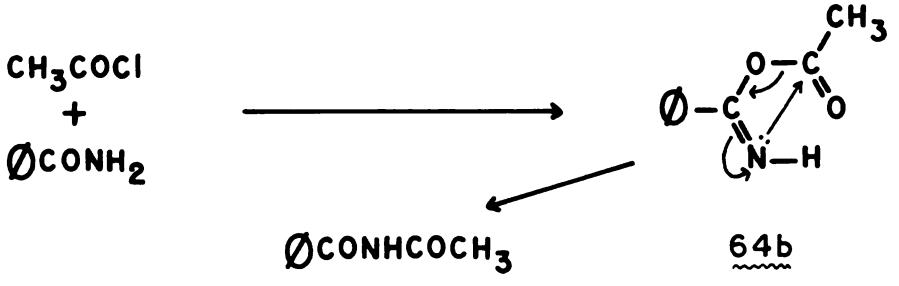


68 58

The first step in this synthesis is the condensation of the acyl chloride 62 with the secondary amide 63 to yield 60a. Titherly and Holden[104] observed that the reaction of benzoyl chloride with acetamide yields acetonitrile and benzoic acid. The formation of CH_3CN ruled out direct N-acylation and was consistent with a mixed anhydride intermediate.



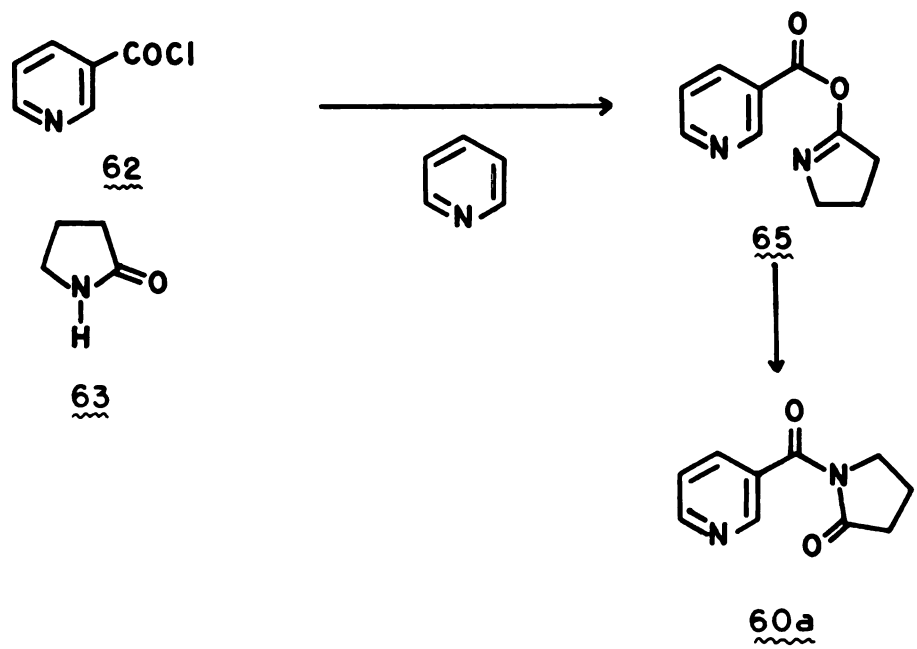
The same authors also observed that the reaction between benzamide and acetyl chloride yields only N-acetylbenzamide.



This was explained[105] by the fact that the breakdown of the intermediate 64a as shown was promoted by the generation of the stable benzoate ion, while the breakdown of 64b in the same manner would produce the much less stable acetate ion. Changes in the nucleophilicity of the nitrogen atom in the intermediate 64 due to changes in the substituent on the α

carbon may also be a factor. Challis and Challis[105] analyzed the data from the reaction of various acyl halides with a number of amides and concluded that the reaction between an amide and an acyl halide generally proceeds by O-acylation and that N-acyl products arise by rearrangement. With primary amides nitrile formation is favored by a reactive acyl halide and by an enhanced stability of the expelled carboxylate ion. With secondary amides where nitrile formation is impossible, O-N rearrangement of the mixed anhydride intermediate occurs. Evidence supporting this type of intermediate is abundant in the literature.[105]

The formation of N-nicotinoyl-2-pyrrolidinone (60a) by the reaction of nicotinoyl chloride and 2-pyrrolidinone (63) is therefore believed to proceed through the O-acyl intermediate 65 as shown below.

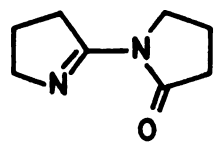


This reaction has been used successfully by Korte and Steinen[99] and by Mundy, et al.[100] to obtain 60a in yields of 50% and 73% respectively based on the amount of nicotinoyl chloride used. However our first attempts to repeat Mundy's procedure gave only a poor yield of a white crystalline solid whose melting point (92°-93°C) did not agree with that reported for N-nicotinoyl-2-pyrrolidinone (104°-105°C[100]). The eims (electron impact mass spectrum) of this crystalline material indicated the molecular weight to be 152 and its NMR spectrum in deuterated chloroform (Figure IV) failed to show aromatic protons in the molecule thus ruling out the presence of a pyridyl moiety. The mass spectrum and NMR spectrum evidence seemed to suggest that the product obtained was a pyrrolidinone dimer. Apparently the thionyl chloride left in the reaction mixture was causing the 2-pyrrolidinone to dimerize.

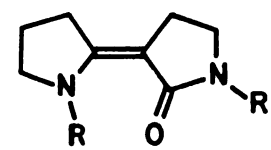
Pyrrolidinone dimers have previously been reported as the product from reactions done under similar conditions. Castagnoli[106] obtained a white crystalline material (mp 57.2°-59.0°C) as a by-product from the reaction of pyrrole with 2-pyrrolidinone in the presence of phosphorous oxychloride. An infrared spectrum of this material was reported to have two strong absorption bands in the double bond region (1714 cm^{-1} and 1622 cm^{-1}). On the basis of the similarity of the mp and ir, this material was believed to be the same as the compound previously obtained

by Brederick[107] from the reaction of 2-pyrrolidinone and phosphorous oxychloride in benzene. Brederick had assigned the structure 66a to this material. However, Castagnoli tentatively assigned it the structure 66b on the basis of its uv (λ max 226 nm, ε 13520) and its absorption in the NH region of the ir (between 3610 cm^{-1} and 3311 cm^{-1}). The structure 66b is also analogous to the structure 66c which is formed by the self-condensation of N-methyl-2-pyrrolidinone in the presence of $POCl_3$.[108]

In spite of the similarity of our conditions to those of Castagnoli and Brederick ($SOCl_2$ vs $POCl_3$ + pyrrolidinone), the dimer we obtained does not appear to be the same. There is nearly 35°C difference in the melting points (93° vs 59°C) and a direct comparison of our nmr spectrum with that given by Castagnoli in the same solvent shows distinct differences in spite of a general similarity. On the basis of our ir, nmr and eims data we have assigned structure 66d to the pyrrolidinone dimer we obtained.

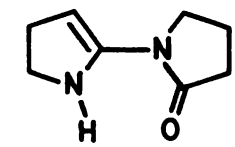


66a



66b: R = H

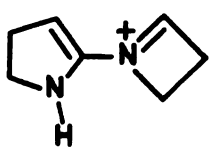
66c: R = CH_3



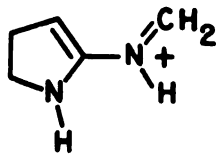
66d

The ir spectrum of 66d showed three equally strong sharp absorption bands at 1720 cm^{-1}, 1630 cm^{-1} and 1400 cm^{-1} which were assigned to the stretching vibrations of a C=O bond, a C=C bond and to the bending vibration of a substituted vinylic C-H bond respectively. Unfortunately the spectrum was taken in $CHCl_3$ and it is not possible to observe whether there is any absorption in the NH region. However the nmr spectrum in $CDCl_3$ (Figure IV) showed a singlet at 3.55 ppm integrating for one proton which was assigned to an exchangeable proton on nitrogen. The nmr also showed the presence of a one proton triplet at 5.05 ppm (J=3 Hz) which was assigned to the vinylic proton. The remaining nmr assignments are given in Table I.

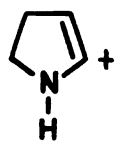
The mass spectrum showed a strong molecular ion (m/e 152, 93.7% of the base) with a base peak at $[M-1]^+$ due to the loss of a hydrogen radical. Other significant peaks were at m/e 123 (22% of the base, loss of CO or C_2H_4 from the $[M-1]^+$ peak), at m/e 97 (87.5% of the base, loss of $O=\dot{C}-CH=CH_2$ from M^+) and at m/e 68 (53% of the base, see below).



m/e 123



m/e 97



m/e 68

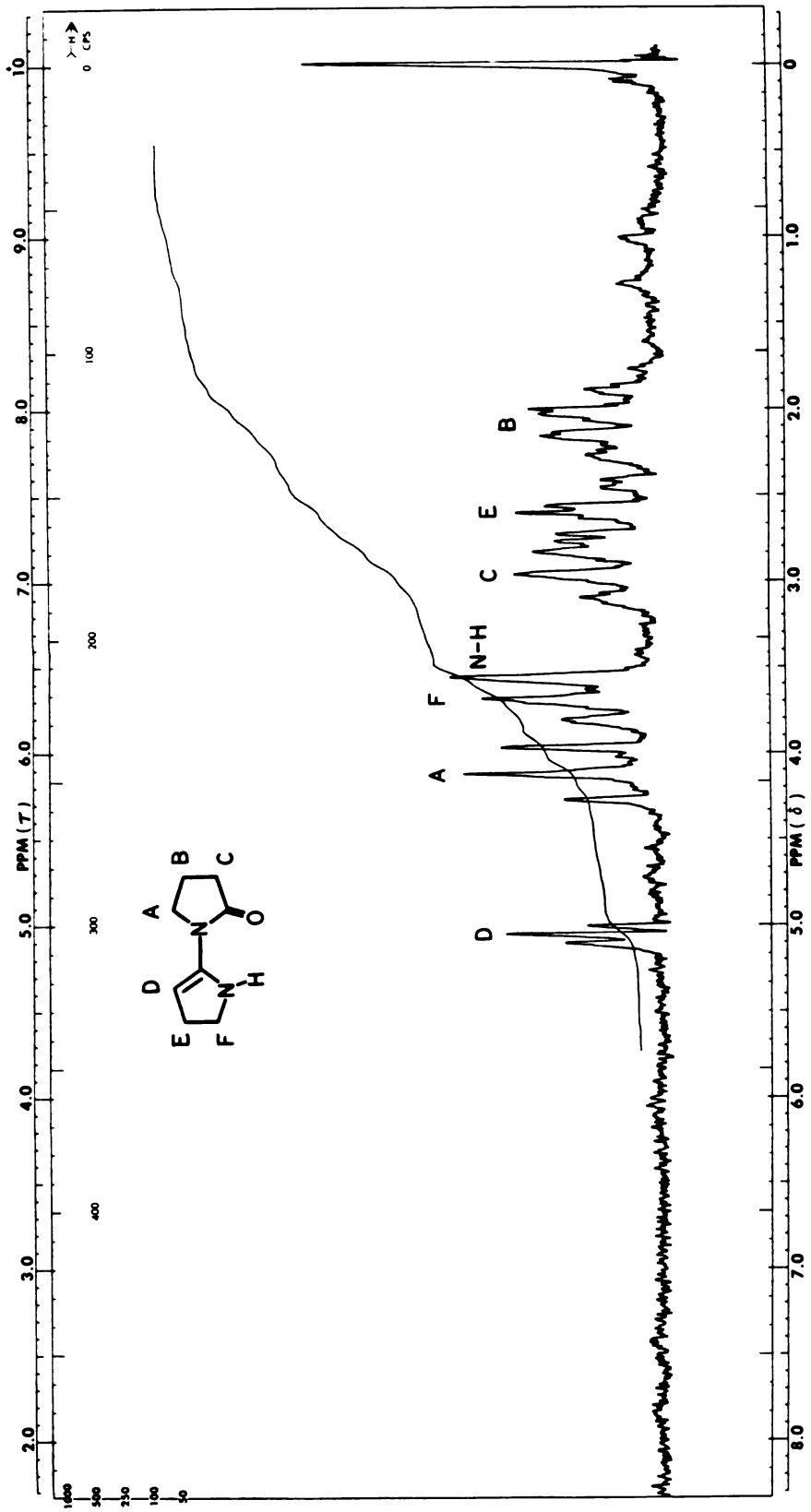
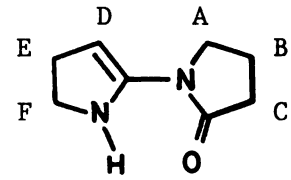


Figure IV. The nmr spectrum of N-($\Delta^{2'}$-pyrrolinyl)-2-pyrrolidinone (60 MHz, $CDCl_3$, TMS).

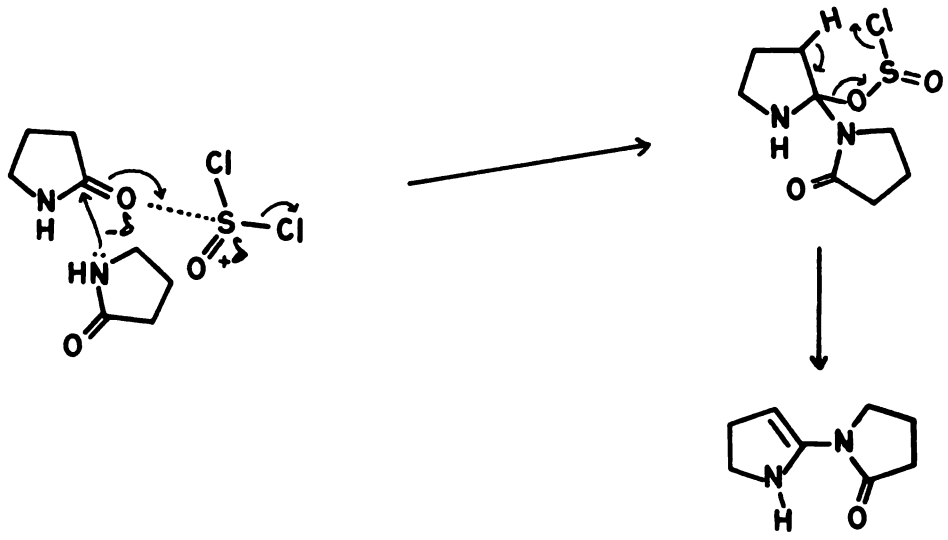
Table I

NMR assignments for N-($\Delta^{2'}$-pyrrolinyl)-2-pyrrolidinone



Protons	Chemical shifts, ppm.
D	5.05 (t, J_{DE} = 3 Hz)
A	4.1 (t, J_{AB} = 9 Hz)
F	3.7 (t, J_{FE} = 8 Hz)
C	2.95 (t, J_{CB} = 8 Hz)
E	2.68 (d of t, J_{ED} = 3 Hz, J_{EF} = 8 Hz)
B	2.1 (m)
N-H	3.55 (s)

A schematic mechanism by which dimerization of 2-pyrrolidinone can occur is as follows:



66d

Therefore it is important that all traces of thionyl chloride be removed from the reaction mixture before the 2-pyrrolidinone is introduced into the reaction mixture. This was accomplished by co-distillation with dry benzene. After the removal of the excess $SOCl_2$, the reaction between nicotinoyl chloride and 2-pyrrolidinone was carried out according to the conditions given in the literature. The course of the reaction was followed by tlc and gc-eims. The results suggested that the major component of the reaction mixture before work up is the desired product N-nicotinoyl-2-pyrrolidinone (60a). However, after acid base extraction

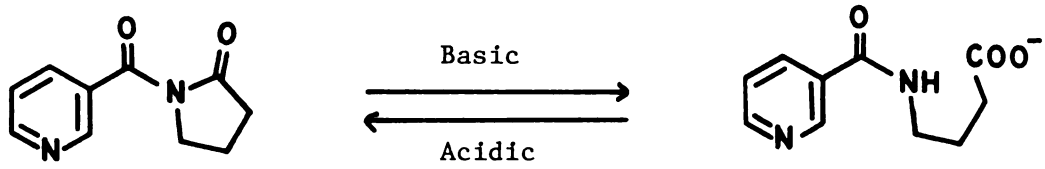
of the reaction mixture the amount of 60a obtained was very small (21%). In order to increase the recovery, we carried out the extractions of 60a in water with dichloromethane over the pH range of 2-11. The results of this experiment as shown in table II suggested that the optimum pH for the extraction is around 4.

Table II

Recovery of N-nicotinoyl-2-pyrrolidinone (60a) by extraction into CH_2Cl_2 from aqueous solutions at various pH's.

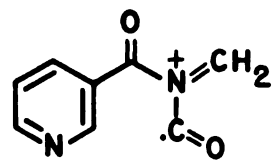
pH	2	3	4	9	11	11→3
% Recovery	63.4	83.6	91.5	52.2	26.8	86.2

An explanation for the fall off in recovery at basic pH is that the product 60a undergoes hydrolysis at one of the two amide linkages. In the extraction shown in the last entry of the Table, the pH of the solution was first made to 11, the resulting solution was allowed to stand for one hour and then the pH was readjusted to 3 and the extraction was carried out. The result was that a high recovery (86%) of 60a was obtained. This indicates that if 60a undergoes ring opening at basic pH the process must reverse at acidic pH.

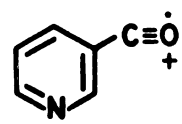


60a

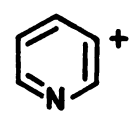
With the modidied amounts of reactants and modified workup procedure the yield of 60a after repetitive fractional crystallization from benzene and from ethyl acetate was 79% based on nicotinoyl chloride and 54% based on nicotinic acid. The product was a white needle-like crystalline solid with a melting point of 103°-105°C (lit.[100] 104°-105°C). The eims showed the correct molecular ion at m/e 190 (35%) and fragment ions which are consistent with the assigned structure. The fragment ion at m/e 162 (24.7%) is due to a loss of C_2H_4 from the molecular ion. The structures of the m/e 106 (100%) and m/e 78 (74.2%) ions are shown below.



m/e 162



m/e 106



m/e 78

The nmr spectrum of 60a in deuterated chloroform (Figure V) displayed between 7.2 ppm - 9.0 ppm the characteristic splitting pattern of the four aromatic protons of a 3-substituted pyridine (see Section

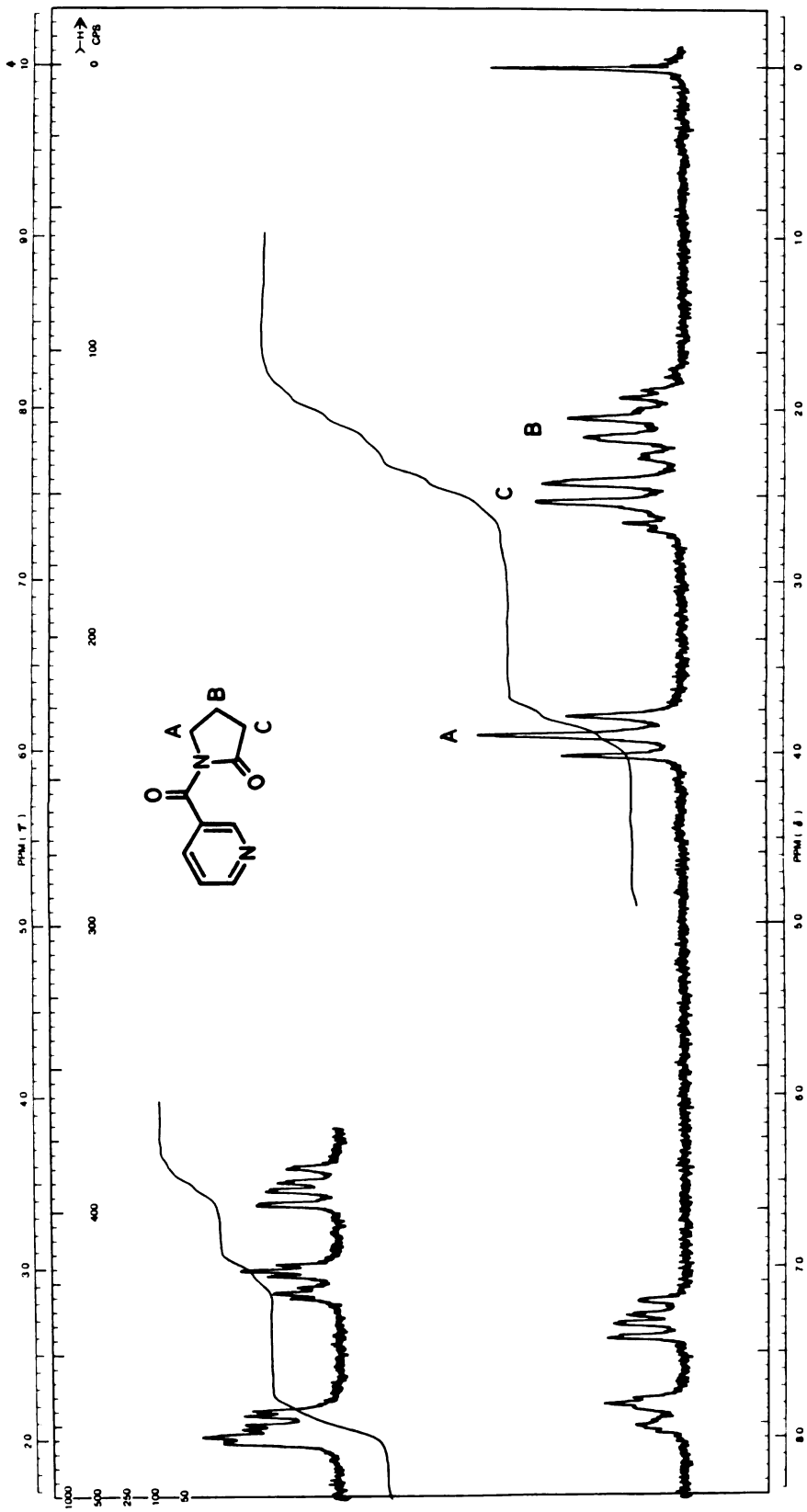


Figure V. The nmr spectrum of N-nicotinoyl-2-pyrrolidinone (60 MHz, $CDCl_3$, TMS).

A.5). A two proton triplet at 3.9 ppm (J=6.5 Hz) was assigned to the two protons α to the pyrrolidinoyl nitrogen and a four proton multiplet from 1.8-2.9 ppm was assigned to the methylene protons at C_3 and C_4.

The preparation of myosmine (58) from N-nicotinoyl-2-pyrrolidinone (60a) consists of the following two steps: (i) a Claisen ester condensation between ethyl nicotinate (61) and 60a followed by (ii) hydrolysis and decarboxylation of the condensation product in concentrated HCl according to a procedure reported by Korte and Steinen.[99] These authors suggested that the base induced reaction of 60a and 61 gave 1,3-dinicotinoyl-2-pyrrolidinone (67) which upon heating at reflux in dilute HCl, gave 3-nicotinoyl-2-pyrrolidinone (68). However no characterization of 67 and 68 was reported.

When the reported procedure was repeated the compound 67 was not isolated. The nmr spectrum of the crude, thick brown oil obtained from the condensation showed a splitting pattern and integration which were different from those expected for 67. The nmr in $CDCl_3$ of the purified white crystalline product (mp 99°-101°C) obtained from the oil is shown in Figure VI. The spectrum displayed between 7.0-9.0 ppm the characteristic signals for four protons of a 3-substituted pyridine. There is one exchangeable proton at 7.68 ppm which is assigned to a proton on the nitrogen. The one proton quartet at 4.4 ppm and the two multiplets from

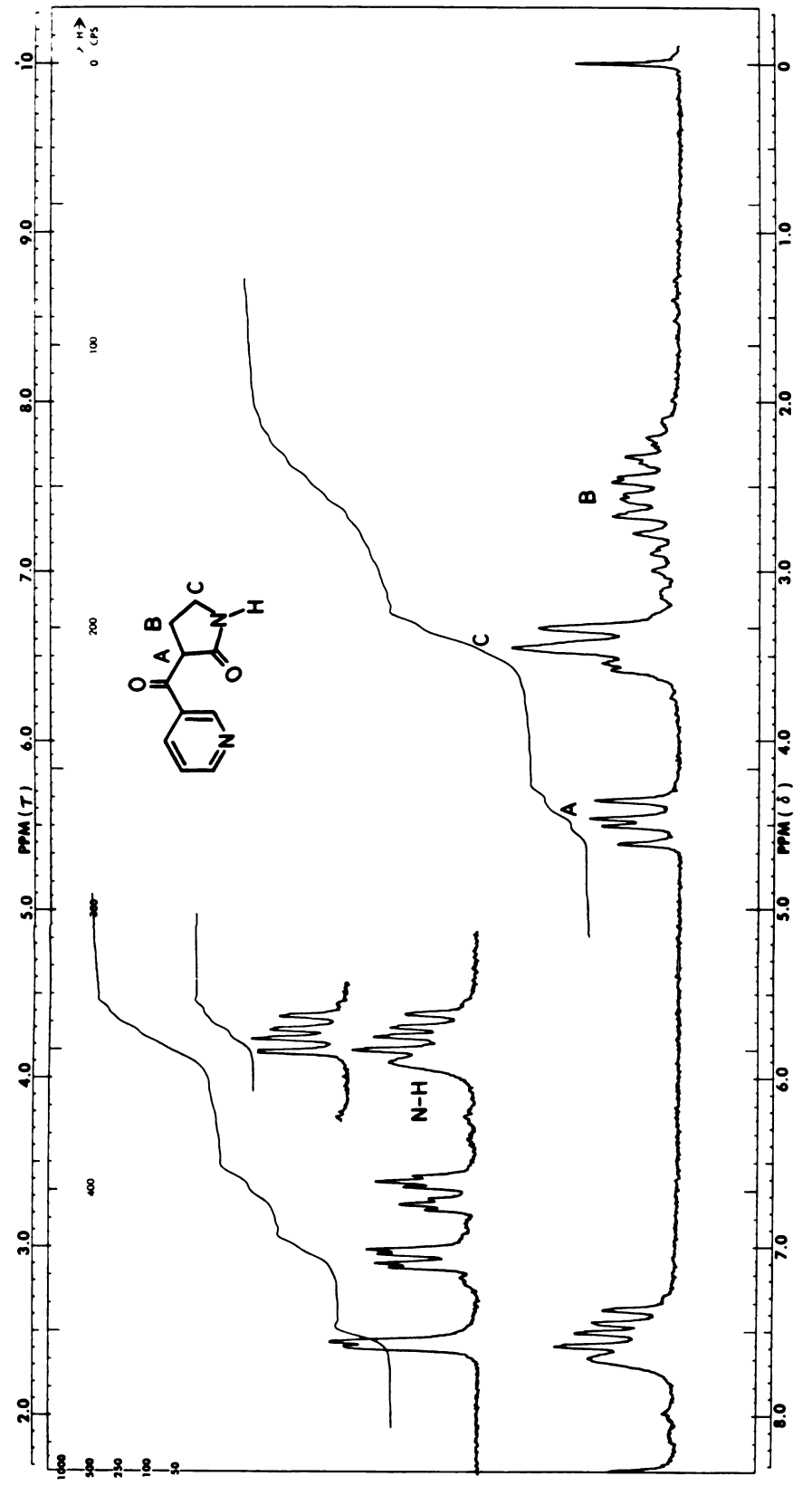
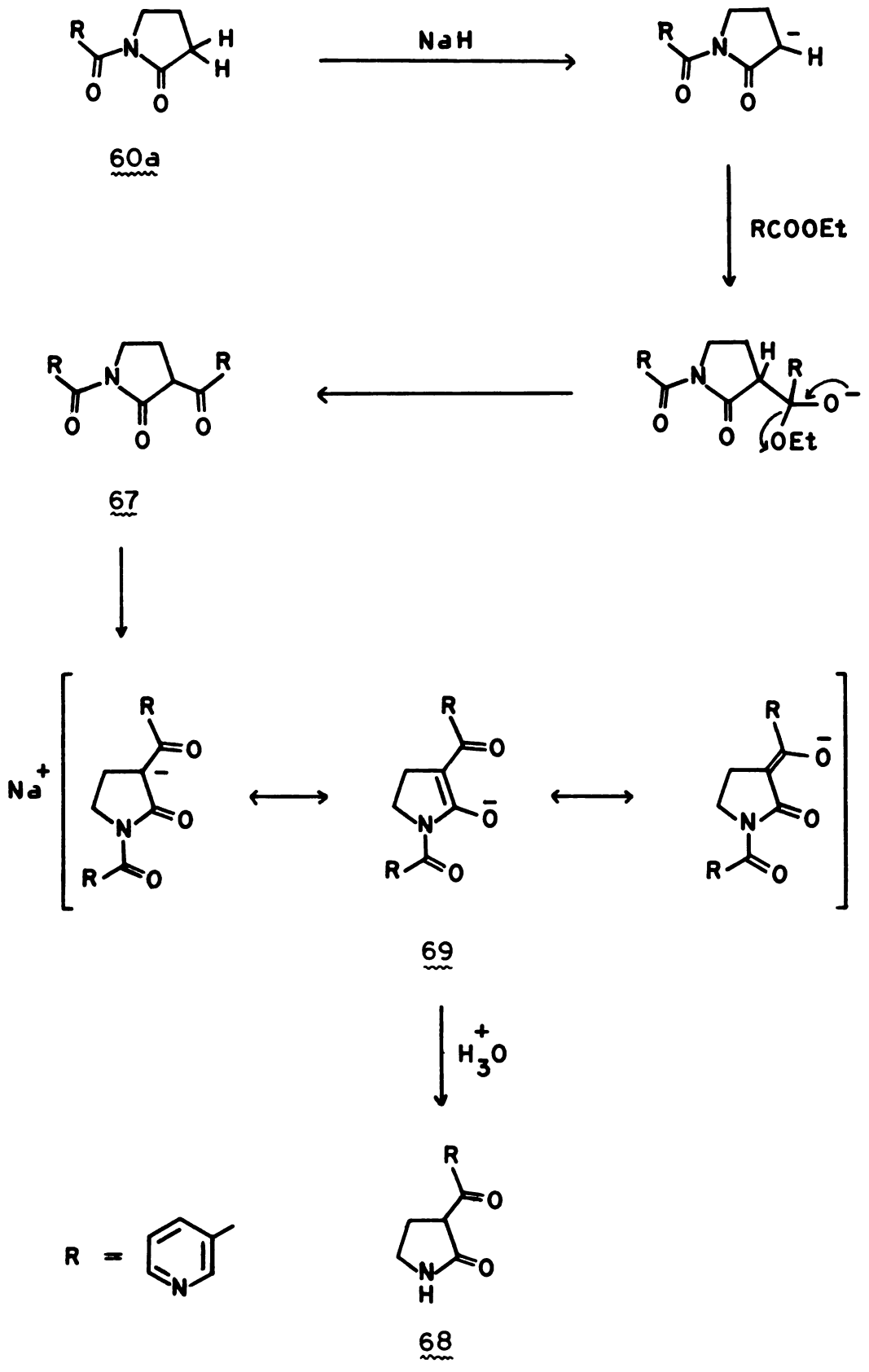
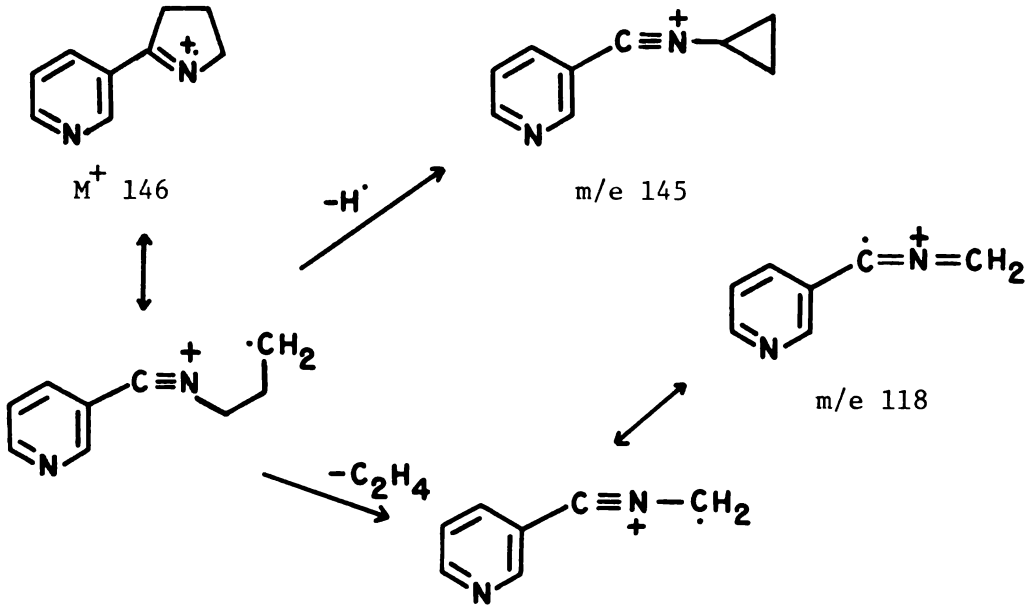


Figure VI. The nmr spectrum of 3-nicotinoyl-2-pyrrolidinone (60 MHz, $CDCl_3$, TMS).

2.0 ppm to 3.7 ppm integrating for four protons are assigned to the protons of the pyrrolidinone moiety. A cims (chemical ionization mass spectrum) showed the molecular weight to be 190 and CHN analysis was consistent with the formula $C_{10}H_{10}N_2O_2$). The ir spectrum showed two strong bands at 3440 cm^{-1} and 3325 cm^{-1} which are assigned to the non-hydrogen bonded and the hydrogen bonded N-H stretching frequencies of a secondary amide.[109] On the basis of these data the condensation product isolated is assigned the structure 3-nicotinoyl-2-pyrrolidinone (68). The isolation of 68 has been the sole evidence for the formation of 67. Probably the amide linkage in 67 is hydrolyzed during the workup to produce 68. A proposed mechanism for the formation of 68 from 60a is shown on the following page.

The acid catalyzed decarboxylation of 3-nicotinoyl-2-pyrrolidinone (68) in concentrated HCl proceeds smoothly to form myosmine which can be recrystallyzed from ether (39°-41°C, lit.[98] 45°C) in 63% yield. The ir spectrum of myosmine showed a medium absorption band at 1620 cm^{-1} due to the stretching vibration of a conjugated C=N bond.[109] The eims of myosmine is very simple. It contains three significant ions at m/e 146 (83%), m/e 145 (43.8%) and a base peak at m/e 118. The mass spectral fragmentation of myosmine according to Duffield, et al.[111] is shown below.


NaH
RCOOEt
OEt
Na+
R =
60a
67
69
68



The nmr spectrum of myosmine in $CDCl_3$ displayed the typical signals of the four aromatic protons characteristic for a 3-substituted pyridine at 7.0-9.0 ppm. Additionally a triplet of triplets at 4.1 ppm, a distorted triplet of triplets at 3.0 ppm and a pentet of doublets at 2.1 ppm each integrating for two protons were observed. The assignments for these multiplets are shown in figure VII.

A possible mechanism to account for the transformation of 3-nicotinoyl-2-pyrrolidinone to myosmine is shown below. The amide linkage of 68 undergoes acid catalyzed ring opening to the β-ketoacid 70 which spontaneously decarboxylates to the ketone 71. This γ-aminoketone 71 then closes to produce myosmine (58).

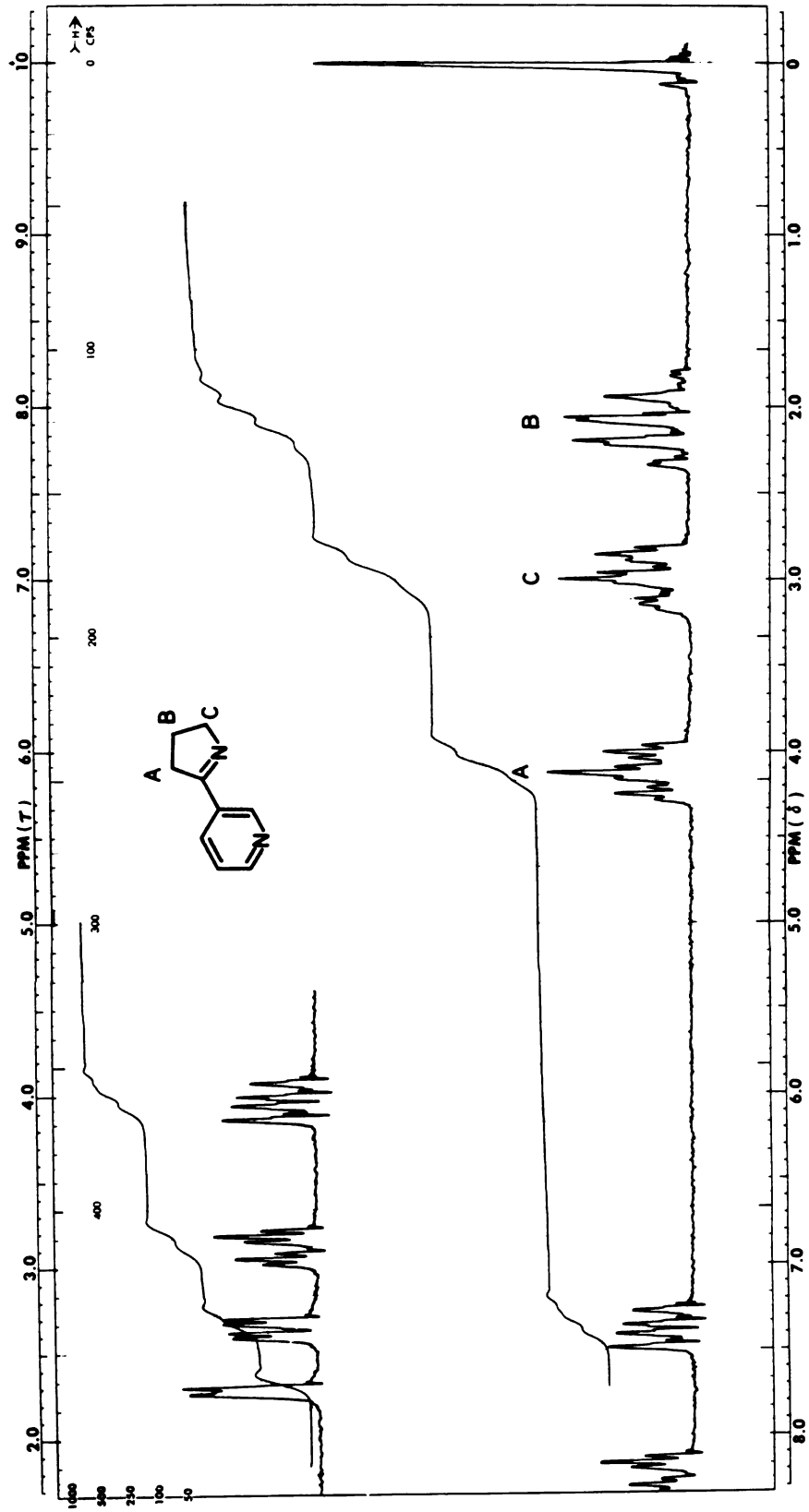
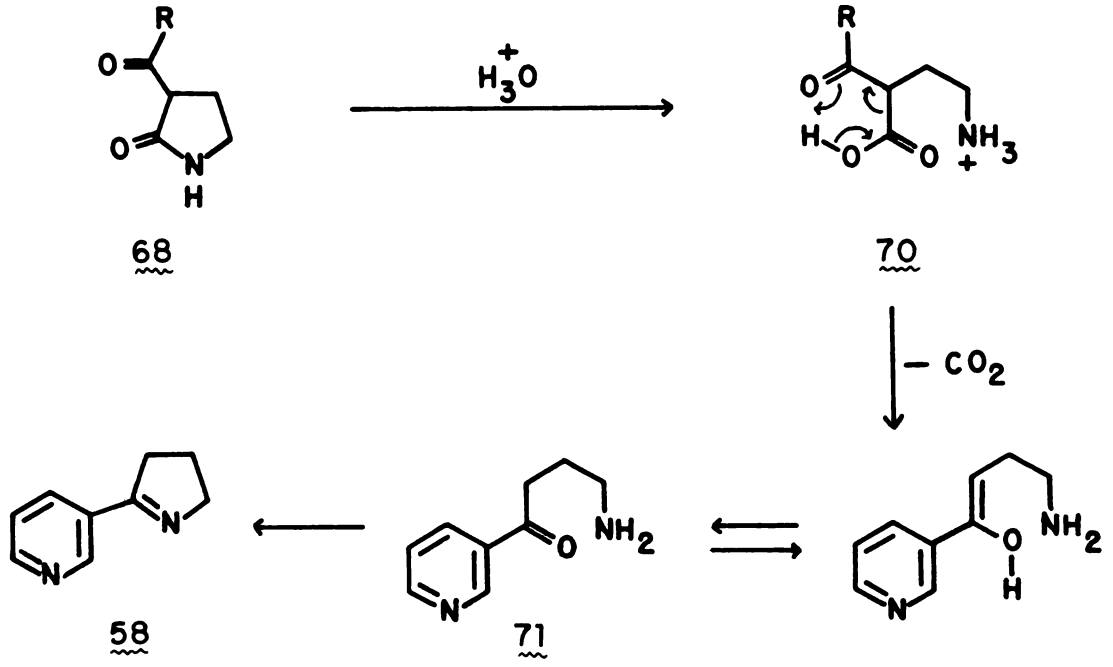
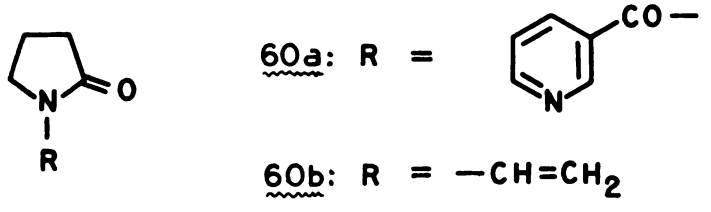
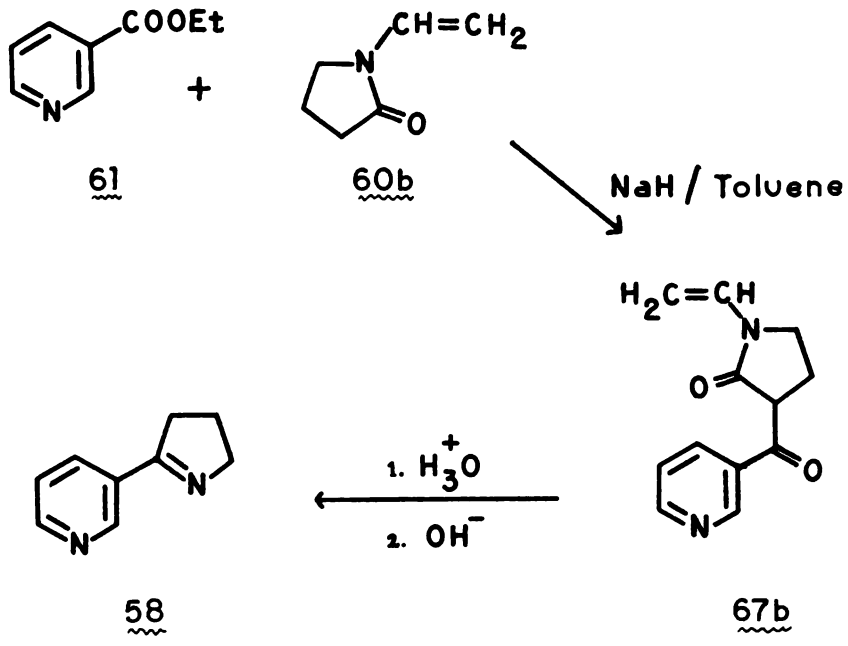


Figure VII. The nmr spectrum of myosmine (60 MHz, $CDCl_3$, TMS).



There has appeared very recently in the literature a similar procedure[110] for the preparation of myosmine which may be superior. In this procedure ethyl nicotinate (61) undergoes base induced ester condensation with N-vinyl-2-pyrrolidinone (60b) instead of with N-nicotinoyl-2-pyrrolidinone (60a). These authors believed that the condensation proceeds via 1-vinyl-3-nicotinoyl-2-pyrrolidinone (67b). However no characterization of 67b was reported.





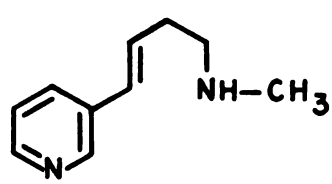
61 60b 67b 58

The yield of myosmine by this route is reported to be 63%. The advantage of this approach is that the N-vinyllactam 60b is commercially available while the N-acyllactam 60a is not and the preparation of 60a is the most difficult and tedious step of the entire synthetic sequence.

2. Nornicotine (2).

Nornicotine is thought to be one of the main metabolites of nicotine (see section C.2.g).[91-93] Additionally, nornicotine is a major tobacco alkaloid in some forms of tobacco.[95] Consequently, the preparation of nornicotine was one of the major synthetic goals.

The chemical synthesis of nornicotine has been known for a long time. The first reported synthesis of nornicotine was by French workers in 1927.[112] In this synthesis, nicotine N'-oxide was transformed into N-acetylnornicotine by treatment with acetic anhydride and free nornicotine was obtained upon adding K_2CO_3. However the properties of nornicotine described by these workers did not agree with those described by German workers in 1930.[113] Their method was to remove the methyl group of nicotine by heating (S)-nicotine with a high boiling organic acid such as benzoic acid or preferably hydrocinnamic acid. This reaction was reported to yield both nornicotine and metanicotine (72). Unfortunately, the nornicotine obtained by this method had an $[\alpha]_D$ of only -5.5°.



72

Methods to prepare optically pure nornicotine were sought by Späth, et al.[114] The first method they developed involved oxidation of (S)-nicotine by $KMnO_4$ to yield crude nornicotine with an $[\alpha]_D^{20} = -76.1°$. Upon recrystallizing this material as the perchlorate salt, the nornicotine recovered gave an $[\alpha]_D^{20} = -83.2°$. The second method used $AgNO_3$ as the oxidizing agent. The crude nornicotine obtained by this method had an $[\alpha]_D^{20} = -40.0°$. The free nornicotine obtained after recrystallization of the picrate salt gave an $[\alpha]_D^{20} = -88.8°$. No optical rotation higher than this value has ever been reported for nornicotine. Unfortunately, the yields of nornicotine by these two methods were less than 10%.

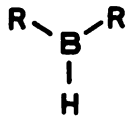
In 1933, American workers reported a seven step synthesis of nornicotine starting with pyridine.[116] This synthesis proceeds via the Grignard reaction between 3-cyanopyridine and γ-ethoxypropyl magnesium bromide followed by oxime formation of the resulting ketone with hydroxylamine. Nornicotine was finally obtained by the cyclization of 1-(3-pyridyl)-1-amino-4-ethoxybutane. The syntheses developed in later years all have myosmine as the key intermediate.[99-102] Hydrogenation of the imino functionality of myosmine affords racemic nornicotine.

Since nornicotine is chiral at $C_{2'}$, we desired a synthetic procedure which would lead to the (R)- and (S)-compounds and which could be adapted to the synthesis of deuterionornicotines. Thus efforts were made to

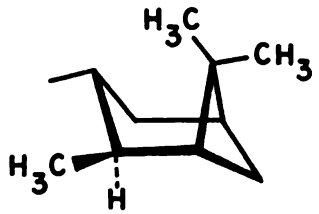
prepare optically active nornicotine by an asymmetric reduction of myosmine.

(-)-Diisopinocampheylborane (73) which is readily synthesized from borane and (+)-α-pinene (74),[117] was reported to reduce olefins to optically active alcohols whose configuration agreed in most cases with those predicted. The optical purities of the resulting alcohols were in the range of 65-91% for cis olefins[117] and 13-22% for trans and hindered olefins.[118] The hydroboration of myosmine with this chiral reagent 73 was carried out according to the procedure outlined in the literature.[117] The yield of nornicotine as determined by a gc analysis was less than 8%. The enantiomeric composition of this nornicotine was determined using pentafluorobenzoyl-(S)-prolyl imidazolide (PFBPI, see Section A.4). This analysis showed that the reduction of myosmine by 73 is not stereoselective.

74



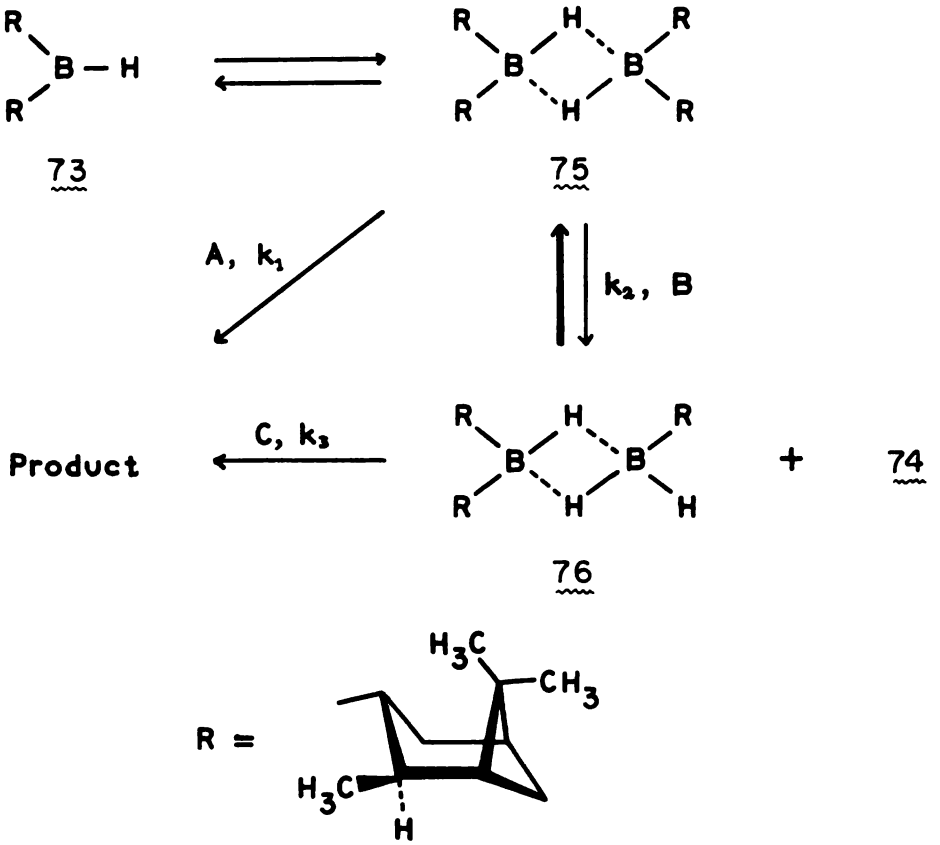
R =



73

Zweifel, et al.[119] found that 73 and its dimer tetraisopinocampheyldiborane (75), appear to exist in equilibrium with triisopinocam-

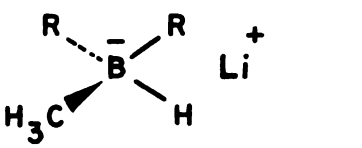
pheyldiborane (76) and (-)-α-pinene (74)



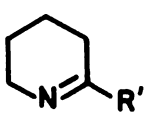
It is to be expected that k_3 is larger than k_1 because 76 is a less hindered molecule than 75. Therefore the rate of reduction is determined by k_1 or k_2. If k_1 is greater than k_2, which will be true when the bond to be reduced is not hindered, the reduction will take place via path A and the product will have high optical purity. However, if the bond to be reduced is hindered, k_2 will be greater than k_1, the reduction will occur via path B and C and the product will have a low optical purity. The reduction of myosmine to nornicotine seems to

follow the second path. When a large excess of 74 was used to suppress the dissociation of the hydroborating reagent (path B) no nornicotine was detected and myosmine was recovered unchanged.

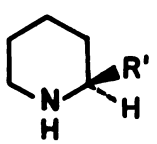
Another hydroborating reagent tried was lithium methyl-(hydro)-dipinan-3α-ylborate (77). This compound was reported to reduce the C=N bond of a series of 2-alkyl-3,4,5,6-tetrahydropyridines (78) to the corresponding (R)-2-alkylpiperidines (79).[120]



77



78



79

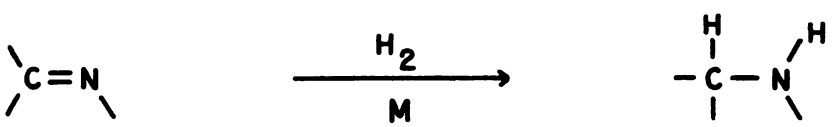
R'=$-CH_3$, $-CH_2CH_3$, $-CH_2CH_2CH_3$

The authors suggested that the carbon atoms of the tetrahydropyridine ring are bulkier than the substituents R' in 78 since the smaller the R' group used the greater was the induced chirality (optical yield: 20-25% when R' = $-CH_3$; 3-4% when R' = $-CH_2CH_3$ or $-CH_2CH_2CH_3$).

It is not clear from a molecular model of myosmine whether the pyridine or the pyrroline moiety is bulkier. Furthermore according to molecular models, as myosmine approaches the boron-hydrogen bond in the

model of 77 severe steric hindrance becomes apparent. Thus it was expected that if nornicotine was formed at all by this method its optical purity would be very low. When the reaction was attempted these expectations were borne out. The course of the reaction was followed by gc over a period of four days. Even at the end of this time the gc analysis showed that less than half of the myosmine had been converted to nornicotine. The recovery of nornicotine upon workup was also poor (<10%) perhaps because of the difficulty in removing the diglyme used as solvent. The derivatization of the product with the glpc chiral reagent trifluoroacetyl-(S)-prolyl chloride (TPC, see Section A.4) showed that there was no stereoselectivity in the reduction. In view of these poor results, attempts at asymmetric reduction were abandoned and other methods of reducing myosmine to nornicotine were sought.

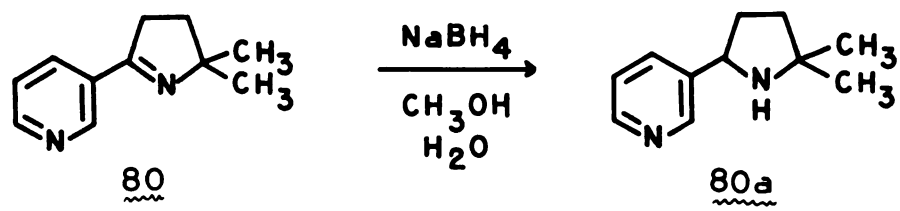
Hydrogenation of imines to amines usually proceeds readily over metal catalysts.[121]



Successful catalytic hydrogenation of myosmine with palladous oxide in ethanol has been reported with a 93% yield of nornicotine.[101] However catalytic deuteration of myosmine in ethyl acetate gave nornicotine

which was only 65% d_1 and 35% d_0.[111] Presumably the low deuterium incorporation is due to acetic acid or ethanol contaminants in ethyl acetate.[143]

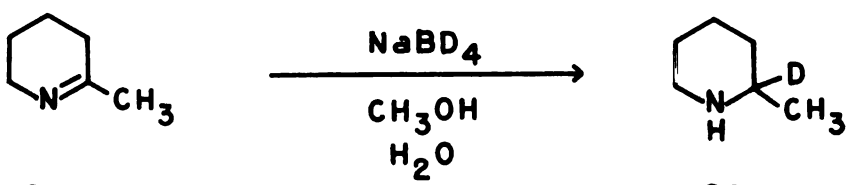
The reduction of imines with sodium borohydride has been reported and in many cases good yields were obtained.[122,123] Among these is the reduction of 5',5'-dimethylmyosmine (80) to the corresponding dimethyl substituted nornicotine 80a.[123]



80 80a

Myosmine was succesfully reduced to nornicotine by $NaBH_4$ in methanol-water according to the literature procedure outlined for 80. A crude yield of 95% was obtained. Since the desired compound is nornicotine-2'-d_1 (2a), conditions for the reduction of myosmine in a non protic solvent with $NaBD_4$ were considered in order to avoid possible deuterium loss. However the work of Craig and Lee[124] has shown that such precautions are unnecessary. These workers found that the reduction of 2-methyl-Δ^1-piperideine (81) to 2-methylpiperidine-2-d_1 (82) with sodium borodeuteride in CH_3OH and H_2O took place without significant loss of deuterium at the 2 position. This result is not surprising in

view of the findings of studies on the exchangeability of borohydrides.[126] Although this exchange does occur competitively with hylrolysis at acidic pH, the rate of exchange at basic pH is very slow. It is known that $NaBH_4$ reacts with water to form hydrogen gas and $NaBO_2$ in small amounts. After a short time the pH of the solution increases to about 9 due to the creation of the metaborate ion and the evolution of hydrogen ceases.[125] Although the formation of a basic solution protects sodium borodeuteride against exchange it can have unexpected side effects as well. When the reduction of 81 by $NaBD_4$ was carried out in CH_3OD and D_2O, an excess deuterium incorporation was observed due to the exchange of the protons on the allylic carbon.[124]

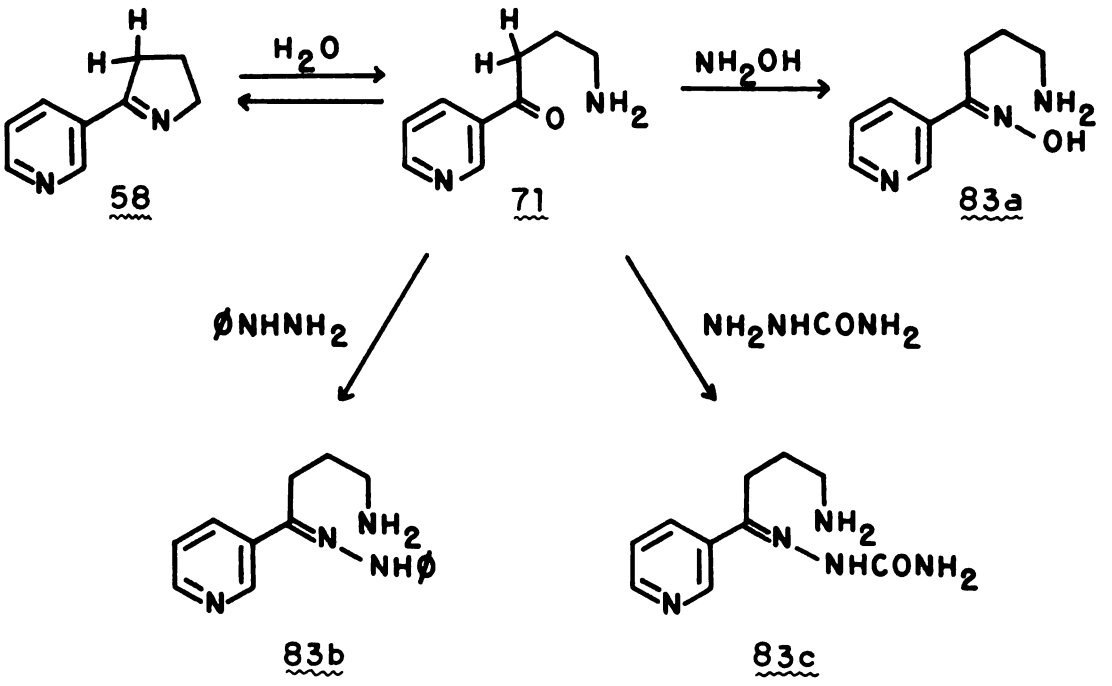


81 82

We were able to make use of these observations not only to prepare nornicotine-2'-d_1 but also through an allylic exchange to prepare nornicotine-3',3'-d_2 (2b) and nornicotine-2',3',3'-d_3 (2c) via myosmine-3',3'-d_2 (58a).

The reduction of myosmine by $NaBD_4$ in CH_3OH and H_2O proceeds smoothly to yield nornicotine-2'-d_1 (2a) in good yield and with the deuterium incorporated exclusively at the 2' position of the pyrrolidine moiety as desired. The nmr spectra of nornicotine-d_0 and -2'-d_1 are shown in figures VIII and IX. The integration indicates about 95% deuterium incorporation which is consistent with the isotopic purity of the $NaBD_4$ used. The eims indicated that not more than 7.7% of nornicotine-d_0 was present.

Haines, et al.[101] reported that myosmine reacts instantaneously with phenylhydrazine, hydroxylamine and semicarbazide to form carbonyl derivatives of the γ-aminoketone 71. Thus it was suggested that myosmine exists in equilibrium in water with its open chain form 71.



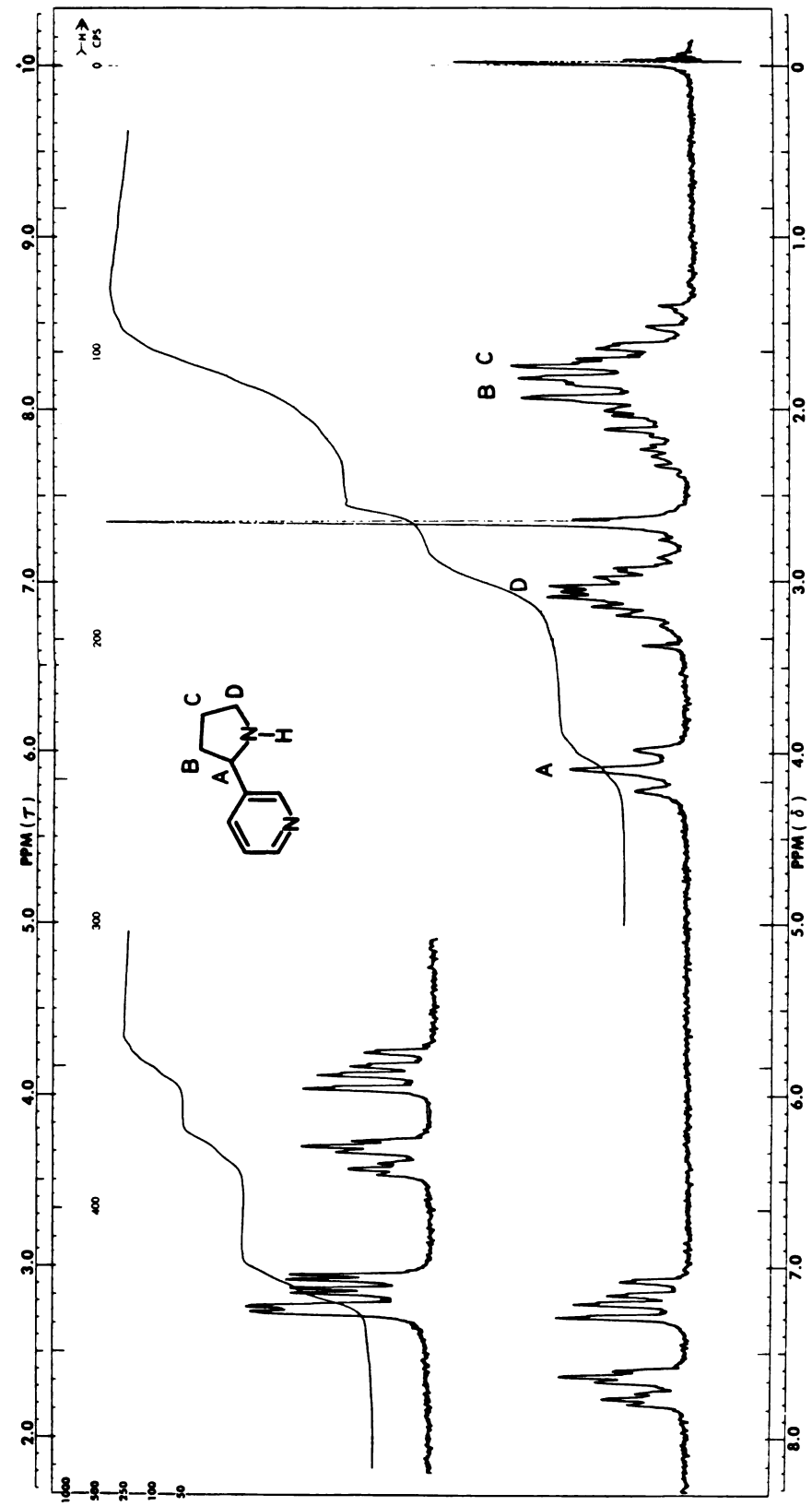


Figure VIII. The nmr spectrum of nornicotine-d_0 (60 MHz, $CDCl_3$, TMS).

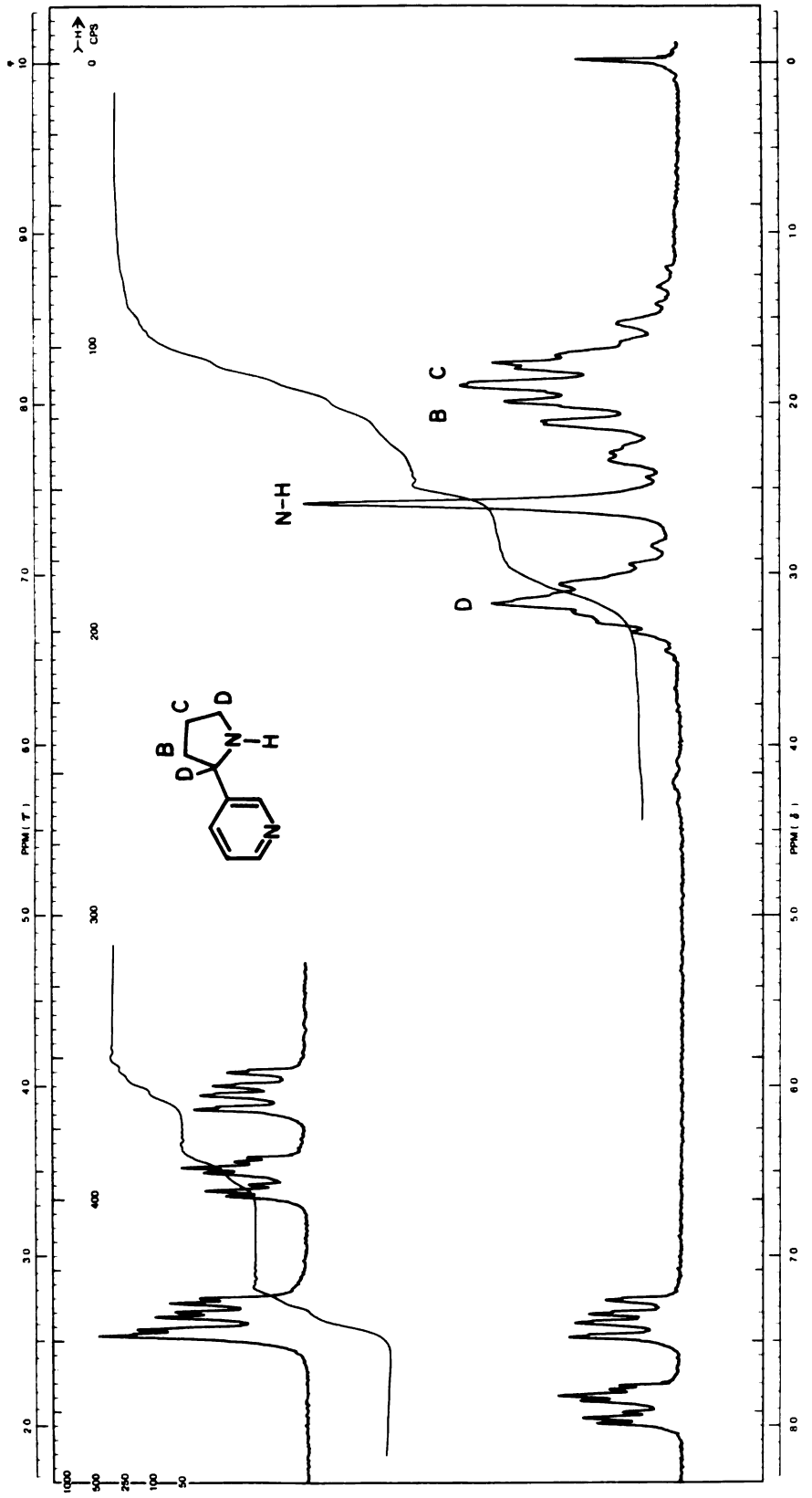


Figure IX. The nmr spectrum of nornicotine-2'-d_1 (60 MHz, $CDCl_3$, TMS).

The open chain form 71 of myosmine possesses two hydrogen atoms next to the carbonyl group, which are acidic enough to be exchanged with D_2O under the influence of a base such as K_2CO_3.[127] The corresponding hydrogen atoms in the cyclic form, myosmine (58), also may be acidic enough to exchange under the same conditions. In any case, an nmr spec- of myosmine in D_2O containing potassium carbonate showed that after one day at 100°C there was a complete conversion to myosmine-3',3'-d_2 (see Figures X and XI).

The eims of myosmine-d_0 and myosmine-3',3'-d_2 shown in Figure XII are very similar. It is only in the region of the molecular ion that the m/e peak shifts are observed. Unfortunately, the M-1 and M-2 ions are quite intense and lead to overlapping ions in a mass spectrum of a mixture of the d_0, d_1 and d_2 compounds. Thus it is difficult to determine the isotopic composition of myosmine-3',3'-d_2 from the eims. The deuterium exchange of myosmine under basic conditions provides an easy way to prepare in high yields labeled nornicotines suitable for metabolic work. Reduction of myosmine-3',3'-d_2 with $NaBH_4$ gives nornicotine-3',3'-d_2 (2b) while reduction with $NaBD_4$ gives nornicotine-2',3',3-d_3 (2c).

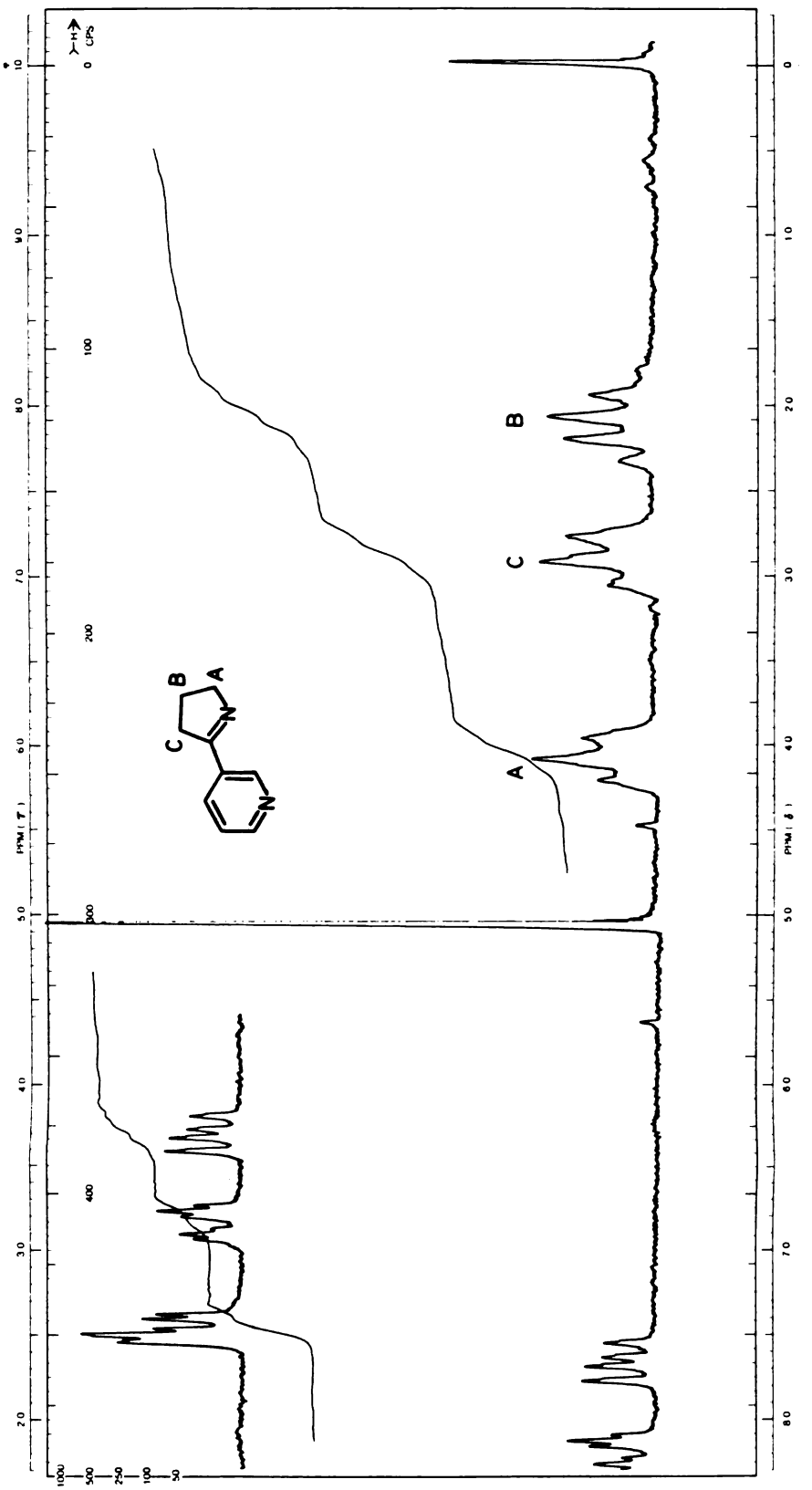


Figure X. The nmr spectrum of myosmine-d_0 (60 MHz, D_2O, DSS).

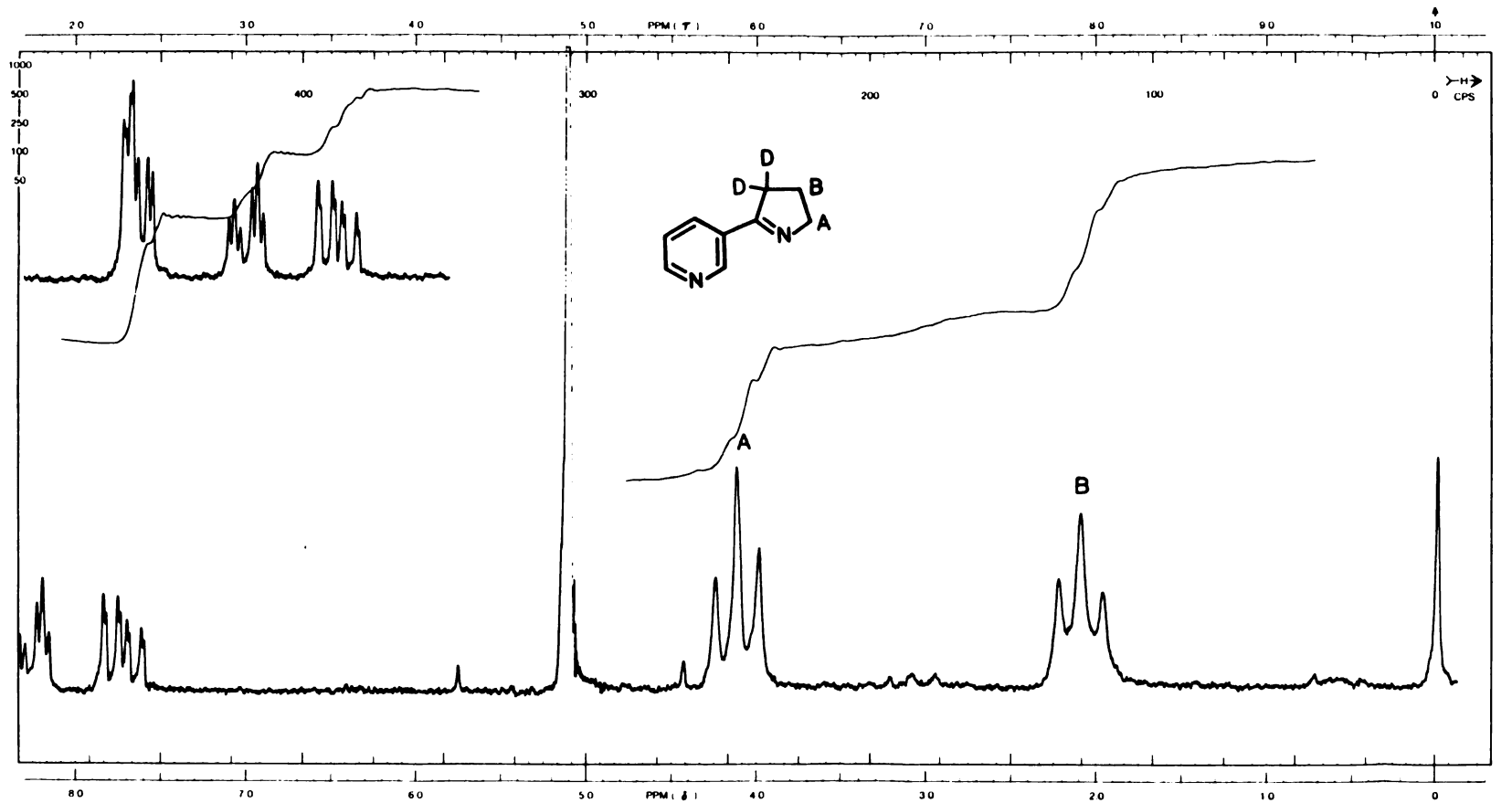


Figure XI. The nmr spectrum of myosmine-3',3'-d_2 (60 MHz, D_2O, DSS).

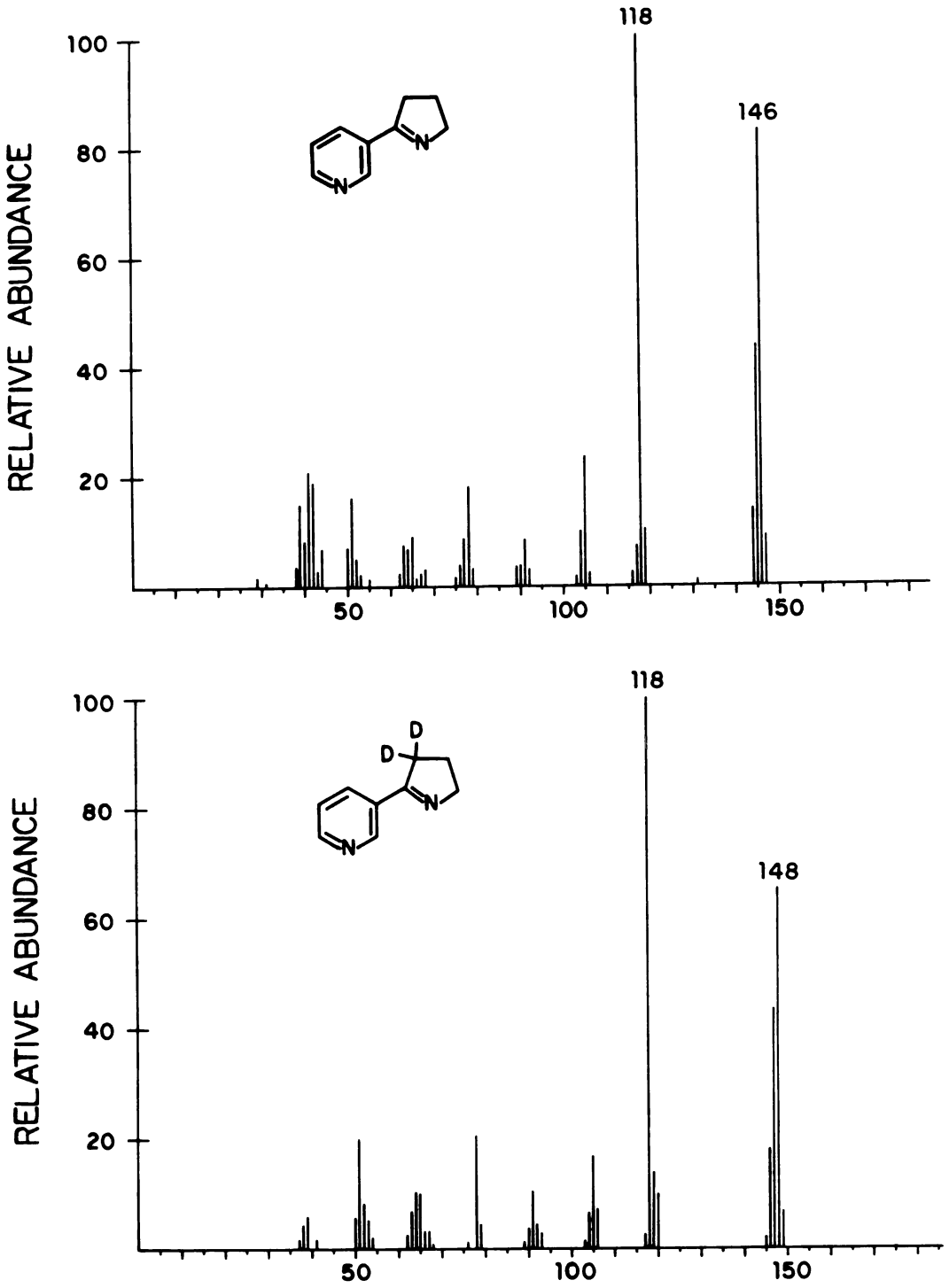
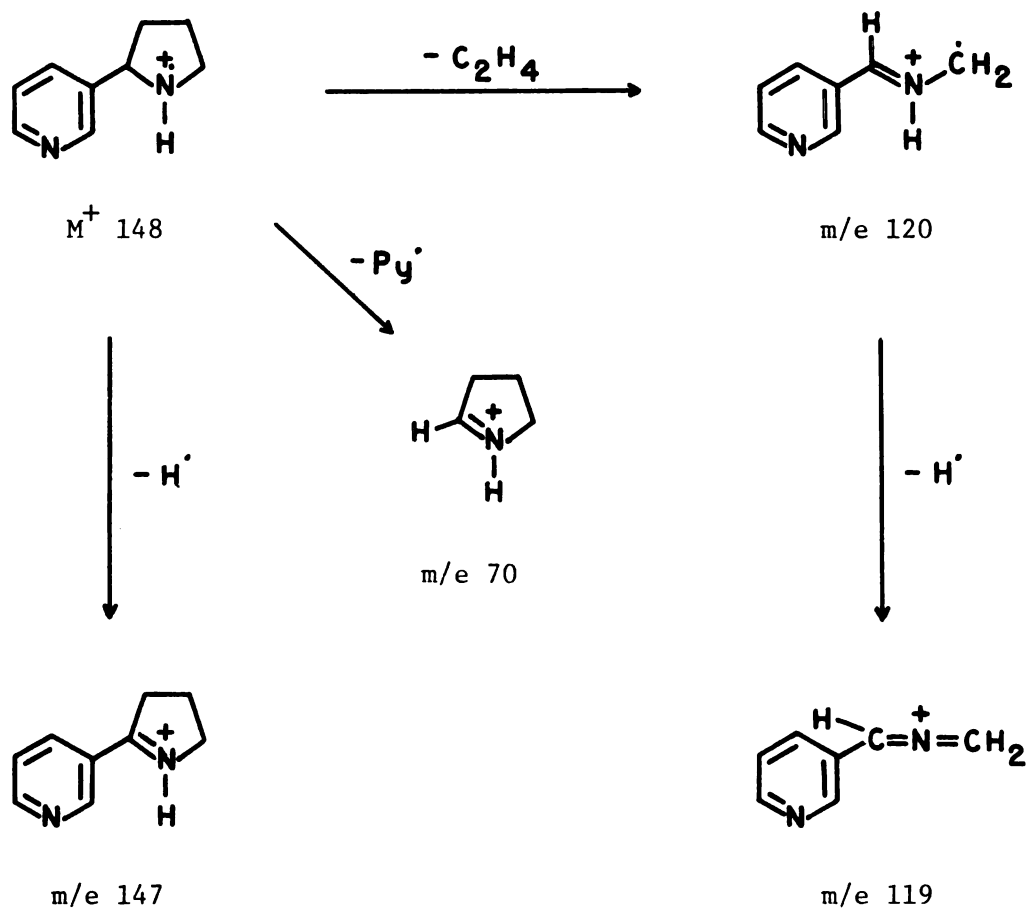


Figure XII. The ei mass spectra of myosmine-d_0 (top) and of myosmine-3',3'-d_2 (bottom).

The mass spectra of nornicotine-d_0, -2'-d_1, -3',3'-d_2 and -2',3',3'-d_3 are presented in Figure XIII and Figure XIV and the intensities of the important ions are shown in table III. The eims of the d_0 and 2'-d_1 species agree well with the spectra reported in the literature.[111] The eims of the 3',3'-d_2 and 2',3',3'-d_3 compounds have not previously been reported. The following scheme shows the mass spectral fragmentation of nornicotine according to Duffield, et al.[111]



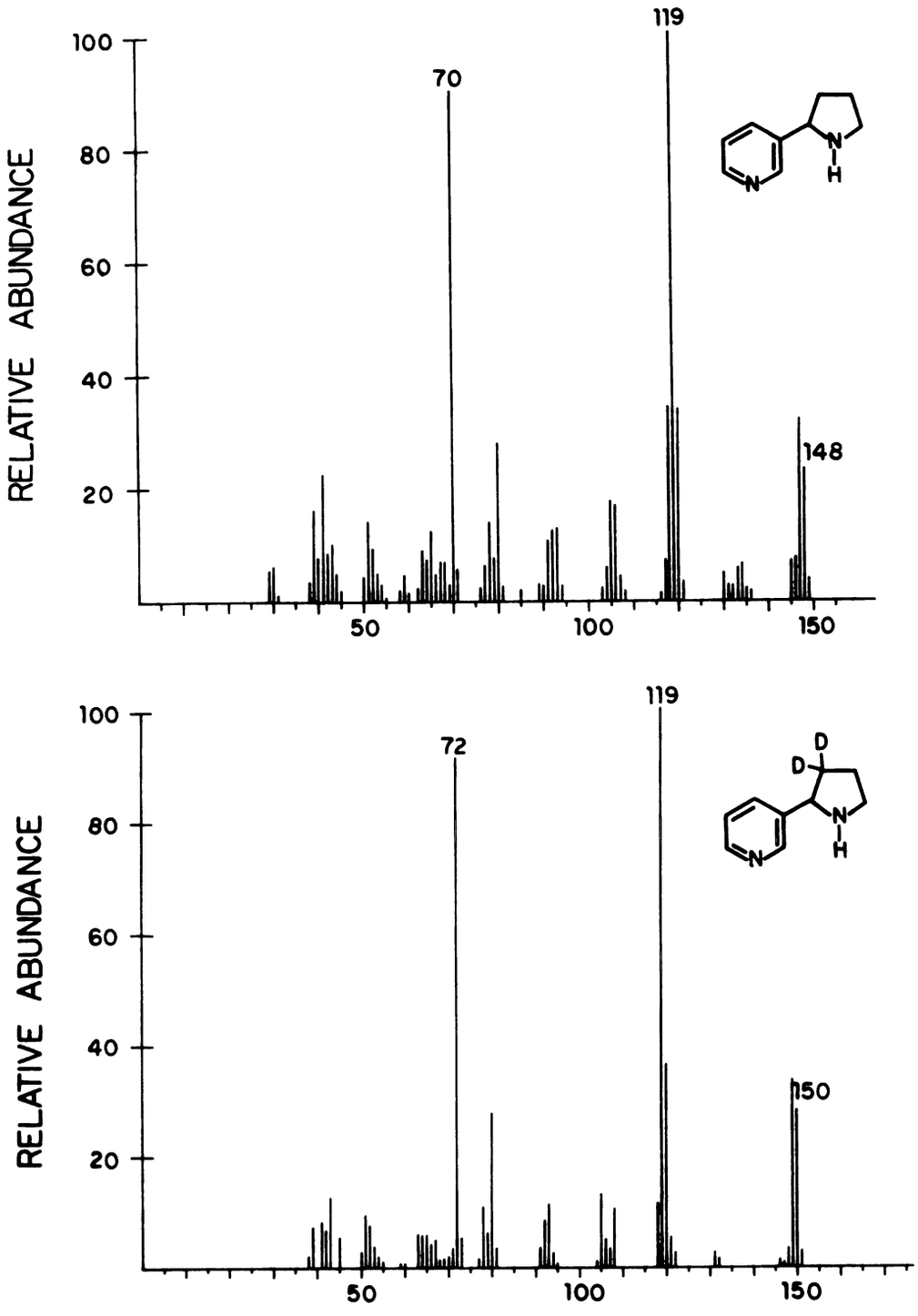


Figure XIII. The ei mass spectra of nornicotine-d_0 (top) and of nornicotine-3',3'-d_2 (bottom).

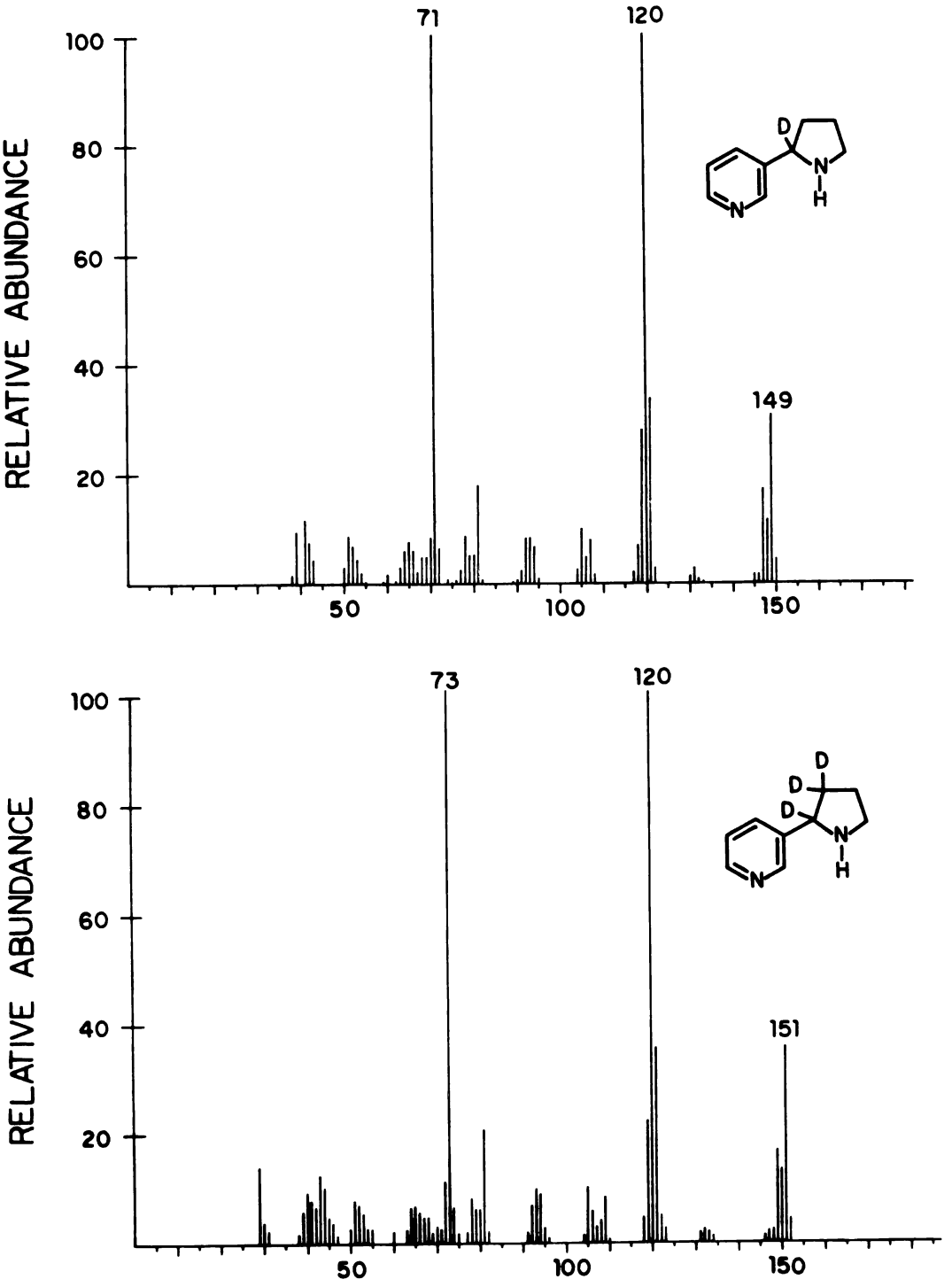
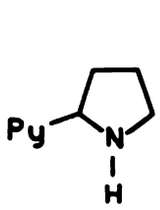


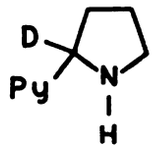
Figure XIV. The ei mass spectra of nornicotine-2'-d_1 (top) and of nornicotine-2',3',3'-d_3 (bottom).

Table III

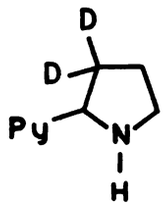
Relative intensities of important m/e peaks in the ei-mass spectra of nornicotines.



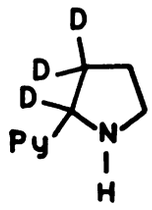
d_0



2'-d_1



3',3'-d_2

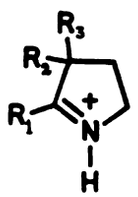


2',3',3'-d_3

m/e	%	m/e	%	m/e	%	m/e	%
148	23.0	149	30.2	150	27.9	151	34.8
147	32.0	148	15.0	149	33.7	150	13.1
146	7.2	147	17.6	148	3.4	149	17.0
145	7.0	146	1.5	147	1.1	148	2.0
119	100.0	120	100.0	119	100.0	120	100.0
70	90.0	71	100.0	72	90.7	73	100.0
69	3.0	70	7.7	71	3.9	72	11.6
		69	4.6	70	2.1	71	2.3
				69	1.6	70	2.3

The eims of nornicotine-d_0 and -3',3'-d_2 show a large m/e ion at M-1 while the eims of nornicotine-2'-d_1 and -2',3',3'-d_3 show prominent m/e ions at M-1 and M-2. This indicates that the loss of one mass unit from the molecular ion of the d_0 compound is not entirely due to the hydrogen at $C_{2'}$ but is also due to hydrogen losses from other parts of the molecule. This is in agreement with Duffield's results[111] which indicate that only 55% of the M-1 peak for nornicotine is due to the hydrogen at $C_{2'}$. This explains the complex patterns which are seen in the region of the molecular ion in the mass spectra of the deuterium labeled nornicotines.

The base peak at m/e 119 is also unsuitable for isotopic composition analyses since it does not shift in the d_2 case. The only prominent fragment which can be used to monitor the relative amounts of d_0, d_1, d_2 and d_3 species is the pyrrolidinium ion 84 at m/e 70.



84 (m/e 70) $R_1 = R_2 = R_3 = H$

84a (m/e 71) $R_1 = D$, $R_2 = R_3 = H$

84b (m/e 72) $R_1 = H$, $R_2 = R_3 = D$

84c (m/e 73) $R_1 = R_2 = R_3 = D$

Unfortunately, the interpretation of the relative ion currents observed in this mass region is not straightforward. The eims of nornicotine-2'-d_1 shows an m/e 70/71 ratio of 7.7%; however it is doubtful that this means 7.7% d_0 species is present. Duffield, et al.[111] reported that only 90% ± 5% of the m/e 70 ion shifts to m/e 71 in nornicotine-2'-d_1 even after correction for the d_0 species present. It should be noted also that the nornicotine-3',3'-d_2 spectrum shows an m/e 70 to m/e 72 ratio of 2.3% even though it is statistically unlikely that any d_0 species is present. Thus it is unlikely that there is more than 4% nornicotine-d_0 present in the nornicotine-2'-d_1 sample.

In the spectrum of nornicotine-3',3'-d_2 an m/e 71/72 ratio of 4.1% is observed. Again it is doubtful that much of the m/e 71 ion is really due to a d_1 impurity.

The mass spectrum of nornicotine-2',3',3'-d_3 shows that the ratio of m/e 72 to m/e 73 is 11.6%. This suggests an isotopic composition of about 94% d_3 and 6% d_2 with most of the deuterium loss being at $C_{2'}$ rather than $C_{3'}$. This is confirmed by the nmr spectrum of nornicotine-2',3',3'-d_3 in $CDCl_3$ (Figure XV) which shows about 8% of the integration for a single hydrogen at $C_{2'}$ (signal at 4.1 ppm).

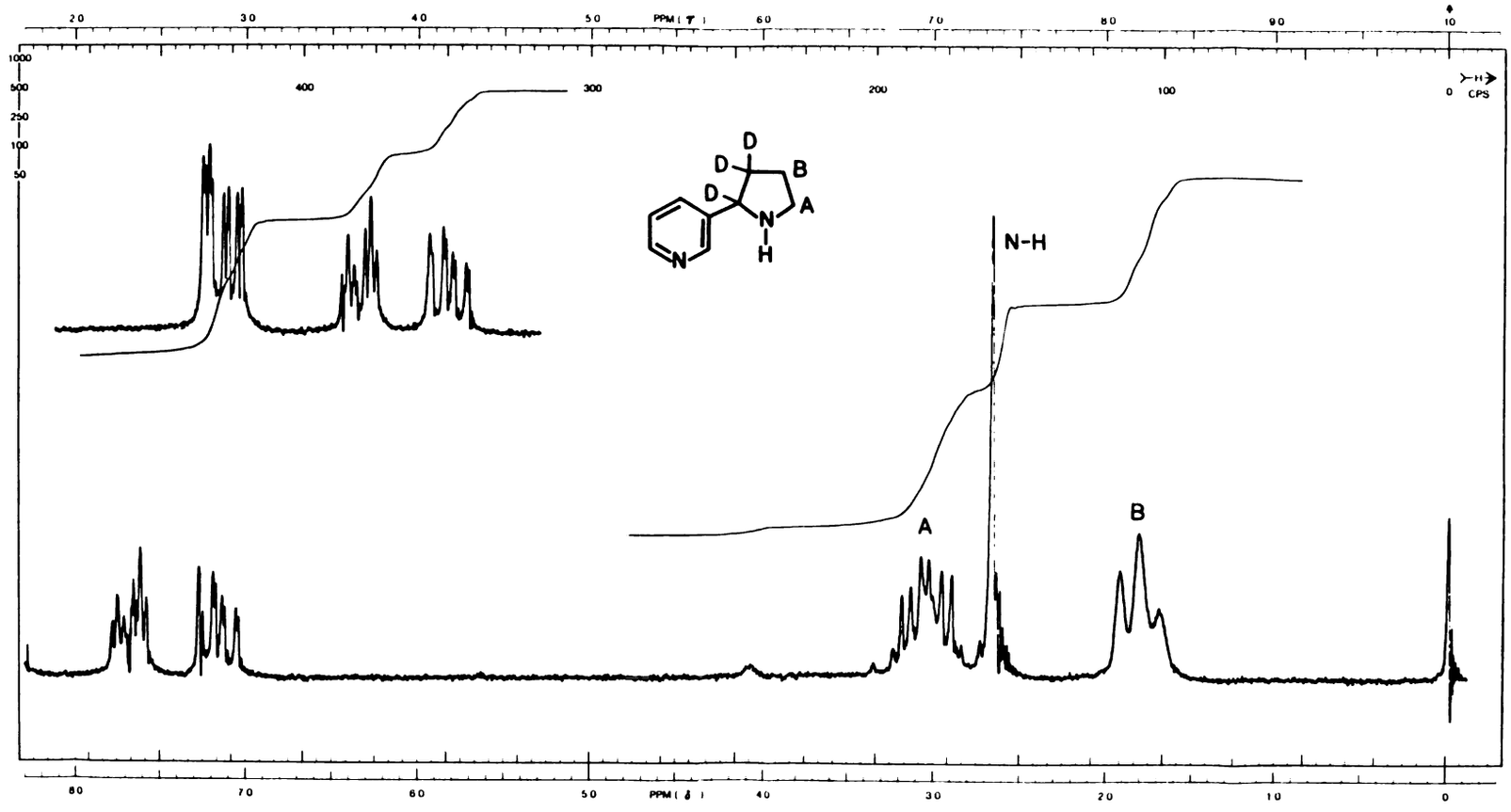
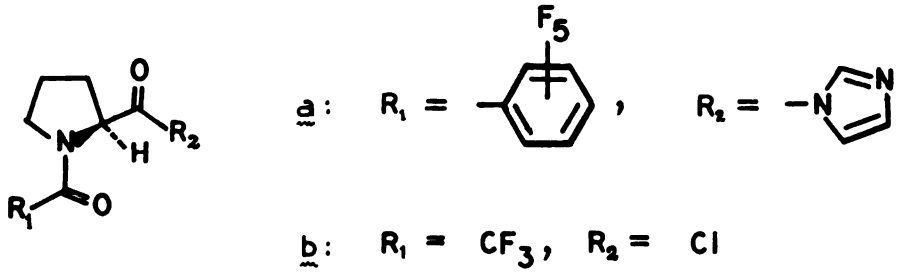


Figure XV. The nmr spectrum of nornicotine-2',3',3'-d_3 (60 MHz, $CDCl_3$, TMS).

3. Derivatization of nornicotine with chiral reagents.

The use of chiral derivatizing reagents has proved useful for the determination of enantiomeric composition and the analysis of the stereoselectivity operating in the metabolism of certain drugs.[128] This method depends upon the derivatization of enantiomers with a chiral reagent to generate diastereomers. With the proper chiral reagent the diastereomeric derivatives can be quantitatively analyzed by gas chromatography.

Initial attempts to prepare diastereomers of nornicotine using pentafluorobenzoyl-S-(-)-prolyl-1-imidazolide (PFBPI, 85a) failed to give sufficient reaction even after several hours at elevated temperature. Fortunately, commercially available N-trifluoroacetyl-S-(-)-prolyl chloride (TPC, 85b) gave excellent results; the derivatization takes less than ten minutes. The following page shows a typical gas chromatogram of the diastereomeric TPC-derivatives of nornicotine. This method was used to follow the attempted resolution of nornicotine discussed in the next section.



85

a: R_1 = , R_2 =

b: $R_1 = CF_3$, $R_2 = Cl$

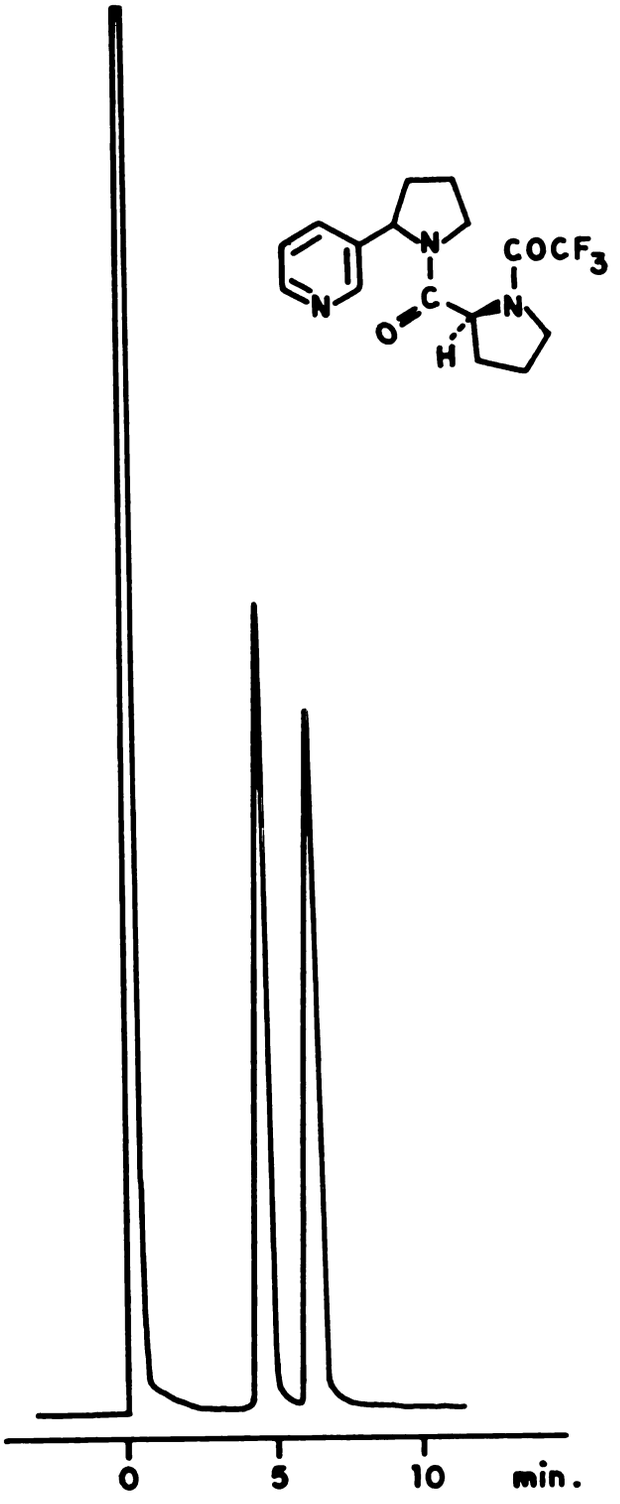


Figure XVI. The gas chromatogram of racemic nornicotine as the diastereomeric TPC derivative on 3% OV-17 at 260°C isothermal.

4. Resolution of nornicotine.

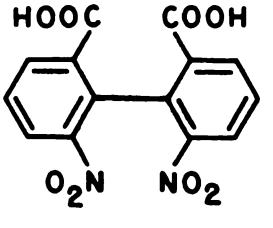
In order to obtain optically pure deuterium labeled (S)-nicotine we required methods for the resolution of either nornicotine or nicotine itself.

The resolution of racemic nicotine with d-tartaric acid was reported first in 1904.[129] However it was found that the tartrate system is particularly difficult to work with because of the various kinds of salts obtainable. Both neutral and acidic tartrates with or without H_2O of crystallization are known. These early workers reported that in ethanol or aqueous ethanol, a mixture of salts is obtained. Therefore recrystallization from ethanol water mixtures is not suitable for a proper resolution even though a high yield of a nicely crystalline material can be obtained. According to these same workers only the bitartrate dihydrate salt is formed in water and it is by forming this salt that nicotine can be resolved. A partially successful attempt was made to repeat the early work. However, it took three weeks for the crystals to appear and the yield was poor (<2%) due to the high water solubility of the bitartrate salt. This result was very discouraging especially considering the small amount of deuterated nicotine available.

In any case there are two major advantages to performing the resolution at the nornicotine step: the course of the resolution can be

followed by the gc method and the resolved nornicotine itself would be useful in metabolic studies.

Optically pure (S)-nornicotine has been usually obtained by fractional recrystallization of the perchlorate salt of the partially resolved nornicotine isolated from tobacco or from the chemical demethylation of natural (S)-nicotine.[114,130] Obviously neither of these methods was suitable for the resolution of the deuterium labeled nornicotines. Thus much effort was spent on developing a suitable acid to resolve nornicotine. The following ten common resolving acids were tried: (+)-malic acid, (-)-mandelic acid, (+)-3-methyladipic acid, (-)-menthoxyacetic acid, di-p-toluoyl-(+)-tartaric acid, (S)-2-pyrrolidinone-5-carboxylic acid, dibenzoyl-(+)-tartaric acid.H_2O, (-)-o-nitrotartranilic acid, (+)-10-camphorsulfonic acid and (+)-tartaric acid. However no satisfactory results were obtained as determined by the TPC derivatization gc method. Finally the resolution of nornicotine with (-)-6,6'-dinitro-2,2'-diphenic acid (86) according to a reported procedure[131] was attempted.



86

The four step synthesis of the acid 86 starting from 2-chloro-3-nitrobenzoic acid was carried out according to a literature procedure.[132] The resolution of 86 with (-)-quinine was accomplished by recrystallization of the salt three times from isopropyl and ethyl alcohol mixtures. A white salt was obtained with a melting point of 224°-226°C. Liberation of the free acid gave a white crystalline solid with $[\alpha]_D = -125°$ (lit.[133] $[\alpha]_D^{27} = -126°$).

The resolution of nornicotine using this acid gave a yellow crystalline solid from methanol (326 mg from 480 mg of total salt) with a melting point of 202°-204°C after one crystallization. Free nornicotine (30 mg, 40.5%) was obtained after decomposition of the diastereomeric salt with saturated potassium carbonate solution. The low recovery of nornicotine could be due to the reaction between nornicotine and the solvents diethyl ether and dichloromethane used for extracting (see Section C.2.c and g). The nornicotine thus obtained gave an $[\alpha]_D = -49°$ (lit.[130] $[\alpha]_D = -88.8°$) which is identical to the result obtained by Späth and Kesztler[131] using the same procedure. They obtained nornicotine of even higher optical rotation by crystallization of the partially resolved nornicotine as the diperchlorate salt.

Unfortunately the TPC derivatizing reagent which was to be used to follow the resolution of nornicotine was not available at the time of

completion of this work. Further work will be required to complete this aspect of the nicotine metabolism program.

5. Nicotine (1).

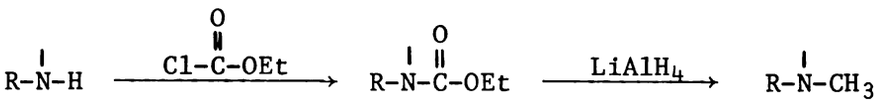
Tertiary amines with at least one methyl group are often conveniently prepared by the Eschweiler-Clark method.[134] This is a reaction between a primary or a secondary amine and formaldehyde followed by reduction with formic acid.

$$R\text{-}\overset{|}{N}\text{-}H \xrightarrow{HCHO} R\text{-}\overset{|}{\underset{+}{N}}\text{=}CH_2 \xrightarrow{HCOOH} R\text{-}\overset{|}{N}\text{-}CH_3 + CO_2$$

Nicotine has been synthesized from nornicotine by this method in high yield.[138 81 89] However this route is not economical for the preparation of nicotine-N-methyl-d_3 (1a) and for this reason the literature method for preparing 1a using trideuteriomethyl iodide in methanol was examined. Craig,[135] who developed this procedure, obtained nicotine-N-methyl-d_3 in only 18.5% yield. Duffield, et al.[111] used this procedure to prepare 1a for the mass spectral fragmentation studies of the tobacco alkaloids. The yield obtained was 33%. The results of our experiments using Craig's procedure were comparable to Duffield's. After workup, a mixture of nicotine and nornicotine was obtained in a one to one ratio. However this material accounted for only about 65% of the starting nornicotine. The rest of the nornicotine was probably further methylated to quaternary salts. Although the reaction is clean, the low yield and the difficulty in separating nicotine from nornicotine made this proce-

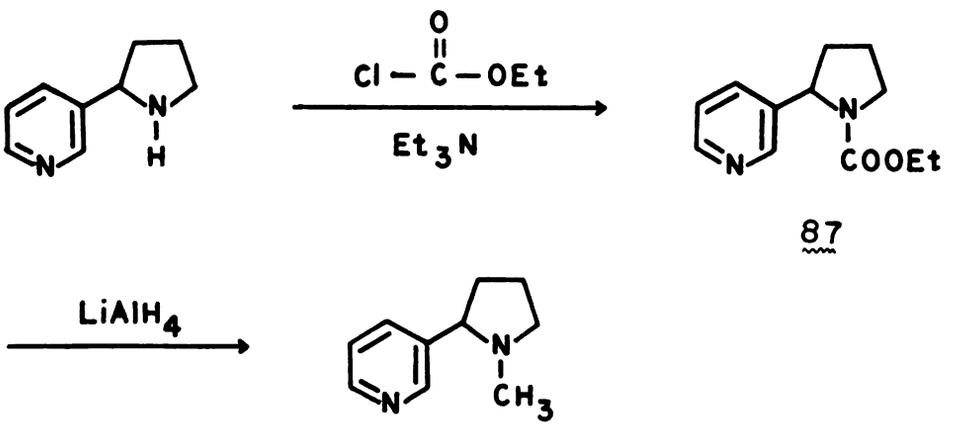
dure unattractive.

Another method of preparing N-methylamines is via the reduction of the corresponding carbamates with lithium aluminum hydride. This reaction usually proceeds in yields of at least 70%.[136]



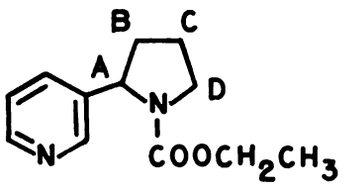
Recently Marshall and McMahon[137] prepared nortriptyline-N-$^{14}CH_3$ using ethyl chloroformate-^{14}C as well as nortriptyline-N-CD_3 and -N-CT_3 using lithium aluminum deuteride and tritide, respectively.

The carbamate reaction sequence was found to work equally well for the synthesis of nicotine. Ethyl chloroformate was added to nornicotine in the presence of triethylamine at room temperature. After filtering off the triethylamine hydrochloride, a bulb to bulb distillation gave the expected carbamate as a colorless liquid in a yield of 80%.



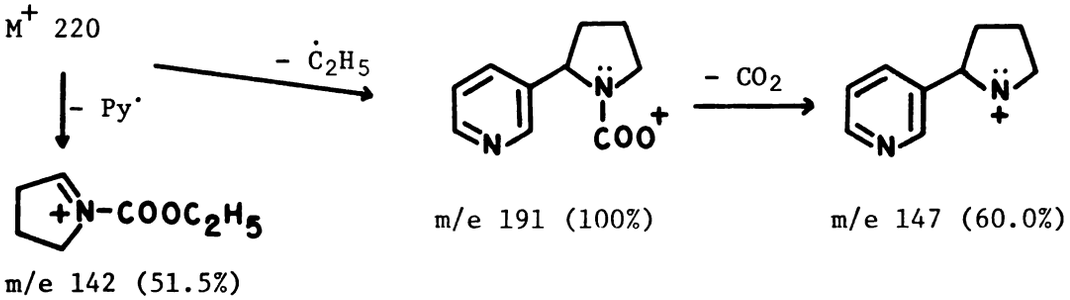
87

The N-ethoxycarbonylnornicotine (87) structure assignment was confirmed by spectral data. The infrared specrum showed an intense band at 1710 cm^{-1} for the stretching vibration of the C=O bond. The nmr spectrum in $CDCl_3$ indicated that the molecule contained a 3-substituted pyridine moiety (signals from 7.0 to 9.0 ppm). The presence of an ethoxy group was indicated by a two proton quartet at 4.0 ppm and a three proton triplet at 1.1 ppm with a coupling constant of 6.5 Hz. The seven protons on the pyrrolidine ring were accounted for by a one proton doublet of doublets at 4.9 ppm (A), a two proton triplet at 3.6 ppm (D) and a four proton multiplet from 1.6 to 2.5 ppm (B & C).



87

The electron impact mass spectrum (Figure XVII) showed the correct molecular weight for N-ethoxycarbonylnornicotine at m/e 220 (34.8%). The fragmentation observed is rationalized by the scheme shown below.



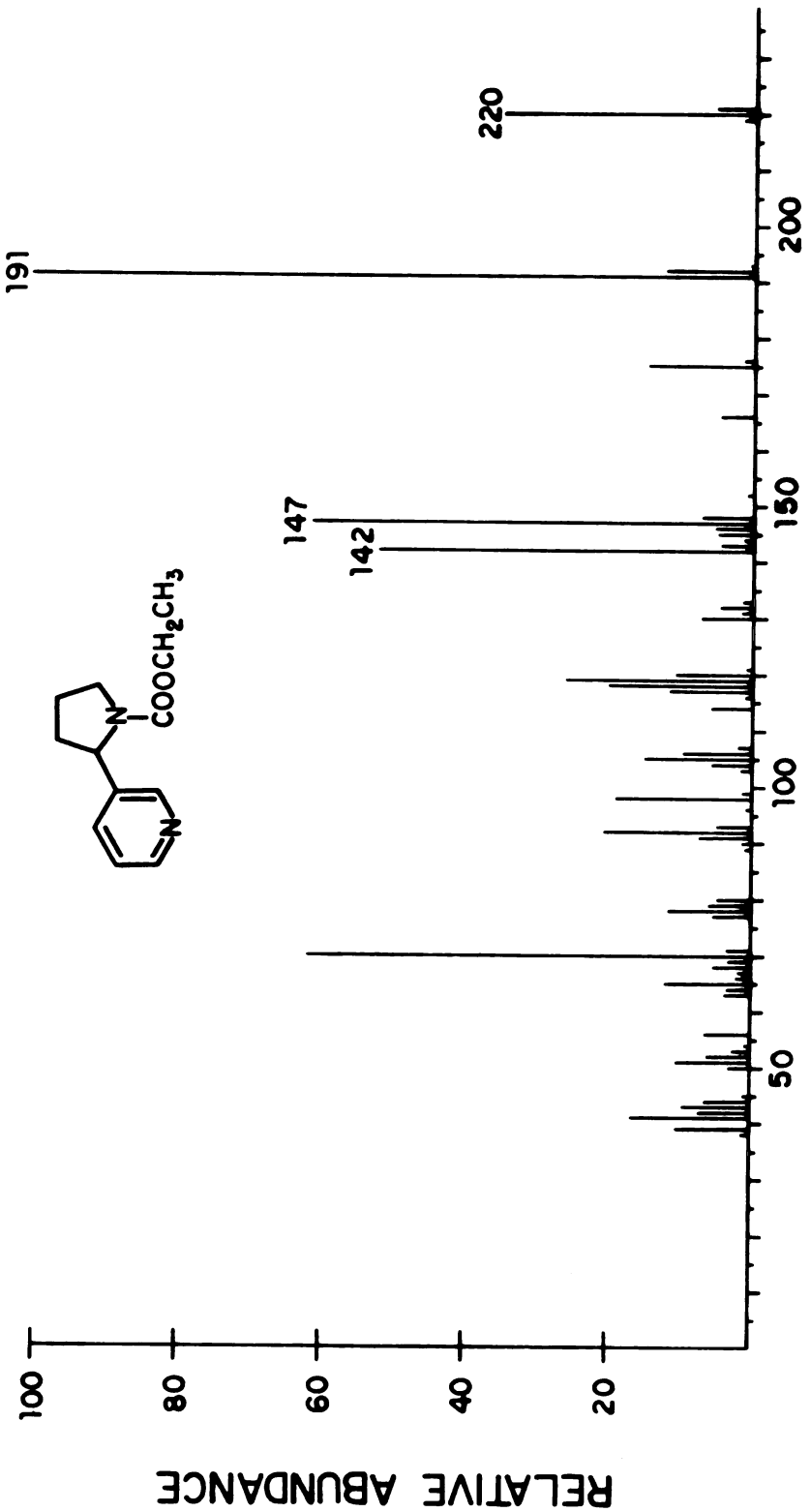
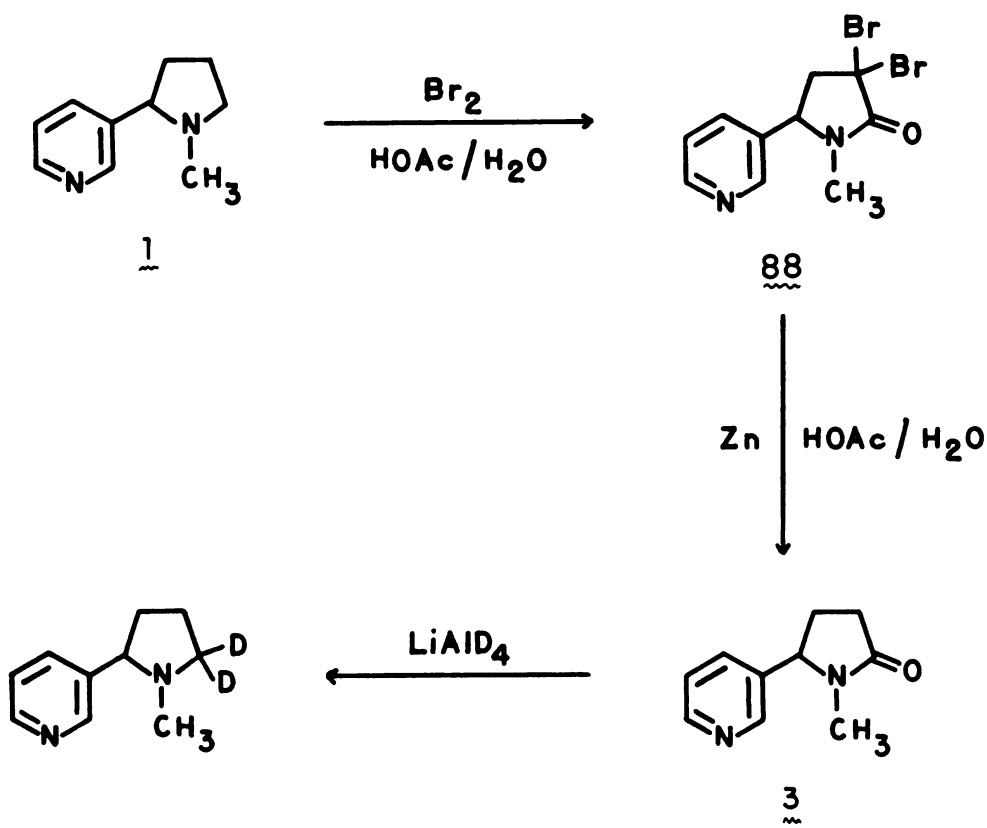


Figure XVII. The ei mass spectrum of N-ethoxycarbonylnornicotine.

The overall yield of nicotine obtained by $LiAlH_4$ reduction of 87 followed by short path distillation was 73%. When lithium aluminum deuteride was used, nicotine-N-methyl-d_3 was obtained. The eims of nicotine-d_0 and -N-methyl-d_3 agree with those reported in the literature.[111] The isotopic composition of nicotine-N-methyl-d_3 as determined by mass spectrometry was found to be 94% d_3 and 6% d_2 (see analysis later).

The synthesis of nicotine with deuterium atoms at C_5' was accomplished by oxidation of nicotine to cotinine[97] followed by reduction of cotinine with lithium aluminum deuteride.



1 88 3

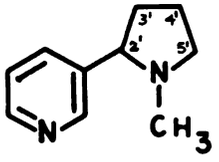
The exact mechanisms of the oxidative bromination of nicotine and the reductive debromination of 3,3-dibromocotinine (88) by zinc are not well understood. Therefore evidence that the three methyl protons and the protons at $C_{2'}$ and $C_{3'}$ in nicotine (C_5 and C_4 in cotinine) are not subject to loss or exchange with the medium during the course of the reaction was sought.

In one experiment the deuterium bromide perbromide salt of 3,3-dibromocotinine (88·DBr·Br_2) obtained from nicotine in deuterated media was converted to cotinine in protio solvents. In another experiment the perbromide salt made in protio solvents was converted to cotinine in deuterated solvents. The two samples of cotinine were analyzed by eims and by nmr for possible exchange of protons or deuterons.

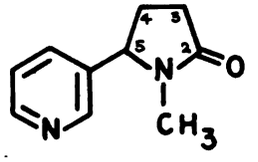
The spectral data of the first cotinine sample were identical to those of cotinine-d_0; no detectable incorporation of deuterium into the molecule had occurred. The ei mass spectrum and the nmr spectrum in $CDCl_3$ of the second cotinine sample, obtained from the debromination of 88 in deuterated solvents, is compared to those of cotinine-d_0 in Figures XVIII-XX. The nmr integration shows that two deuterium atoms had been introduced into the cotinine molecule and the mass spectrum shows a shift of two mass units in both the molecular ion and base peak. These spectra of dideuteriocotinine are identical to those of cotinine-

3,3-d_2 obtained from the deuterium exchange of cotinine in D_2O in the presence of K_2CO_3.[139] That the two deuterium atoms were incorporated into C_3 of cotinine can be seen by the nmr splitting pattern. The complicated nmr splitting pattern of the six protons on the pyrrolidinone ring of cotinine-d_0 (Figure XIX) has simplified into a typical splitting pattern of an ABX system:[140] four lines (2 doublets) at 4.68 ppm for the X proton on C_5 and eight lines (two of the lines are obscured by the N-CH_3 signal, 2 quartets) at 2.33 ppm and 2.20 ppm for the A and B protons on C_4. A first order analysis gives J_{AB} = 13 Hz, $\Delta\nu_{AB}$ = 12.5 Hz , J_{AX} = 8.5 Hz and J_{BX} = 6 Hz. As expected, 3,3-dibromocotinine (88) gives a similar nmr spectrum (Figure XX) with J_{AB} = 14 Hz, $\Delta\nu_{AB}$ = 13.5 Hz, J_{AX} = 8 Hz and J_{BX} = 6 Hz; δ_X = 4.75 ppm, δ_A = 3.38 ppm and δ_B = 3.25 ppm.

The mass spectrum shows that cotinine-3,3-d_2 obtained by reduction of 88 in deuterated solvents, was about 95% d_2 and 5% d_1. This enrichment at C_3 of cotinine can be enhanced to 99.8% d_2 by incubating further in heavy water in the presence of K_2CO_3.



1



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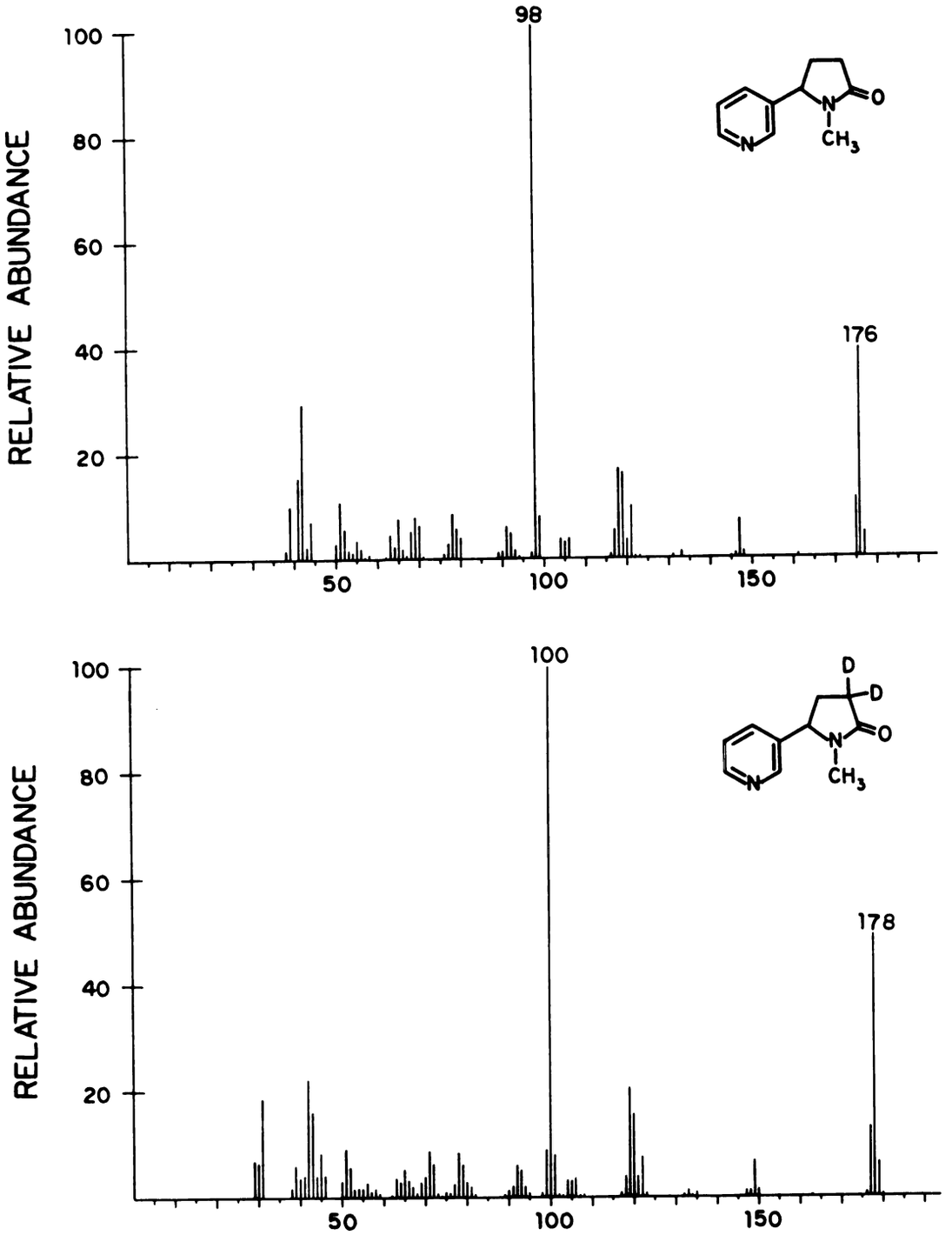


Figure XVIII. The ei mass spectra of cotinine-d_0 (top) and of cotinine-3,3-d_2 (bottom).

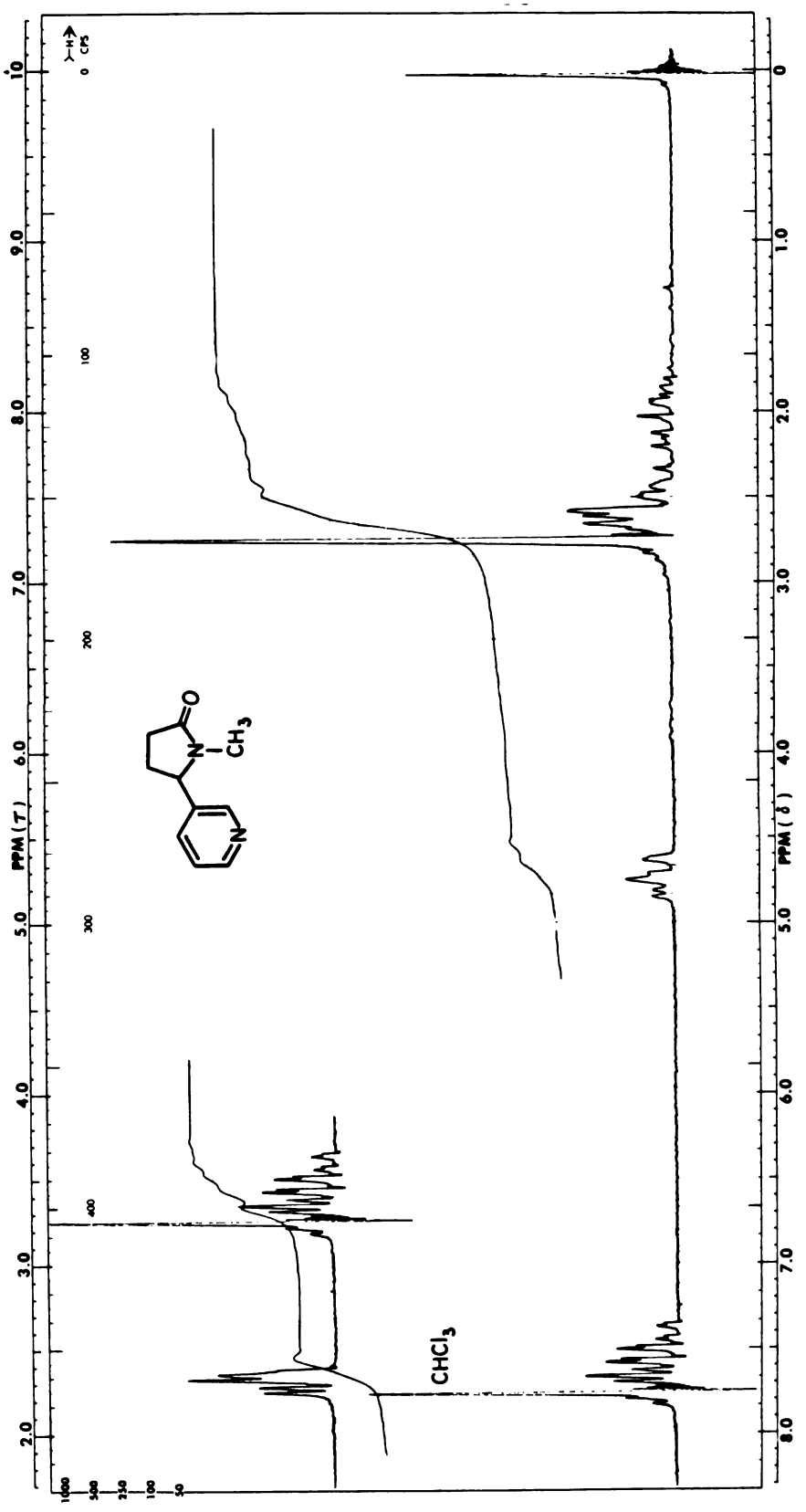


Figure XIX. The nmr spectrum of cotinine (60 MHz, $CDCl_3$, TMS).

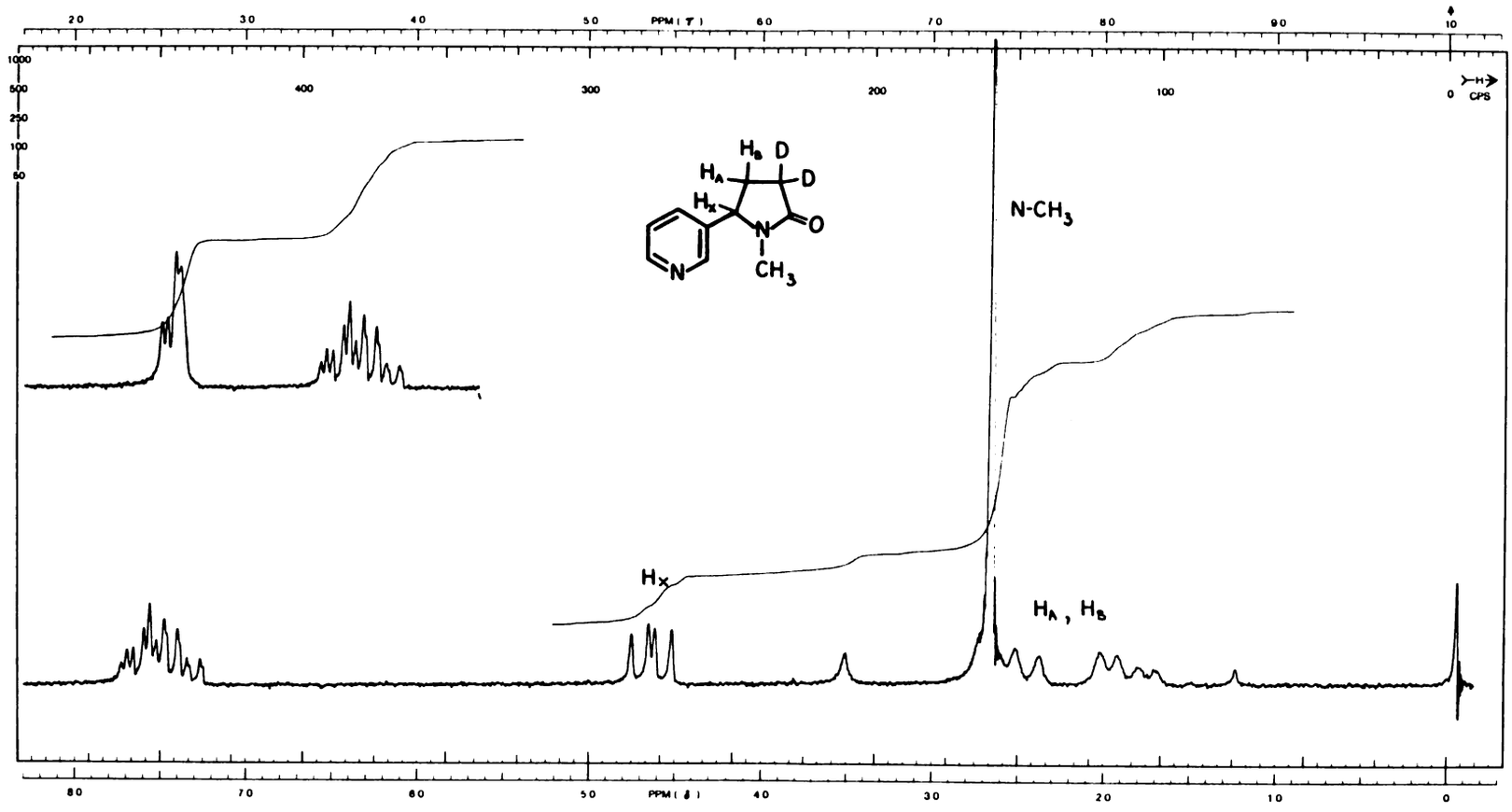


Figure XX. The nmr spectrum of cotinine-3,3-d_2 (60 MHz, $CDCl_3$, TMS).

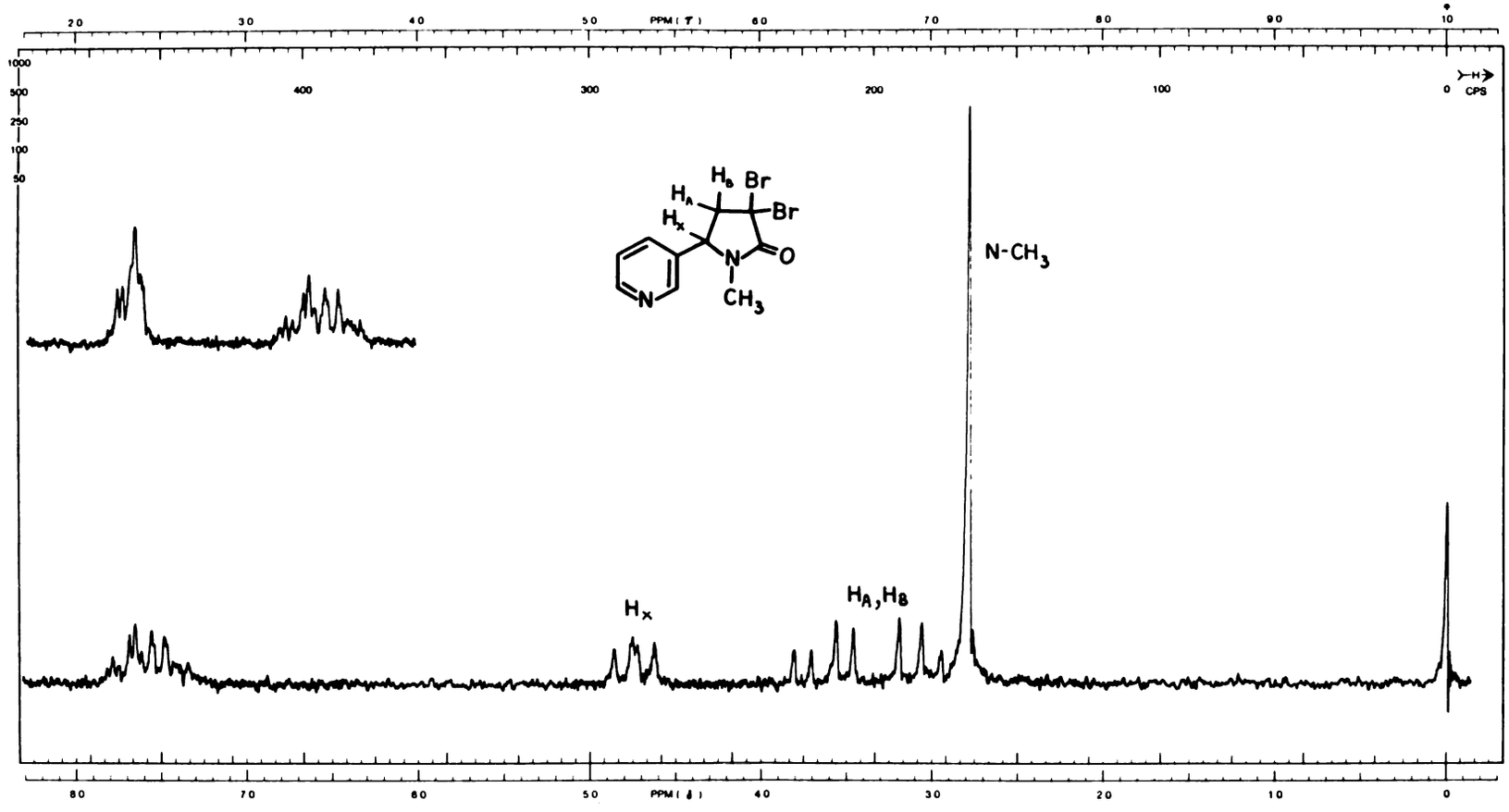
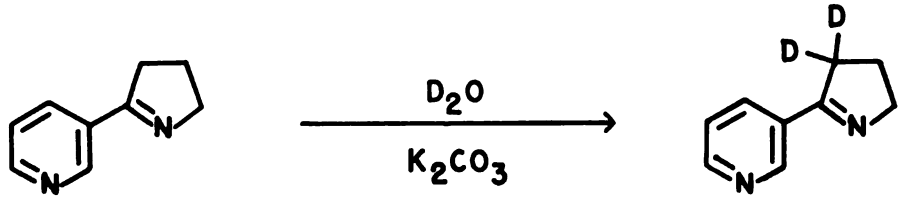
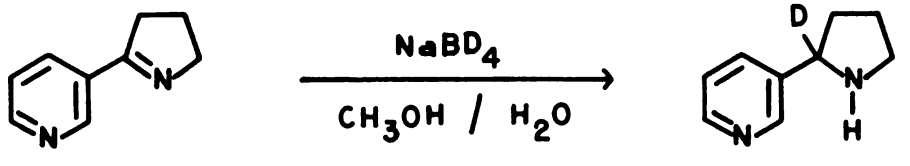


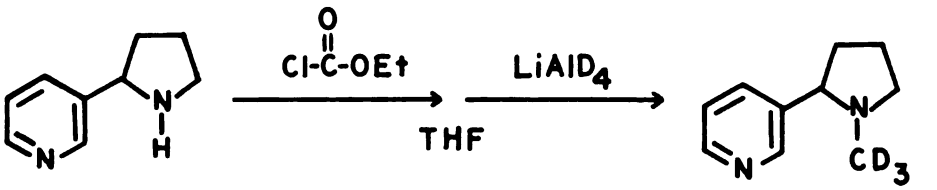
Figure XXI. The nmr spectrum of 3,3-dibromocotinine (60 MHz, $CDCl_3$, TMS).

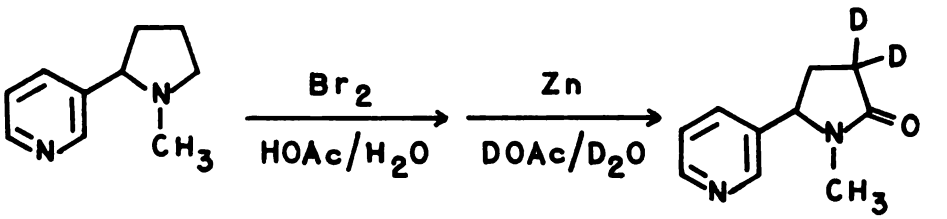
The two foregoing experiments indicate that only the protons at positions $C_{4'}$ and $C_{5'}$ of nicotine (C_3 and C_2 of cotinine) are affected in the two step conversion of nicotine to cotinine. Thus any deuterium labels already at $C_{2'}$, $C_{3'}$ or the N-methyl group of nicotine will be unaffected by the chemical conversions required to put labels at the $C_{4'}$ and $C_{5'}$ positions.

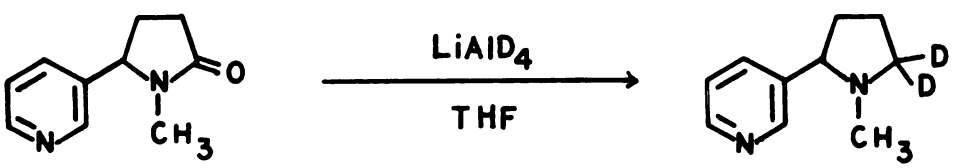
The above results together with those discussed earlier and with the lithium aluminum deuteride reduction of cotinine allow the synthesis of nicotine or cotinine labeled at any position on the five membered ring moiety. The reactions below illustrate the key steps.









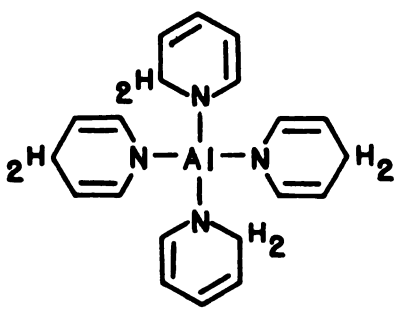


Thus all the deuterated nornicotines and nicotines used in the metabolic studies were prepared by one or a combination of these steps.

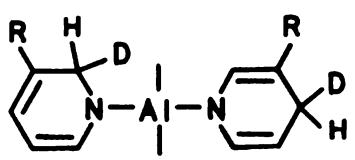
Unfortunately, there is a slight complication in the syntheses of deuterated nicotines. It was observed that in any step involving a lithium aluminum deuteride reduction, a slight excess of the expected amount of deuterium incorporation was obtained. It was found that this is due to a reaction of $LiAlD_4$ with the pyridine portion of the molecule.

Lansbury[141] observed that when $LiAlH_4$ was dissolved in pyridine at room temperature the solution immediately turned orange. Nmr and ir spectra of this solution indicated that there were no Al-H bonds but there were signals corresponding to 1,2- and 1,4-dihydropyridine groups bonded to aluminum. Thus this worker concluded that pyridine reacts with aluminum hydride to form a mild reducing agent, tetrakis-(N-1,2- or N-1,4-dihydropyridyl) aluminate (89a).

In the reduction of N-carbethoxynornicotine to nicotine or of cotinine to nicotine, a yellow or orange color was also observed when these compounds were introduced into the $LiAlH_4$ or $LiAlD_4$ suspension in THF. It seems likely that the pyridine portion of these molecules can also react with aluminum deuteride to form a complex (89b) similar to that formed by pyridine itself.



89a



89b

Apparently, this complex is formed and is subsequently air oxidized to regenerate the original pyridine compound. The net result is a partial incorporation of a deuterium into the pyridine moiety.

When nicotine-d_0 was incubated with $LiAlD_4$ in THF, a similar partial incorporation of deuterium was observed. Figure XXII shows the nmr spectra of the pyridyl protons of nicotine-d_0 and the nicotine recovered from the reaction with $LiAlD_4$ in THF.

In the nmr spectrum of nicotine-d_0 the proton D is split into two triplets since it has one close neighbor C (J_{DC} = 8 Hz) and two far neighbors A and B ($J_{AD} = J_{BD}$ = 2 Hz). In the spectrum of the partially deuterated sample this signal has collapsed into two doublets which indicates that one of D's far neighbors has been replaced by a deuterium atom. The signal for proton C in nicotine-d_0 shows two doublets with the coupling constants expected for a 3-pyridyl proton with two close neighbors D (J_{CD} = 8 Hz) and B (J_{CB} = 4.8 Hz).[109] These signals show additional fine splitting due to the proton A (J_{AC} = 1 Hz). In the spectrum of the partially deuterated sample only the fine splitting has been affected which indicates that it is proton A which has been replaced. Although the signals for proton B are less obscured by the signal for proton A in the partially deuterated sample, no other change is seen in the two doublets for proton B (J_{BC} = 4.8 Hz, J_{BD} = 2 Hz) in

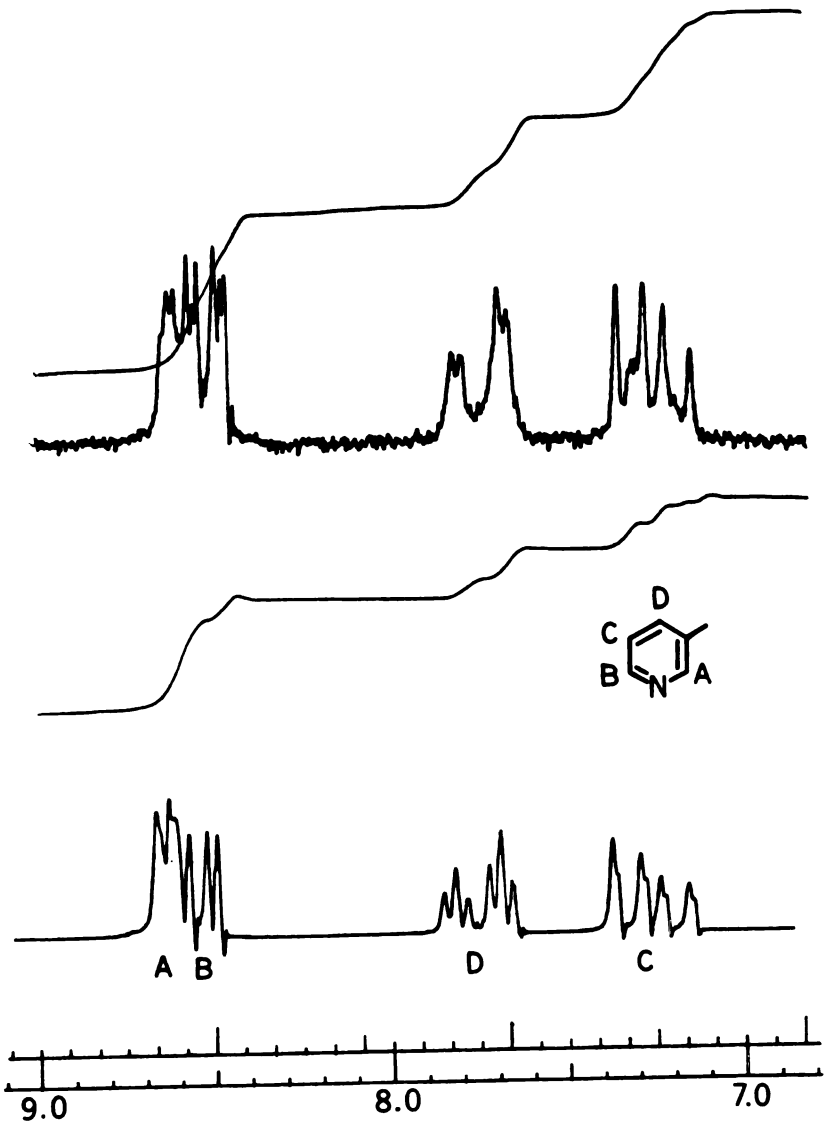


Figure XXII. The nmr spectra (60 MHz, $CDCl_3$, TMS) of the pyridyl protons of nicotine-d_0 (bottom) and of nicotine recovered from the reaction of nicotine-d_0 (top) with $LiAlD_4$ in THF.

going from the nmr spectrum of nicotine-d_0 to that of the partially deuterated nicotine. This is due to the lack of any coupling between protons A and B. There is an obvious loss in the intensity of the signal for proton A in the partially deuterated sample. The integration confirms this; the ratio of A+B : D : C : remaining H's is 1.2 : 0.7 : 0.8 : 10, which indicates there is a slight replacement of deuterium for hydrogen at the other positions as well. Thus the nmr splitting pattern and integration indicates that it is largely the proton at $C_{2'}$ which is replaced. This excess deuterium incorporation in the synthetic samples amounted to as much as 20%. Fortunately, this extra deuterium incorporation does not interfere with the mass spectral analyses since we were able to rely on the fragment ions arising from the loss of the pyridine moiety.

The ei mass spectra of nicotine-d_0, nicotine-5',5'-d_2, nicotine-2',5',5'-d_3 and nicotine-N-methyl-d_3 are shown in Figures XXIII and XXIV and the intensities of important m/e peaks in the mass spectra are shown in Table IV.

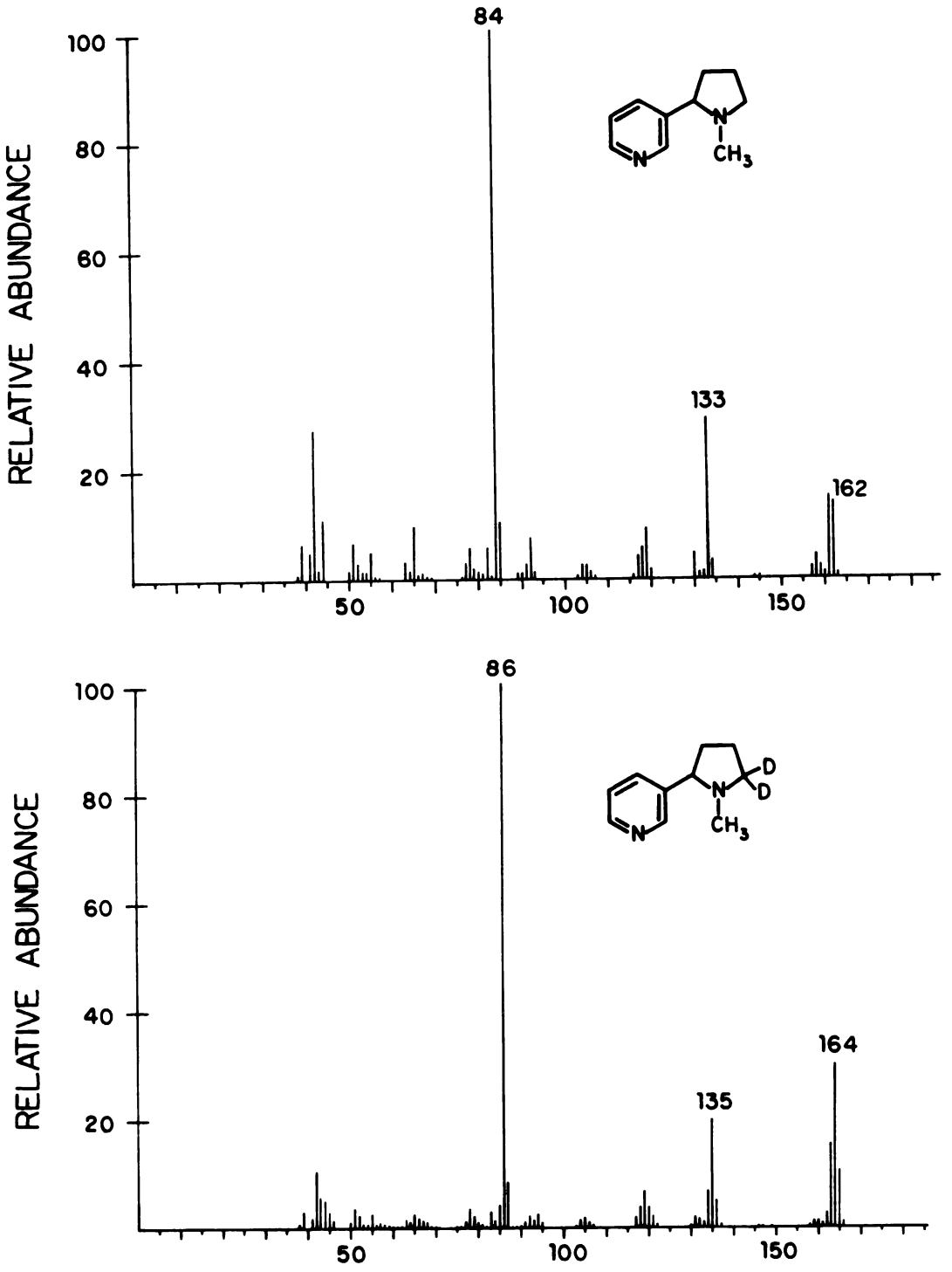


Figure XXIII. The ei mass spectra of nicotine-d_0 (top) and of nicotine-5',5'-d_2 (bottom).

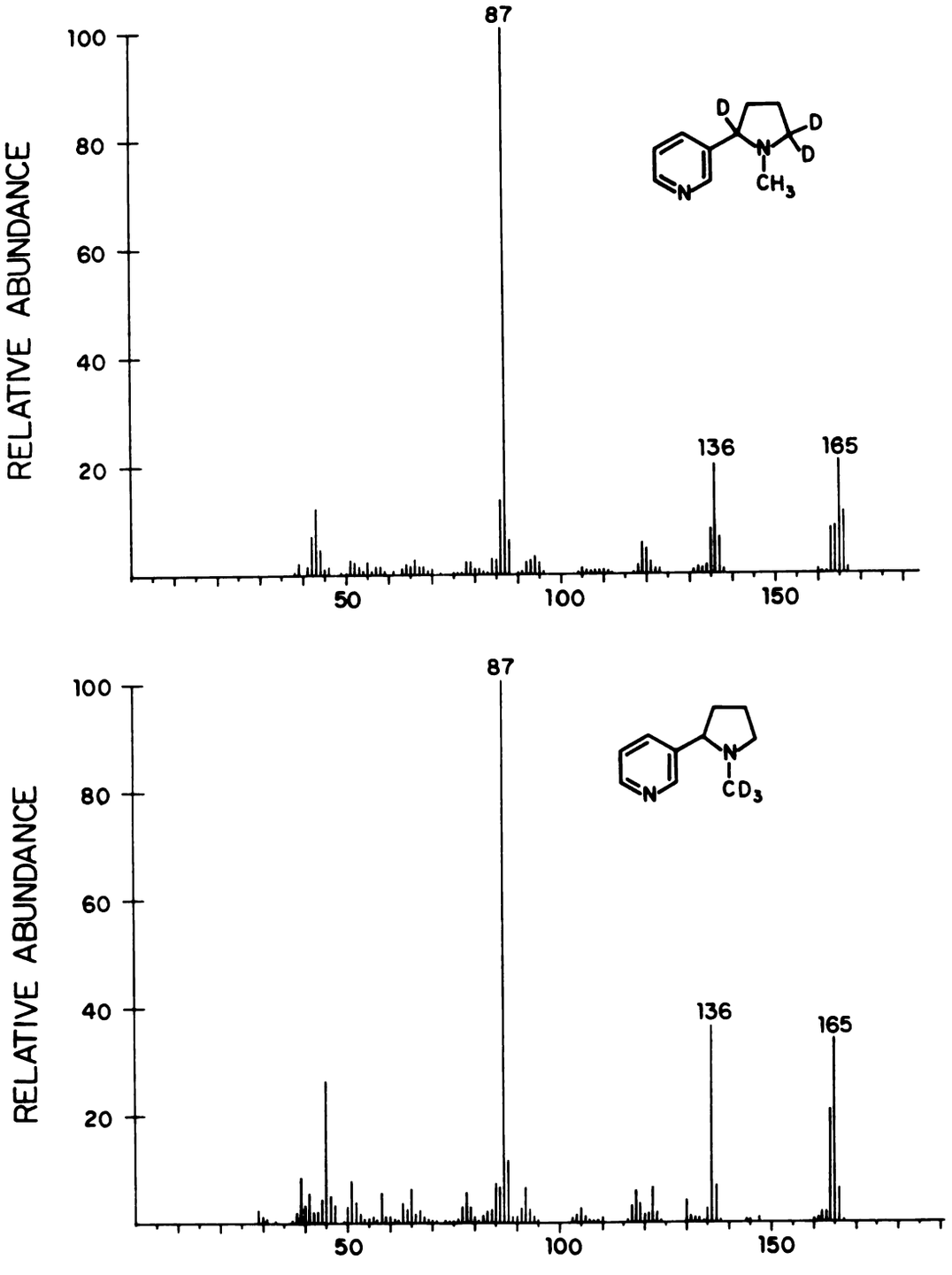
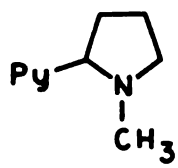
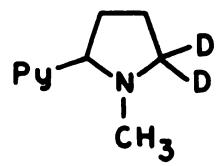


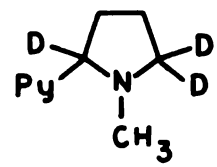
Figure XXIV. The ei mass spectra of nicotine-2',5',5'-d_3 (top) and of nicotine-N-methyl-d_3 (bottom).

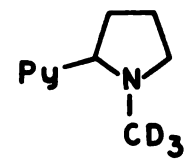
Table IV

Relative intensities of important m/e peaks in the ei-mass spectra of nicotines.

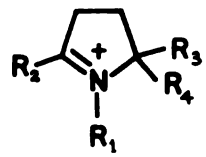








d_0		5',5'-d_2		2',5',5'-d_3		N-methyl-d_3	
m/e	%	m/e	%	m/e	%	m/e	%
162	13.8	164	30.8	165	20.7	165	33.8
161	14.6	163	12.3	164	8.5	164	20.7
		162	1.6	163	8.0	163	1.5
				162	0.1	162	1.5
133	29.2	135	20.7	136	20.0	136	36.0
84	100.0	86	100.0	87	100.0	87	100.0
83	0.6	85	4.6	86	13.8	86	6.7
82	6.0	84	1.5	85	2.7	85	9.0
81	1.2	83	3.0	84	3.0	84	1.6



90 (m/e 84) $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$

90a (m/e 87) $R_1 = CD_3$, $R_2 = R_3 = R_4 = H$

90b (m/e 86) $R_1 = CH_3$, $R_2 = H$, $R_3 = R_4 = D$

90c (m/e 87) $R_1 = CH_3$, $R_2 = R_3 = R_4 = D$

The base peak in the spectrum of nicotine-d_0 occurs at m/e 84 due to the loss of the pyridine moiety to form the pyrrolidinium ion 90. According to Duffield, et al.[111] who obtained nicotine-N-methyl-d_3 through the methylation of nornicotine with CD_3I, the m/e 84 peak is quantitatively transferred to the ion at m/e 87 (100%, 90a) in the eims of the -N-CD_3 species. Thus the extent of deuteration can be calculated directly from the mass spectrum using the base peak.

In the mass spectrum of nicotine-d_0, the loss of a proton from the fragment 90 to give m/e 83 was 0.6%. Therefore the peak at m/e 86 (6.7%) in the spectrum of nicotine-N-methyl-d_3 indicates that there was about 6.0% of nicotine-N-CD_2H present. This suggests that each deuterium in the N-CD_3 moiety has a 2% chance of being a hydrogen which is consistent with the isotopic purity of the lithium aluminum deuteride used (98%). From this reasoning it is apparent that the probability of two deuterium atoms being replaced by hydrogen is very small. The following distribution was calculated assuming a random 2% replacement of

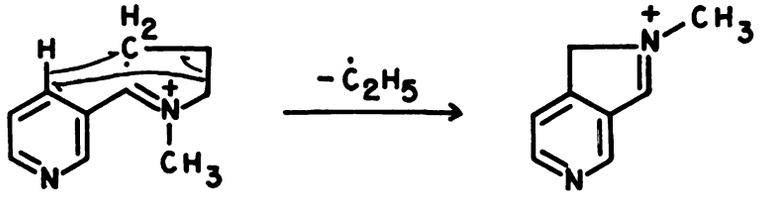
hydrogen for deuterium:

Nicotine-N-CD_3	94.%
Nicotine-N-CD_2H	5.7%
Nicotine-N-CDH_2	0.12%
Nicotine-N-CH_3	0.008%

The eims of nicotine-5',5'-d_2 showed an m/e 85 to m/e 86 (90b) ratio of 4.6%, which again is consistent with the isotopic purity of the $LiAlD_4$ used (98%D). Thus the isotopic composition of nicotine-5',5'-d_2 is 96% d_2 and 4% d_1. However the eims of nicotine-2',5',5'-d_3 showed ratios of m/e 84, m/e 85 and m/e 86 to m/e 87 (90c) of 3.0%, 2.7% and 13.8% respectively. It seems statistically unlikely that there is really 3% of the d_0 species present and probably the m/e 82 (6%) ion of nicotine-d_0 has shifted partially to m/e 84 and partially to m/e 85 in nicotine-2',5',5'-d_3.

The m/e 133 ion in the eims of nicotine-d_0 is quantitatively transferred to m/e 136 in the eims of nicotine-N-methyl-d_3. However, in the case of nicotine-5',5'-d_2 and -2',5',5'-d_3 only about 65% of the m/e 133 ion is transferred to m/e 135 and m/e 136 respectively. This result is similar to those obtained by Duffield, et al.[111] These authors also reported that the m/e 133 ion did not shift in the eims of nicotine-4',4'-d_2. This information rules out the loss of the nitrogen atom with

its attached methyl group as a source of the m/e 133 ion. The formation of this ion can be explained in terms of the loss of an ethyl radical composed of $C_{3'}$ and $C_{4'}$ together with an extra hydrogen from the pyridine moiety. A possible structure for this m/e 133 ion was proposed[111] as follows:

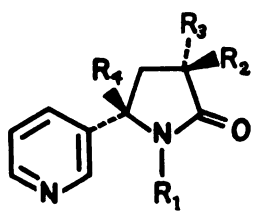


m/e 161 m/e 133

B. The case of 5-hydroxycotinine (52), a cotinine metabolite.

The following sections of this dissertation will deal with the metabolism of nicotine and nornicotine by metabolic hydroxylation at the carbon atoms bonded to the pyrrolidinyl nitrogen. As part of our studies on α hydroxylated metabolites of tobacco alkaloids we have also investigated the structure of a previously reported cotinine metabolite.

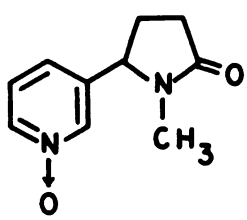
Cotinine (3), the major mammalian metabolite of nicotine, has been reported to be metabolized by both N-oxidation and by α-carbon oxidation. The known metabolites are cotinine-N-oxide (54),[90] cotinine isomethonium ion (53),[89] demethylcotinine (49),[78,80-84] trans-3-hydroxycotinine (50)[68,85,86] and a metabolite resulting from hydroxylation at C_5.[88] The structure of this metabolite is the subject of discussion in this section.



3 : $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$

49 : $R_1 = R_2 = R_3 = R_4 = H$

50 : $R_1 = CH_3$, $R_2 = OH$, $R_3 = R_4 = H$



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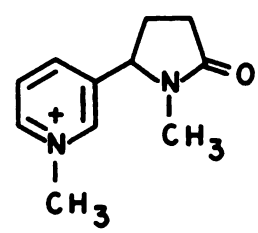
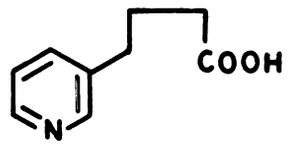


53

In 1959, McKennis and his coworkers[87] obtained a urinary metabolite from dogs treated with cotinine. This metabolite was shown to be amphoteric. Upon hydrolysis, it yielded methylamine and the oxo-acid 91b which was mistakenly assigned the structure 91a. This acid upon Wolff-Kishner reduction gave γ-(3-pyridyl)butyric acid (92). The metabolite and the oxo-acid were also shown to form oximes with hydroxylamine. Unfortunately, the structures of these oximes were also incorrectly assigned as 93a and 94a.



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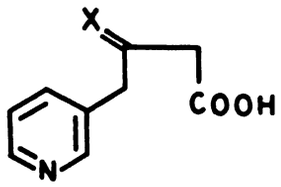
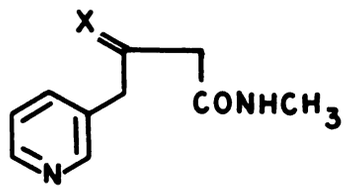


91a : X = O

94a : X = NOH

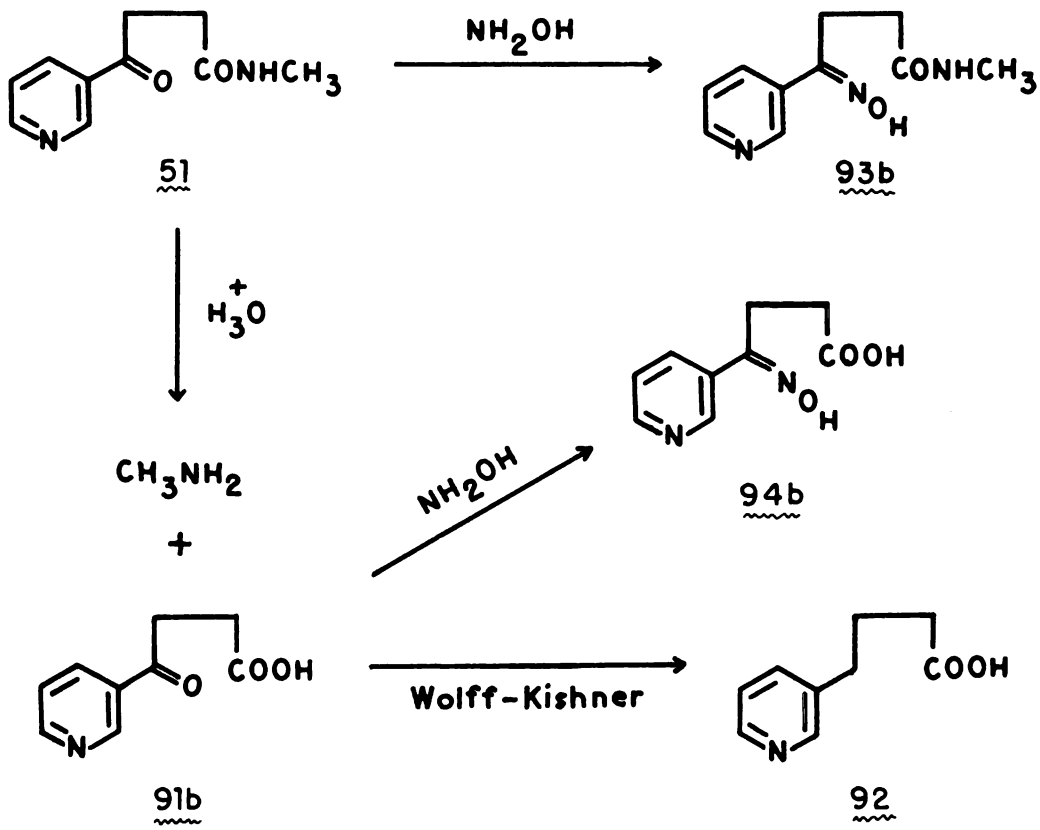


93a : X = NOH

95 : X = O

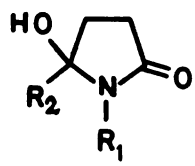
Thus on the basis of this information the metabolite was originally assigned the incorrect structure γ-(3-pyridyl)-β-oxo-N-methylbutyramide (95). In 1963[88] McKennis corrected the mistaken structure assignments

for the degradation products of the metabolite and amended the structure of the metabolite to γ-oxo-N-methylbutyramide 51. This assignment was confirmed by the synthesis of the metabolite from synthetic γ-(3-pyridyl)-γ-oxobutyric acid 91b and methylamine.



In 1965, McKennis and coworkers[142] suggested, with little experimental justification, that the ketoamide 51 might exist in equilibrium with the cyclic form 5-hydroxycotinine (52). However they still preferred the open chain ketoamide 51 because the metabolic sample they obtained showed the properties of the open chain structure including the

ability to form an oxime.



52 : $R_1 = CH_3$, R_2 = 3-pyridyl

96a : $R_1 = R_2 = CH_3$

96b : $R_1 = CH_3$, R_2 = phenyl

Later Dagne[143] studied the metabolism of cotinine in the monkey and in rats and provided evidence supporting the hydroxylactam structure 52 rather than the ketoamide 51 for the same metabolite isolated by McKennis. Dagne also was able to synthesize the metabolite 5-hydroxycotinine (52).[143] He reported that the ir spectrum showed only one sharp intense band in the carbonyl stretching region near 1700 cm^{-1}. By comparing the literature ir spectra of compounds 96a and 96b[144] with the corresponding spectrum of the cotinine metabolite, Dagne assigned this band to a lactam carbonyl rather than an amide carbonyl.

Dagne also observed that the nmr spectrum of this compound in $CDCl_3$ showed no splitting of the nitrogen methyl protons (see Figure XXV) by the nitrogen proton whereas secondary N-methylamides in general do display such coupling.[109] Therefore he tentatively concluded that the metabolite did not bear an $-NHCH_3$ group.

However when Dagne's in vivo experiments with monkey were repeated

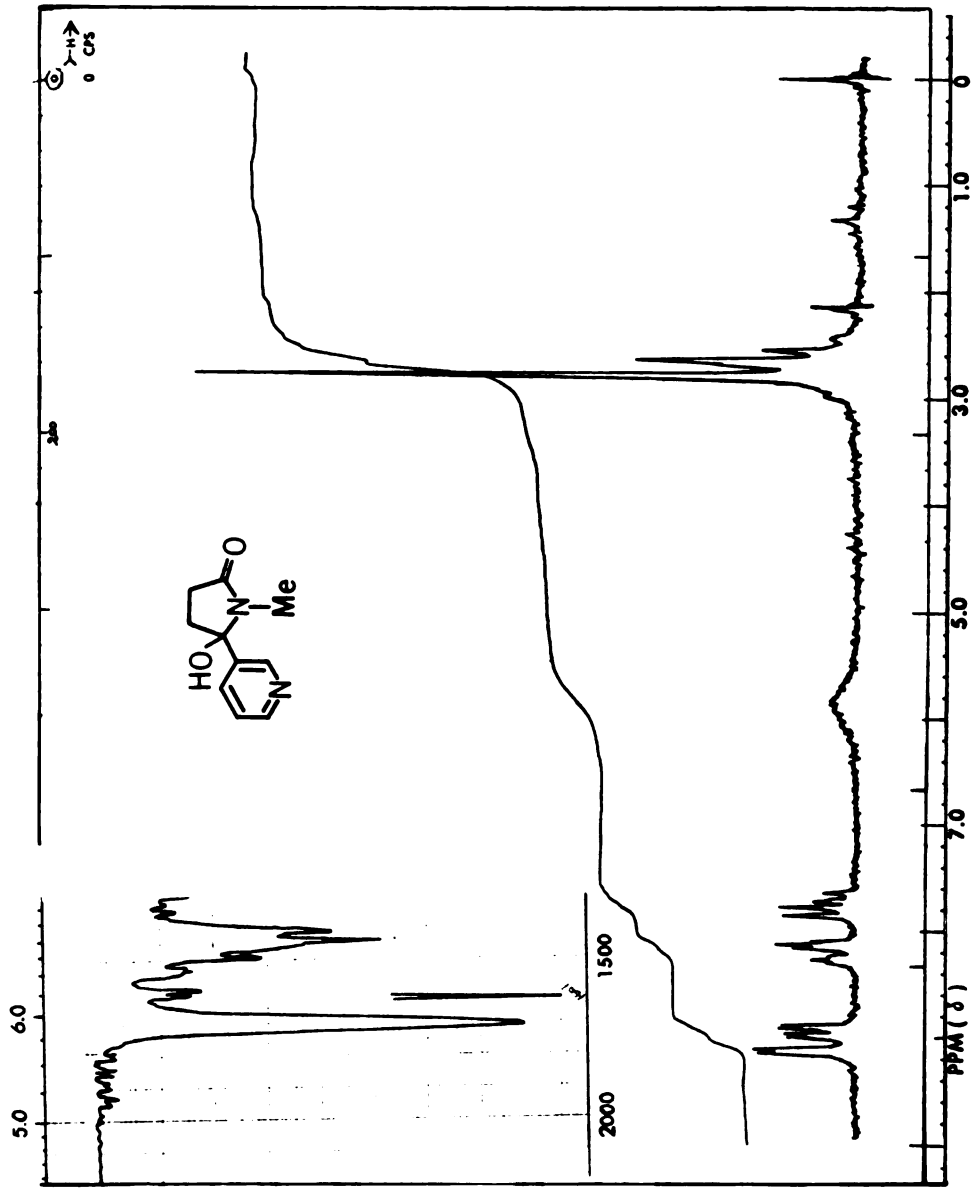


Figure XXV. The ir spectrum ($CHCl_3$) and the nmr spectrum (60 MHz, $CDCl_3$, TMS) of 5-hydroxycotinine as reported by Dagne.

by other workers in our laboratory,[145] in only one experinent was the mass spectrum of this metabolite the same as that reported.[143] In three other trials new major peaks appeared in the mass spectra in addition to those seen in Dagne's spectrum. This led us to retake the eims of Dagne's synthetic 5-hydroxycotinine sample. The spectrum obtained no longer resembled that presented in his dissertation but it did agree with the spectra of the metabolite obtained from the more recent metabolic work. The nature of these differences suggested the possibility of a slow equilibrium between the open and closed forms of 5-hydroxycotinine.

In order to investigate this possible equilibrium more fully we undertook a more careful study of the structure of synthetic 5-hydroxycotinine. We began this study by synthesizing 5-hydroxycotinine by the method of Dagne as shown in Figure XXVI.

Commercially available (S)-nicotine was converted into (S)-3,3-dibromocotinine (88). In methanolic KOH this compound undergoes an interesting series of reactions to yield 5-methoxy-5-(3'-pyridyl)-2-pyrrolinone (97). The methyl group was cleaved in aqueous hydrobromic acid. Catalytic hydrogenation of 5-hydroxy-5-(3'-pyridyl)-2-pyrrolinone (98) gave 5-hydroxycotinine (52) as a white crystalline solid.

The infrared spectrum in $CHCl_3$ of this freshly made material is

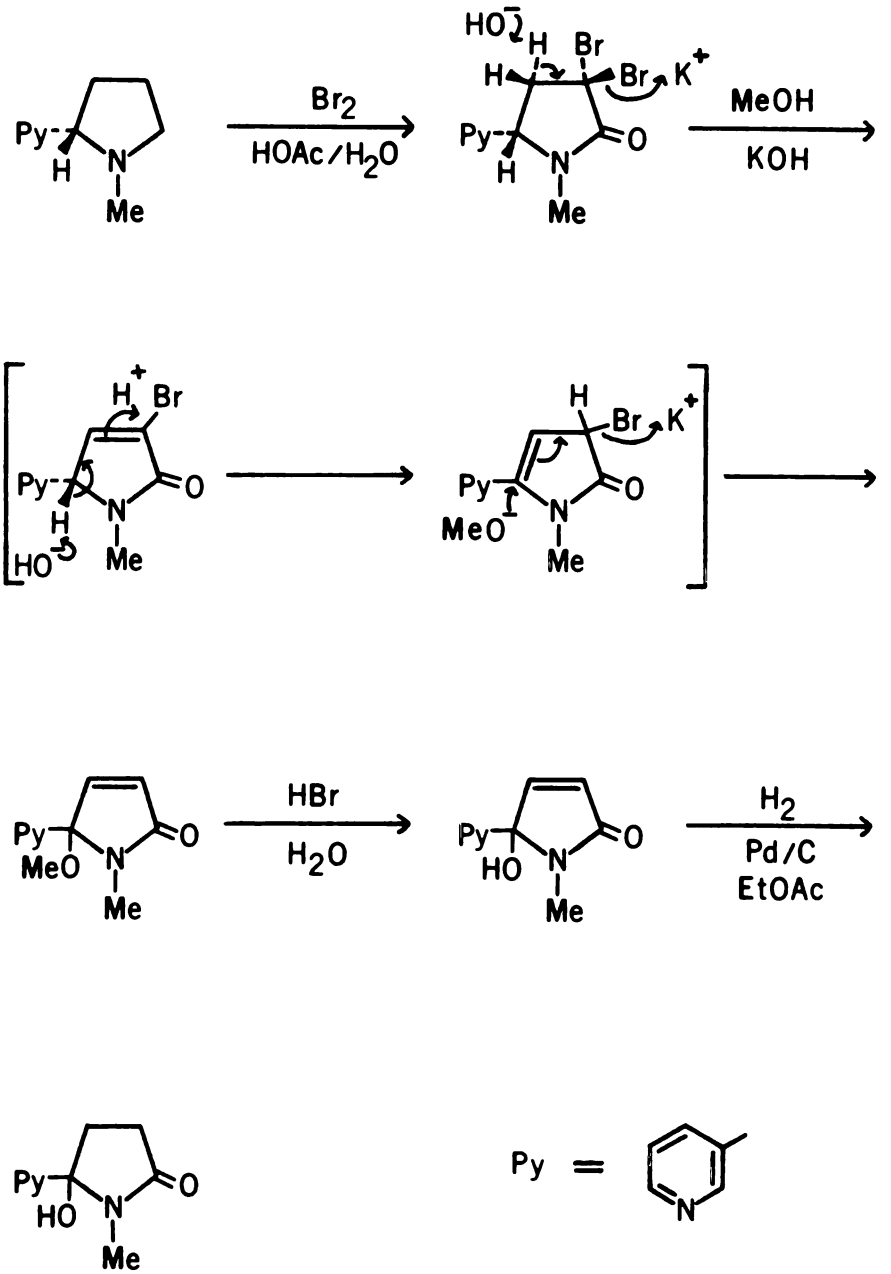


Figure XXVI. Synthetic scheme for the synthesis of 5-hydroxycotinine.

superimposable on the spectrum obtained by Dagne for both the synthetic and metabolic samples. A broad band which appears at 3300 cm^{-1} is difficult to assign because a broad absorption in this particular region can arise from a stretching vibration of an intermolecularly hydrogen bonded OH in "polymeric structures".[109,146] It can also arise from a stretching vibration of a hydrogen bonded secondary amide NH.[109,146] There is one intense absorption band at 1685 cm^{-1} assigned to an amide carbonyl stretching vibration. The absence of a second carbonyl absorption band between 1625-1750 cm^{-1} and the absence of a NH bending vibration in the region 1550-1510 cm^{-1} seems to rule out the presence of the open ring structure 51. This conclusion was further supported by the presence of a sharp single, band of medium intensity at 1430 cm^{-1} which is assigned to the in plane bending vibration of OH in a tertiary alcohol.[109,146]

The uv spectrum displays a broad absorption centering at 300 nm (ε 385) which may be assigned to a carbonyl chromophore directly attached to an auxochrome ($-N-CH_3$)[146] and three fused absorption bands at 265 nm (ε 960), 258 nm (ε 1270) and 253 nm (ε 1150) characteristic of an isolated pyridine ring.[146] It does not seem that there is any significant amount of the open ring structure present since a carbonyl chromophore directly connected to an aromatic system will cause a shift of the

aromatic absorptions and also of its own absorption toward the red.[146,147]

The nmr spectrum in $CDCl_3$ of the freshly prepared synthetic sample was identical to that reported by Dagne; no splitting of the N-methyl group was observed. The eims of the freshly prepared synthetic sample was also identical to that reported by Dagne. However, although the fragmentation observed can be rationalized as the closed ring structure it is more consistent with the open ring structure. The difficulties in interpreting the eims will be discussed further below.

Thus the spectral data largely supports the lactam structure 52. Furthermore in N,O-bis-(trimethylsilyl)-acetamide/pyridine this compound forms a TMS derivative whose eims shows the following major m/e peaks:

m/e 249	$M^+ - 15$	(10.4%)
m/e 186	$M^+ - 78$	(100%)
m/e 175	$M^+ - 89$	(55.2%)

The prominent ion at m/e 175 is best explained as a loss of trimethylsilyloxy ($M^+ - OSiMe$), a loss which is likely to occur only from the TMS derivative of the closed ring compound 52.

However the synthetic 5-hydroxycotinine upon standing for several weeks changed from a white crystalline solid to a colorless gum. Changes were also observed in the eims and in the nmr spectrum (see below). The changes observed in the eims are summarized in Table V.

Table V

A comparison of the electron impact mass spectra of freshly prepared and aged samples of synthetic 5-hydroxycotinine.

m/e	fresh-%	aged-%
192	2.0	14.5
175	8.3	10.0
174	29.1	16.6
163	38.5	20.8
134	17.7	16.6
114	25.0	100.0
106	100.0	71.8
86	22.9	17.7
79	50.4	81.2
78	100.0	49.5
58	45.8	25.0

Figure XXVII shows the eims fragmentation patterns which might be expected for 51 and 52 on the basis of their structures and by comparison to other nicotine related alkaloids. Unfortunately the expectations described in Figure XXVII are not consistent with the observations of Table V. In particular there seems to be little doubt in the assignments of the base peaks, yet these assignments would argue that the freshly prepared sample is the open form 51 rather than the closed ring form 52 as previously thought. On the other hand loss of H_2O from the molecular ion (M^+ - 18, m/e 174) of the open ring structure 51 is extremely difficult to rationalize while the same loss from the closed ring structure 52 is quite reasonable. This would argue that the initial assignment of the freshly prepared sample as the closed ring structure 52 was correct after all. Most of the other fragment ion intensities listed in Table V with the possible exception of m/e 78 and m/e 79 ions are consistent with the structure assignments derived from the base peaks. Thus the bulk of the eims evidence would seem to argue against the closed ring structure 52 for the freshly prepared sample.

On the other hand the changes observed in the nmr argue for the closed ring structure 52 as the structure of the freshly prepared sample. Figures XXVIII and XXIX show the nmr spectra in $CDCl_3$ of the freshly prepared and aged samples. When an aqueous solution of freshly prepared

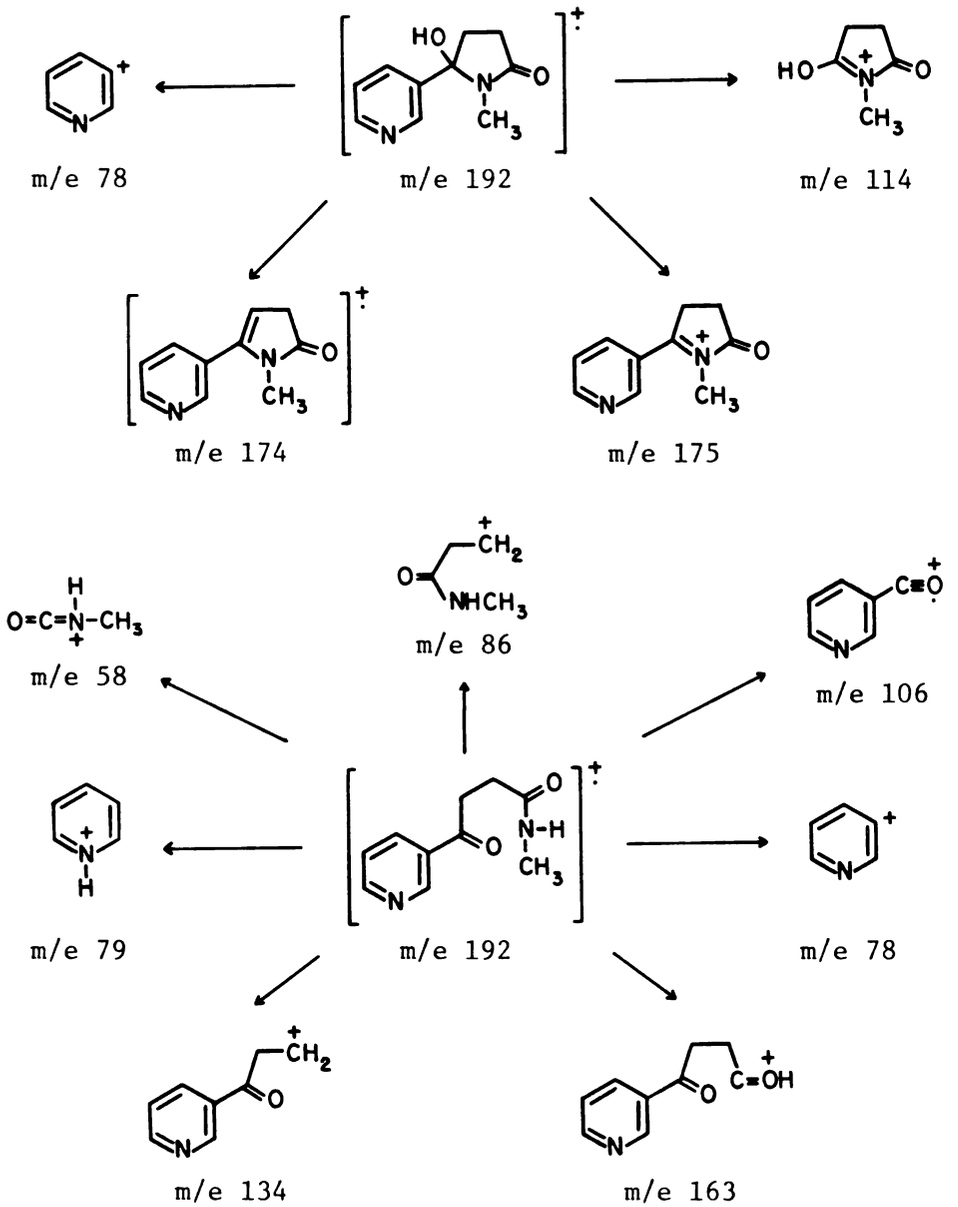


Figure XXVII. The theoretical mass spectral fragmentation for 5-hydroxycotinine and γ-(3-pyridyl)-γ-oxo-N-methylbutyramide.

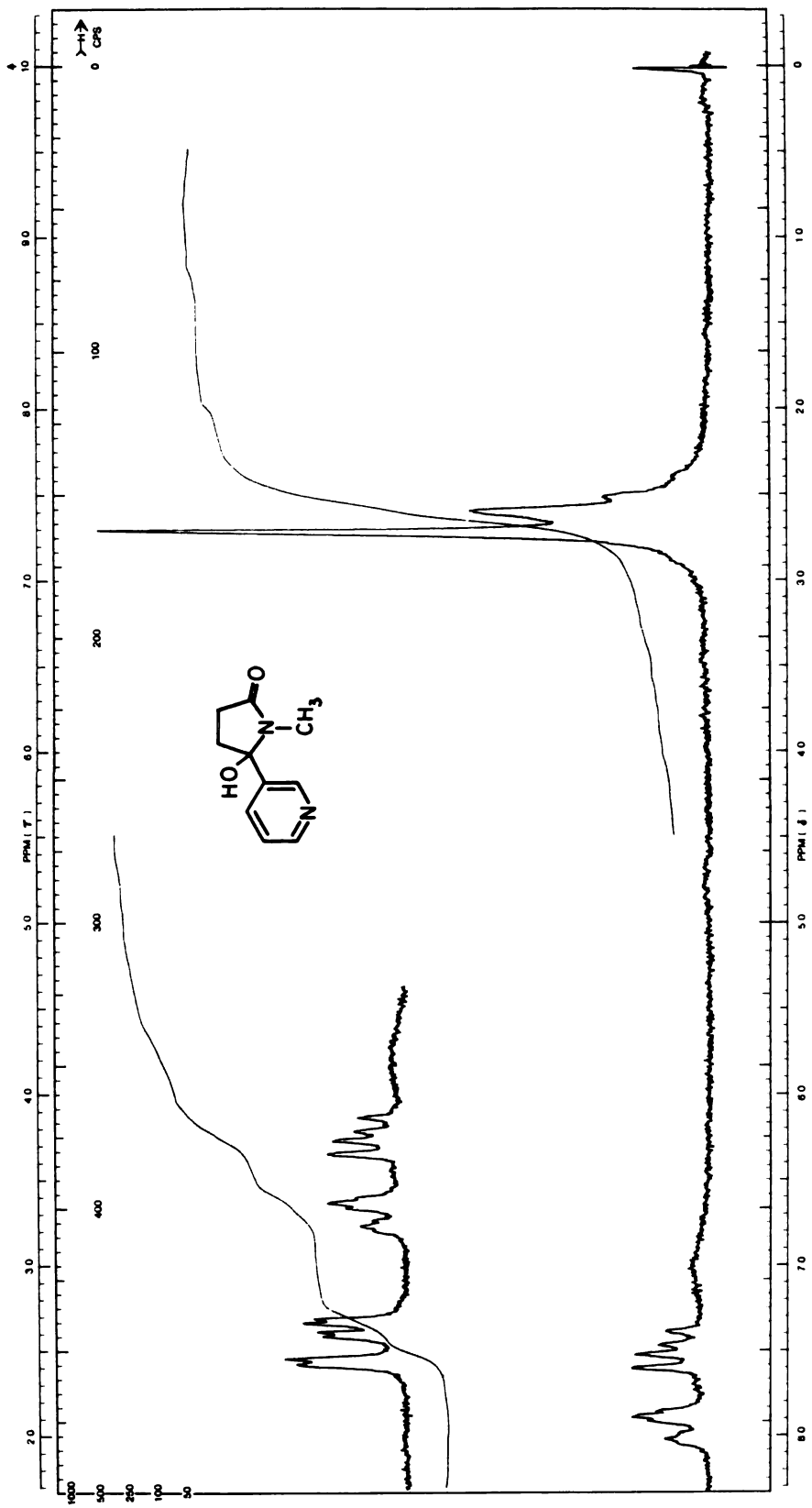


Figure XXVIII. The nmr spectrum of freshly prepared 5-hydroxycotinine (60 MHz, $CDCl_3$, TMS).

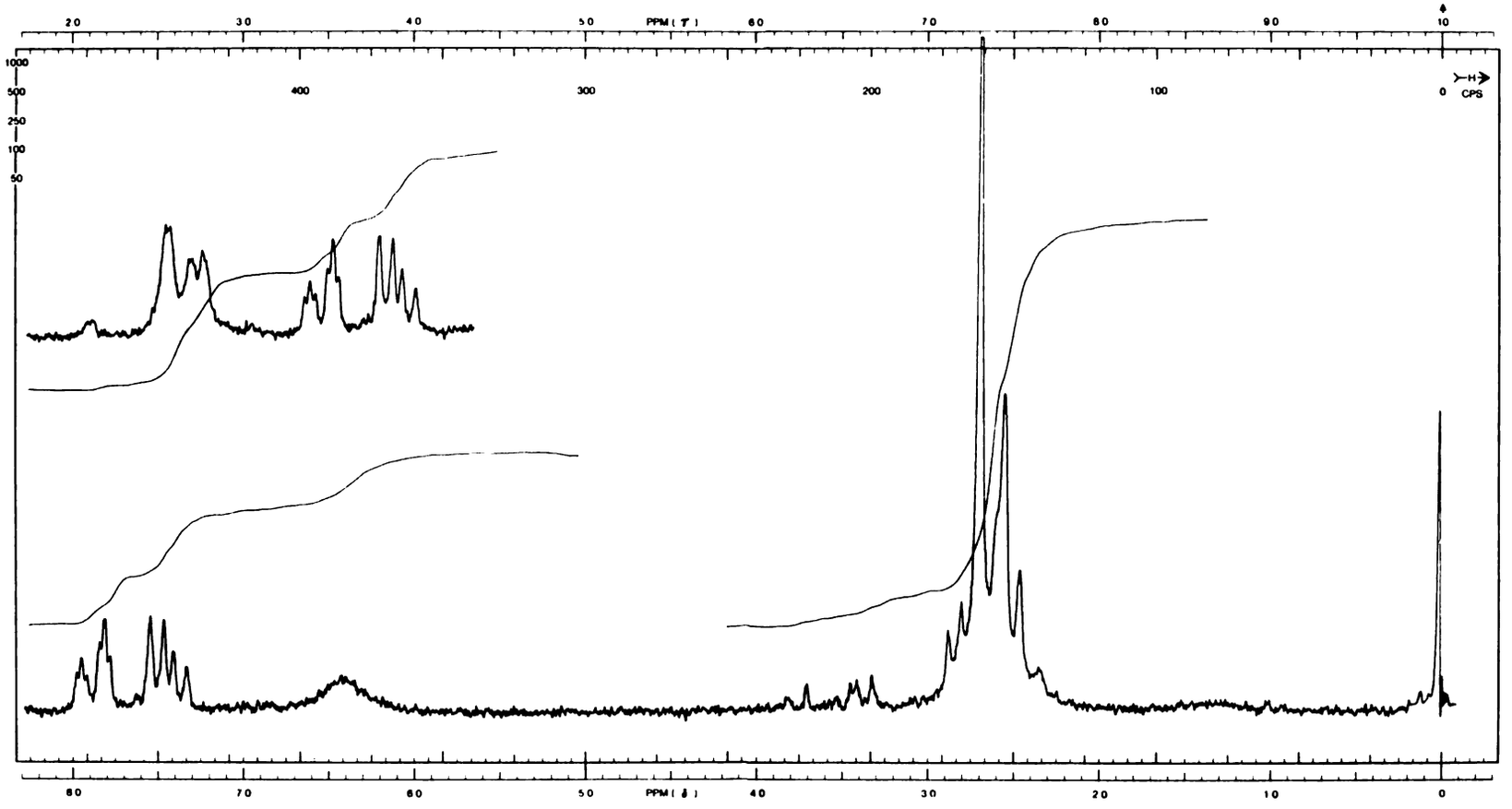


Figure XXIX. The nmr spectrum of an aged sample of 5-hydroxycotinine in $CDCl_3$ (TMS, 60 MHz).

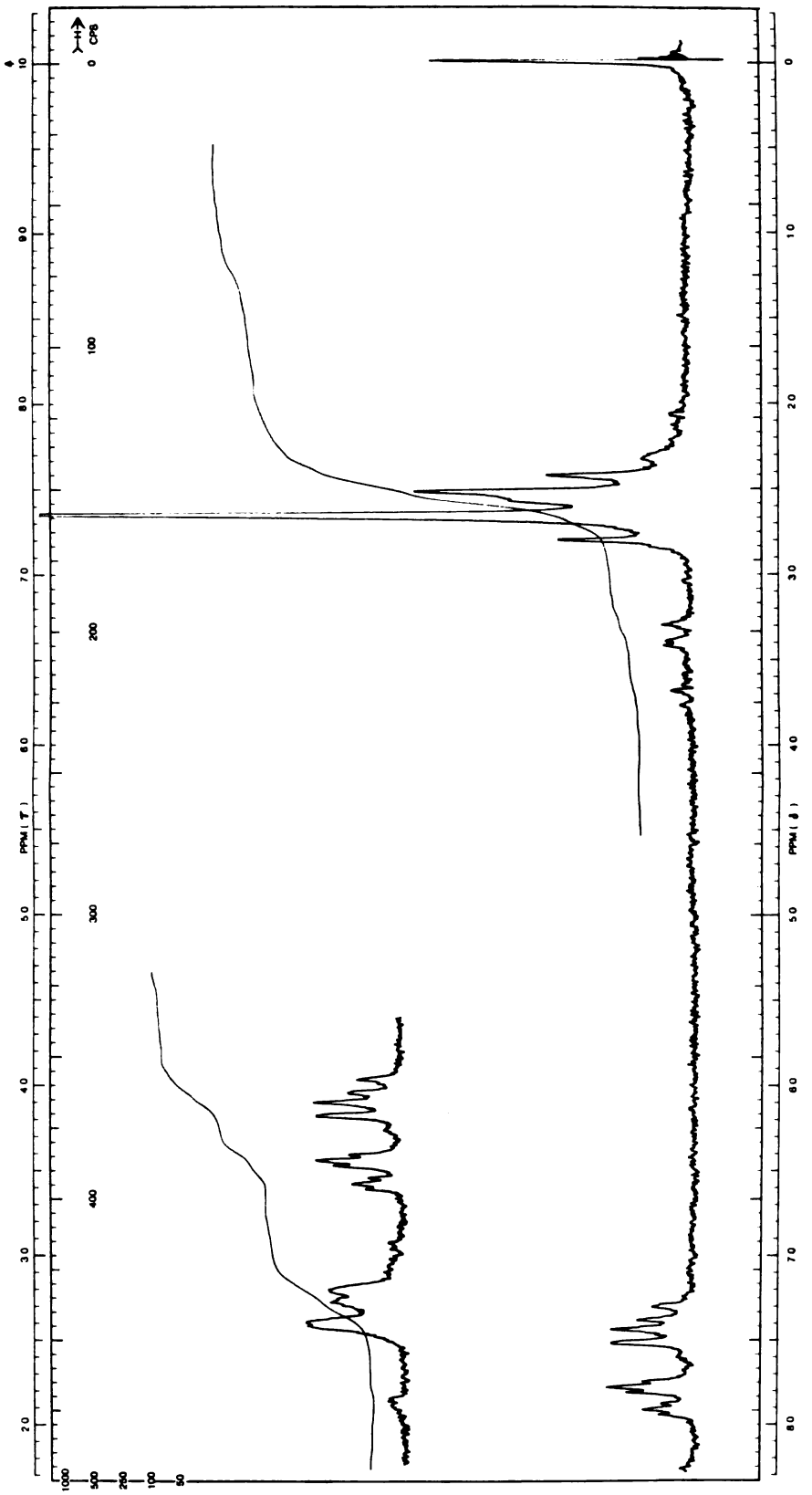


Figure XXIX-A. The nmr spectrum of an aged sample of 5-hydroxycotinine in $CDCl_3$ and one drop of D_2O (TMS, 60 MHz).

5-hydroxycotinine (52) was treated with dilute sodium hydroxide the same changes were observed on a much shorter time scale. This is shown by the nmr spectra presented in Figure XXX. The spectrum A is of 5-hydroxycotinine in water. When one drop of 5% NaOH was added no significant changes were observed within 30 minutes except for the signal at 2.9 ppm which doubled in height (spectrum B). After 20 hours, the material was extracted into chloroform, dried over Na_2SO_4 and an nmr spectrum was taken in $CDCl_3$. Two new signals at 7 and 11 Hz downfield from the intense signal for N-CH_3 of the lactam form (52) were observed in the spectrum D as compared to the spectrum C of pure 5-hydroxycotinine in $CDCl_3$. When a drop of D_2O was added these two signals slowly collapsed into one singlet with a chemical shift average of the two original ones (spectra E and F). Obviously these two signals were caused by protons which were spin-spin coupled with a nearby exchangeable proton. The secondary -N-CH_3 group of the open ring ketoamide 51 fits this behaviour. The splitting of the N-methyl protons was not observed in H_2O probably because of the rapid rate of exchange of the -NH in water. After 24 hours the spectrum G was obtained. A decrease in height of the signal at 2.9 ppm indicates that in chloroform the ketoamide 51 slowly closes to reform 5-hydroxycotinine.

This interpretation of the nmr behaviour of 5-hydroxycotinine in

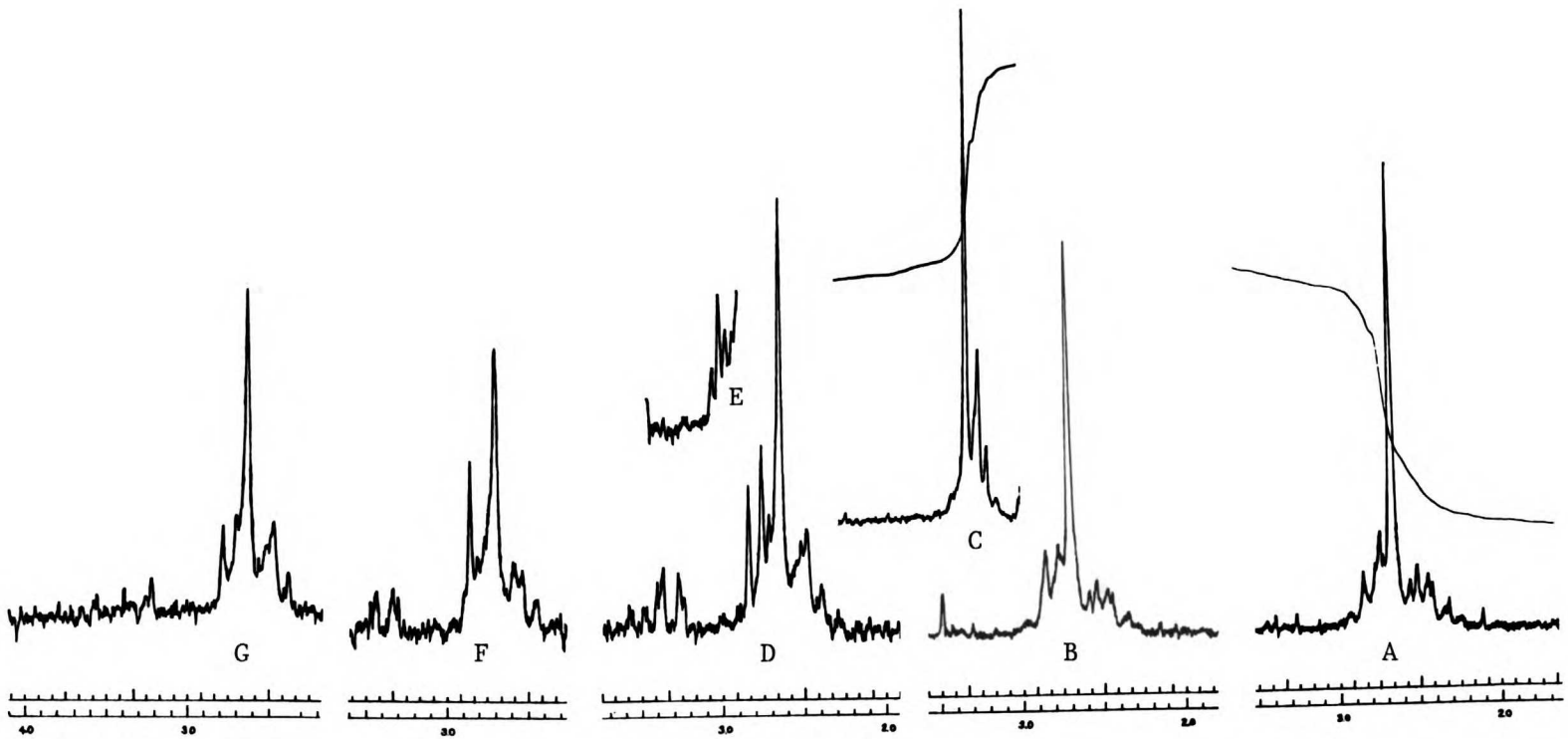
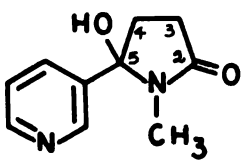


Figure XXX. The 60 MHz nmr spectra of (A) Freshly prepared 5-hydroxycotinine in H_2O; (B) Freshly prepared 5-hydroxycotinine in H_2O and one drop of 5% NaOH; (C) Freshly prepared 5-hydroxycotinine in $CDCl_3$; (D) 5-Hydroxycotinine in $CDCl_3$ after the freshly prepared compound had been incubated for 20 hours in H_2O containing one drop of 5% NaOH; (E) and (F) The same as D but about one minute and 5 minutes after one drop of D_2O had been added; (G) The same as D but 24 hours after one drop of D_2O had been added.

aqueous base found further support in a similar experiment carried out in D_2O. When 5-hydroxycotinine in D_2O was treated with 4% NaOD in D_2O the non-aromatic portion of the nmr spectrum was seen to collapse over a period of 20 seconds into an intense singlet at 2.62 ppm integrating for 5 protons (3 from the methyl protons and 2 from methylene protons). An eims of this material confirmed the incorporation of three deuterium atoms into the structure (2 methylene protons and one exchangeable proton). Evidently there was rapid exchange of two methylene protons with D_2O under the influence of NaOD. This result suggests at least two possibilities. The deuterium exchange took place directly at C_3 of the closed ring structure 52 in the basic medium in the same manner that cotinine is known to undergo base catalyzed exchange[139] or the exchange took place at C_β of the open ring form 51 which under the influence of aqueous base was in rapid equilibrium with the closed ring form 52.



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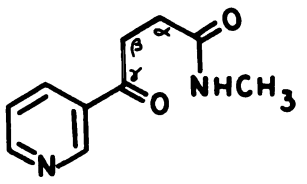


51

These two possibilities were investigated by following the deuterium exchange of cotinine and of freshly prepared 5-hydroxycotinine under the same conditions by nmr integration. The base catalyzed deuterium

exchange of cotinine was found to be a very slow process; no noticeable change in the integration was observed even after 68 hours. This slow rate of exchange is in agreement with the rate of exchange of cotinine in D_2O in the presence of K_2CO_3 where it is known that several days at 100°C are required for complete exchange.[139] Since the protons of cotinine itself do not exchange significantly under conditions where the exchange of 5-hydroxycotinine takes place within seconds, the exchange of the proton at C_3 of 5-hydroxycotinine seems unlikely. This would imply that the exchange is occurring at C_4 which is reasonable only in the open ring form 51.

Thus in summary, from what we know about enzymatic α-carbon hydroxylation of tertiary amides it seems likely that the initially formed metabolite is the closed ring form 5-hydroxycotinine (52). However, what we have found suggests that the closed ring form 52 is in equilibrium with the open ring form γ-ketoamide 51 and the rate and position of this equilibrium is greatly affected by the presence of base and the polarity of the solvent in which the metabolite is dissolved. It should be noted that if the equilibrium between the open chain form 51 and the closed ring form 52 is dependent on the polarity of the solvent, the material isolated in a non polar solvent may not reflect the true structure of the metabolite. This is particularly true when the work up

procedure involves the use of strong acid or strong base as was the case with McKennis.[87, 88]

In the following sections the discussion turns to an exploration of the *in vitro* metabolism of nornicotine and nicotine. In these compounds the products of α-carbon hydroxylation not only have the possibility of equilibration between open and closed ring structures but also have the possibility of equilibrium in water between the elimination and addition of H_2O and OH^-.

C. Metabolic studies.

1. Metabolism of nornicotine (2).

a. Historical.

Nornicotine is an important tobacco alkaloid. The average tobacco used in cigarettes contains 15-20% as much nornicotine as it does nicotine, and in some varieties of tobacco the amount of nornicotine present exceeds the amount of nicotine.[95,148] Nornicotine is also important because it is thought to be a major metabolite of nicotine[84,91-93] in mammals. In spite of this, nornicotine has received little attention especially in regard to studies in mammals. The early mammalian studies on nornicotine were mainly concerned with its pharmacologv and were extensions of studies on nicotine.

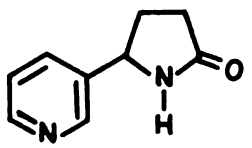
Nornicotine was known as early as 1904.[149] Both enantiomers of nornicotine are found in plants[63] and the optical rotation of the nornicotine obtained from this source varies over a wide range. Späth and Zajic[130] have resolved partially active nornicotine fron tobacco plants by fractional crystallization of the perchlorate salt. They obtained nornicotine with an $[\alpha]_D^{23}$ as high as -88.8°. This is considered to be the optically purest nornicotine yet obtained, since no one has ever obtained a higher optical rotation for nornicotine.

The pharmacological studies of nornicotine are dated as early as

1904.[149] Most of these studies were done with racemic mixtures obtained synthetically or with partially resolved nornicotine isolated from plants. Qualitatively, the effects of nornicotine are about the same as those of nicotine.[150] Quantitatively, the relative toxicity of nornicotine as compared to nicotine depends upon the routes of administration and on the animal species. According to Larson and Haag,[151] both racemic nornicotine and (S)-nornicotine are about as toxic as (S)-nicotine by the intraperitoneal route in rabbits but they are only half as toxic as nicotine by the same route in mice. However, by the intravenous route nornicotine is twice as toxic as (S)-nicotine in rabbits. There are few data on the toxicity of nornicotine in humans.

The metabolism of nornicotine has not yet been extensively investigated and due to the similarity of its structure with that of nicotine, nornicotine has been assumed to be metabolized in a similar fashion. Only two studies on the metabolism of nornicotine in mammals have been reported. Wada, et al.[94] obtained from the chloroform extract of the alkalinized urine from dogs given nornicotine three pyridine containing compounds two of which were identified by their R_f values as demethylcotinine (49) and unmetabolized nornicotine. The third metabolite was not identified. These authors also reported the isolation of γ-(3-pyridyl)-γ-aminobutyric acid (56) from the aqueous phase of the extraction.

Papadopoulos[92] obtained similar results from *in vivo* and *in vitro* metabolic studies of nornicotine with rabbits and rabbit liver homogenate fractions. He showed that nornicotine is metabolized into demethylcotinine (49) and at least one unidentified metabolite.



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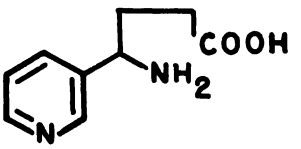

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A study of nornicotine excretion patterns was made by Hucker and Larson.[152] Nornicotine isolated from tobacco was given to anesthetized dogs by infusion and the 24 hour urine was collected. The amount of nornicotine in the urine was determined colorimetrically (cyanogen bromide method). The percentage of nornicotine excreted unchanged increased with increasing dose and reached a maximum of 60% at 15 mg/kg. Nornicotine was found to be completely excreted within 16 hours following cessation of administration. According to these authors. the rate of metabolism of nornicotine is independent of the dose within the range studied (up to 25 mg/kg). However the results obtained from this study were based on the assumption that unmetabolized nornicotine is all excreted into the urine.

b. Identification of nornicotine metabolites.

All our in vitro experiments on nornicotine metabolism were carried out with the 10,000 x g supernatant fractions or the microsomal fractions (100,000 x g pellets) from rabbit liver. The incubations were done in pH 7.4 phosphate buffer in the presence of the required cofactors: NADPH and magnesium ion. Racemic nornicotine-d_0, -2'-d_1 and -3', 3'-d_2 were used as the substrates of the incubation in air at 37°C for one hour. The incubates were extracted directly into dichloromethane and were analyzed by tlc (silica gel, EtOH : CH_3COCH_3 : C_6H_6 : conc. NH_4OH = 5 : 40 : 50 : 5) followed by gc-eims (2% carbowax KOH column) of the material from each tlc band.

The tlc analysis of the extract from the incubation of nornicotine-d_0 with the microsomal fraction showed the presence of four bands on fluorescent plates with R_f values of 0.08 (band I), 0.24 (band II), 0.34 band III) and 0.50 (band IV) relative to the solvent front (7.65 cm).

Band I was identified as unmetabolized nornicotine on the basis of its R_f value, gc retention time and ei-mass spectrum.

Band II was present in the least amount judging by its faintness and did not give a discernible gc peak. This suspected metabolite remains unidentified.

Band IV was identified as myosmine on the basis of its R_f value

gc retention time and ei-mass spectrum. Figure XXXI compares the eims of synthetic myosmine to that of the metabolic sample. This identification was confirmed by the incubation of nornicotine-3',3'-d_2 which gave an eims for band IV that was identical to the mass spectrum of synthetic myosmine-3',3'-d_2.

A gc-eims of the methanol extract from band III showed it to be a mixture of two compounds. One of the two was identified as nicotinamide which probably is formed from the breakdown of NADPH during the incubation. The eims of the other compound is very similar to that of myosmine (see Figure XXXII) with a molecular ion at m/e 146 and a base peak at m/e 118. The differences between the two spectra are in the relative intensities of the ion at m/e 91 and of the M^+ and $[M-1]^+$ ions. This indicates that this unknown is a pyrroline, an isomer of myosmine. When the extract of band III was treated with N,O-bis-(trimethylsilyl)-acetamide overnight at room temperature the gc-eims analysis showed that the retention time of the unknown had not shifted and the eims obtained was unchanged. Thus the unknown does not form a TMS derivative which rules out the presence of an N-H or O-H in the structure. The same gc-eims analysis also showed a shift in the retention time of the nicotinamide gc peak and the eims obtained was consistent with the mono TMS derivative of nicotinamide.

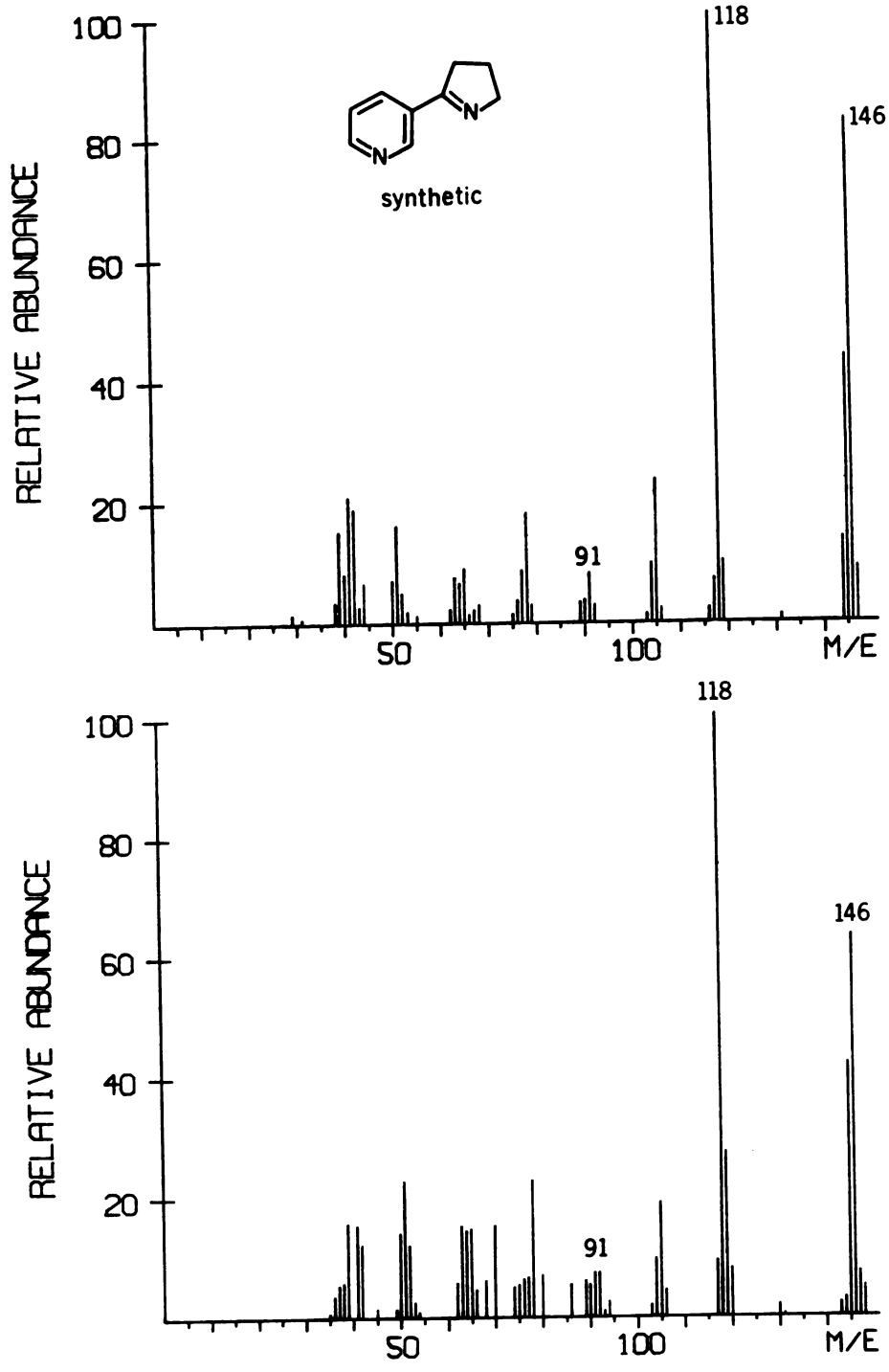


Figure XXXI. The ei mass spectra of synthetic myosmine (top) and of metabolic myosmine (bottom).

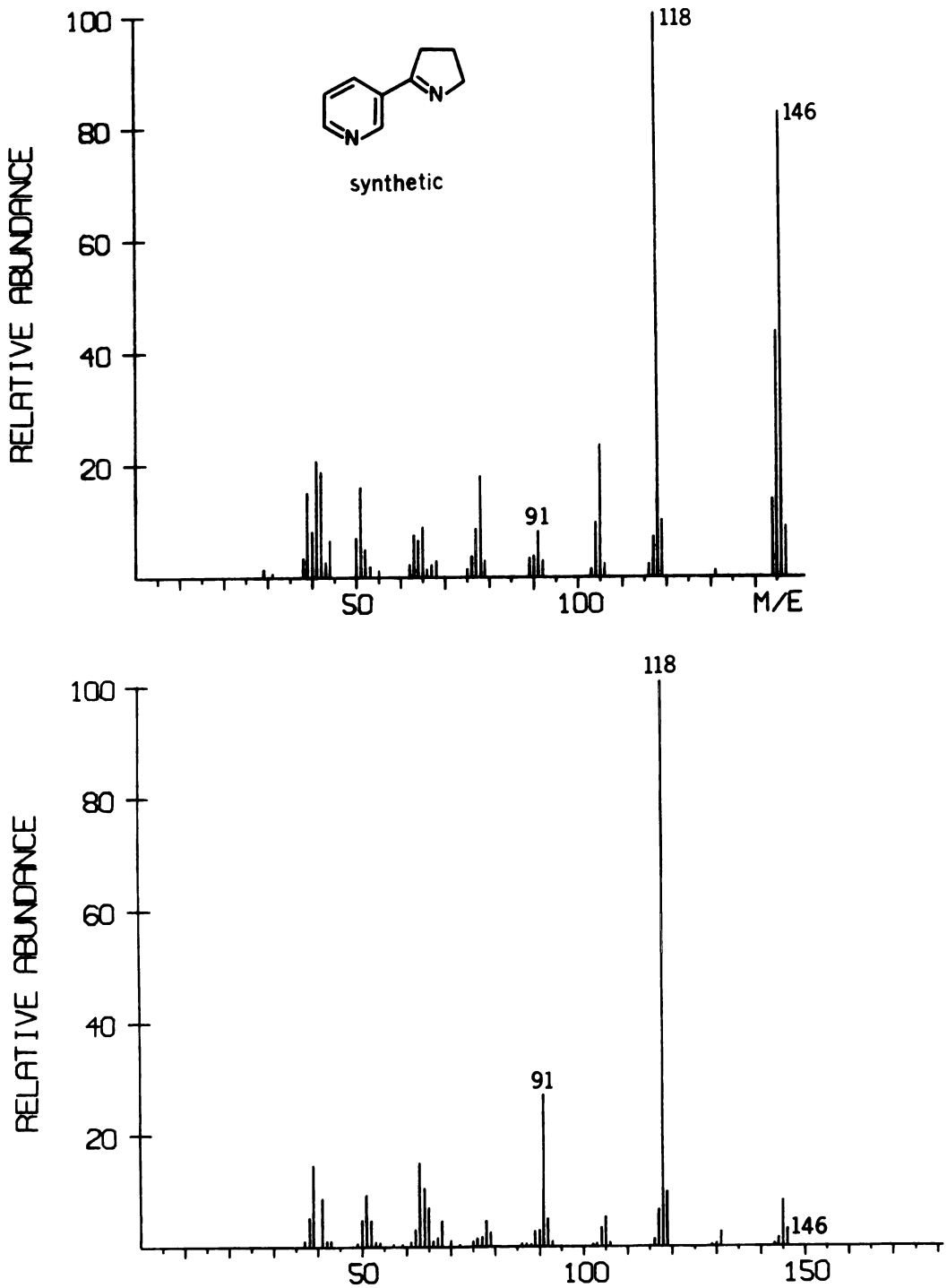
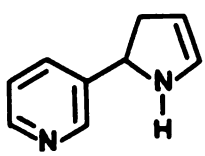


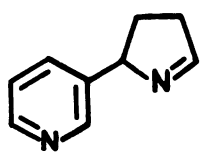
Figure XXXII. The ei mass spectra of synthetic myosmine (top) and of metabolic 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline (bottom).

The use of labeled substrates narrowed the possible sites of metabolic alteration. The eims of the unknown metabolite obtained from the incubation of nornicotine-2'-d_1 showed a peak shift of one mass unit higher in every major ion (m/e 147, 119 and 92) while the eims of the unknown from metabolism of nornicotine-3',3'-d_2 showed a shift to two mass units higher in the molecular ion alone. The fragment ion at m/e 118 and m/e 91 did not shift. Since there was no loss of deuterium in forming the unknown metabolite from either of these substrates, metabolic alteration at either the 2' or 3' position of the five membered ring was ruled out. As expected, metabolism of nornicotine-2'-d_1 gave myosmine which was unlabeled.

The experiments with labeled substrates limit the possible structures for the unknown metabolite to two:



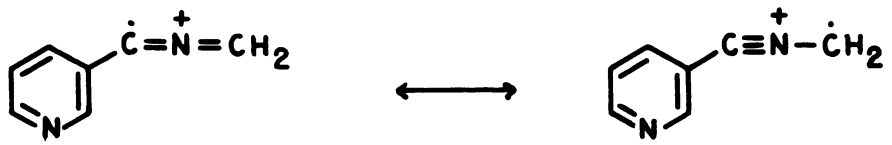
97



98

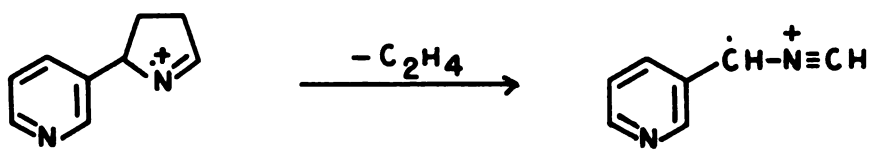
The structure 97 can be ruled out on the basis of the result from the trimethylsilylation experiment and on the basis of the mass spectral fragmentation pattern observed for the metabolite. The loss of 28 mass units from the molecular ion of myosmine to form the m/e 118 fragment

has been shown to be due to the loss of C_2H_4 (positions 3' and 4' of the five membered ring).



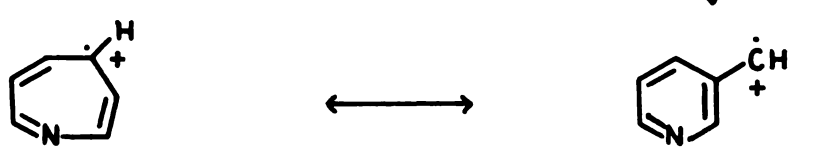
m/e 118

Since the unknown metabolite also shows a base peak at m/e 118 a similar fragmentation is probably involved. This would be difficult to account for with structure 97 but could easily be accounted for by the fragmentation pattern for 98 shown below.



M^+ 146 (8%)

m/e 118 (100%)



m/e 91 (27%)

These fragment assignments are consistent with the peak shifts observed with nornicotine-2'-d_1 and nornicotine-3',3'-d_2 mentioned above.

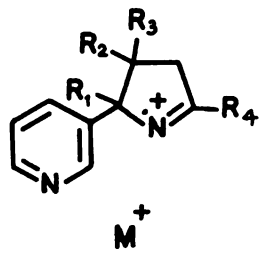
The above structure assignment for the nornicotine metabolite was

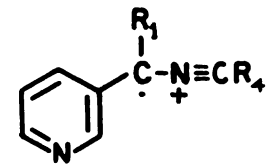
confirmed by the studies on the metabolism of nicotine in the presence of cyanide ion (see Section C.2). In addition to the cyanonicotines and cotinine, a fourth nicotine metabolite was observed by gc-eims when dichloromethane was used as the extracting solvent (see Figure XXXIV). When nicotine-d_0 was used as the substrate, an ei-mass spectrum showed this metabolite to be identical to the nornicotine metabolite 98. Presumably this metabolite is formed in two steps via nornicotine. When nicotine-5',5'-d_2 and -2',5',5'-d_3 were used as substrates the eims of 98 showed the molecular ion and the base peak had shifted one and two m/e units higher respectively. The ion at m/e 91 did not shift in the eims of 98 from nicotine-5',5'-d_2 but shifted to m/e 92 in the eims of 98 from nicotine-2',5',5'-d_3. Thus in each case a deuterium has been lost from the 5' position of the parent alkaloid in the formation of the metabolite. This confirms the structure of the new metabolite as 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline (98).

Table VI summarizes the eims of the Δ^5-pyrroline 98 obtained from the metabolism of nornicotine-d_0, -2'-d_1 and -3',3'-d_2 by the microsomal fraction and from the metabolism of nicotine-5',5'-d_2 and -2',5',5'-d_3 by the 10,000 x g supernatant fraction in the presence of cyanide ion.

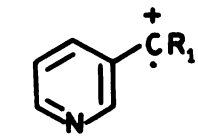
Table VI

The eims of deuterated 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline.





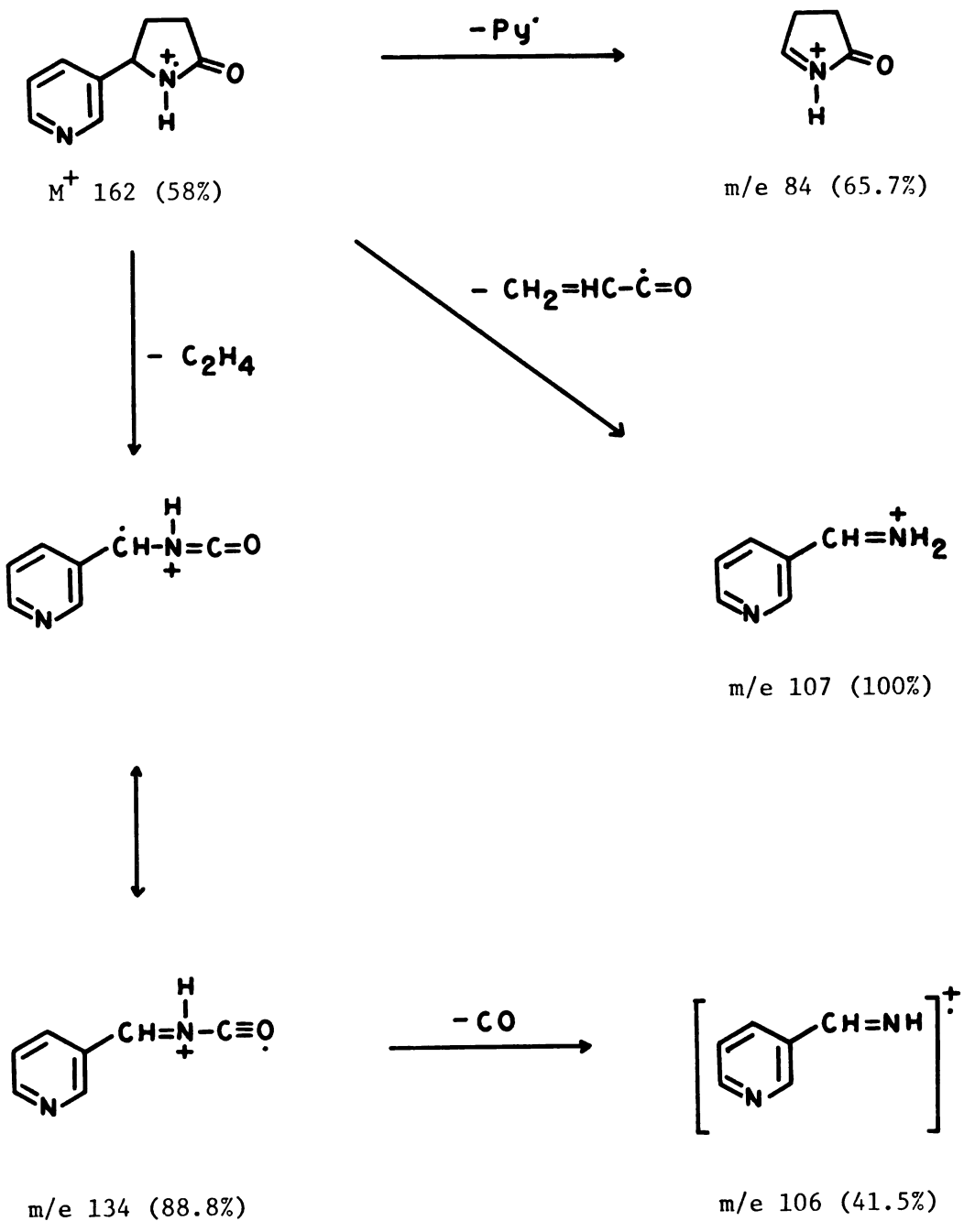
A



B

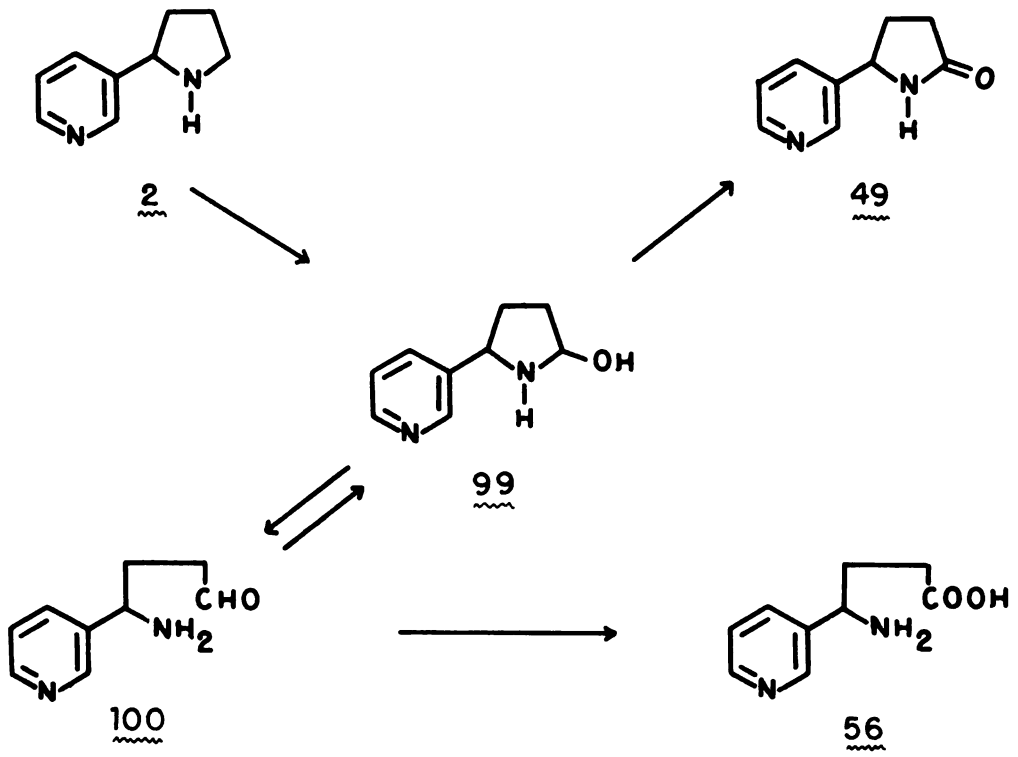
Substrate	Metabolite						
	R_1	R_2	R_3	R_4	M^+	A	B
Nornicotine-d_0	H	H	H	H	146	118	91
Nornicotine-2'-d_1	D	H	H	H	147	119	92
Nornicotine-3',3'-d_2	H	D	D	H	148	118	91
Nicotine-5',5'-d_2	H	H	H	D	147	119	91
Nicotine-2',5',5'-d_3	D	H	H	D	148	120	92

The metabolism of nornicotine by the microsomal fraction did not show the formation of demethylcotinine, a known metabolite of nornicotine.[92,94] However when the metabolism of nornicotine was carried out using the 10,000 x g supernatant fraction, demethylcotinine was observed by gc-eims. The fragment ion assignments for demethylcotinine are shown below.


-Py·
M+ 162 (58%)
m/e 84 (65.7%)
- C2H4
- CH2=HC-Ċ=O
m/e 107 (100%)
m/e 134 (88.8%)
-CO
m/e 106 (41.5%)

c. Pathways leading to nornicotine metabolites.

The observation from our experiments that demethylcotinine was seen only when the metabolic studies were carried out with the 10,000 x g supernatant fractions seems to suggest that the metabolic conversion of nornicotine to demethylcotinine, like the conversion of nicotine to cotinine,[7] consists of at least two steps via the formation of a 5'-hydroxy intermediate. The second step requires a soluble enzyme which is not present in the microsomal fraction.

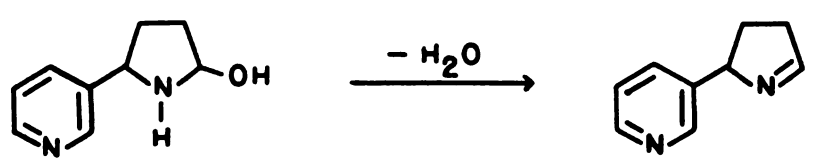


The supposed intermediate 5'-hydroxynornicotine (99) may undergo ring opening to form γ-(3-pyridyl)-γ-aminobutyraldehyde (100) which is

then oxidized by aldehyde dehydrogenase to the corresponding butyric acid 56.

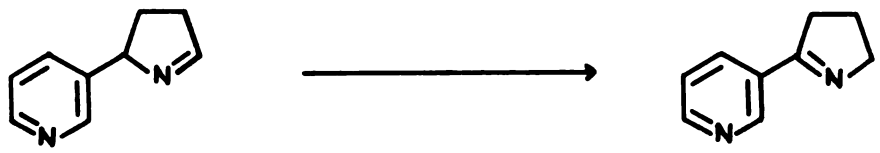
The suggestion[94,154] that the γ-aminoaldehyde 100 and γ-amino acid 56 may be intermediates in the metabolic formation of demethylcotinine (49) from nornicotine although plausible has found little support. There is no evidence that either of these compounds is enzymatically or spontaneously converted into demethylcotinine. In fact the only report where the acid 56 has been shown[94] to undergo ring closure to form demethylcotinine was done in a nitrogen atmosphere and at 150°C that is, not under physiological conditions.

The postulation of the carbinolamine intermediate 5'-hydroxynornicotine (99) is also consistent with the formation of the Δ^5-pyrroline metabolite 98 which can be derived from 99 by the loss of a mole of H_2O.



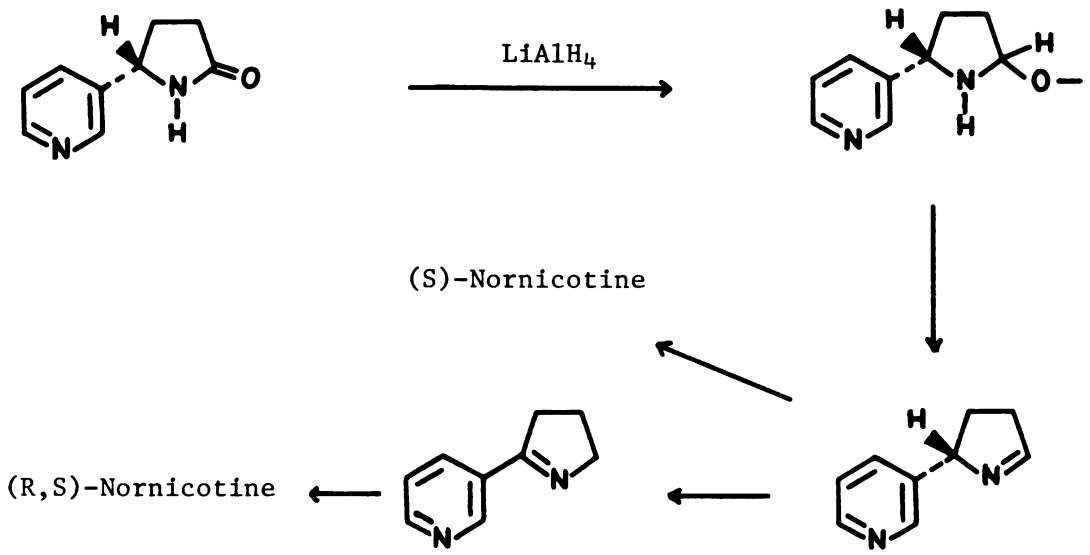
99 98

Similarly, myosmine (a Δ^1-pyrroline) may arise from the corresponding 2'-hydroxynornicotine or it may be formed from the Δ^5-pyrroline by a tautomeric shift.



98 58

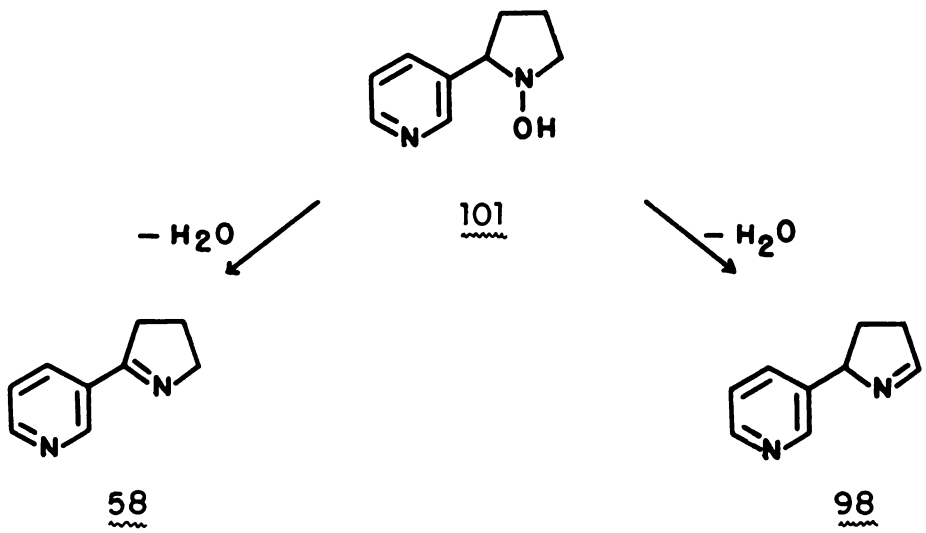
This conversion of the Δ^5-pyrroline to the Δ^1-pyrroline was proposed to account for the partial racemization of the nornicotine obtained by the $LiAlH_4$ reduction of (S)-demethylcotinine.[94]



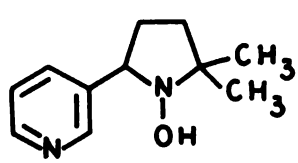
Our metabolic studies do not rule out this conversion although they do rule out the reverse conversion of myosmine to the Δ^5-pyrroline 98. When myosmine was incubated under the same conditions used for the nornicotine metabolic studies, none of the Δ^5-pyrroline was formed and

myosmine was recovered unchanged. The labeling experiments also show that the Δ^5-pyrroline 98 is not formed from myosmine; the Δ^5-pyrroline obtained from the metabolism of nornicotine-2'-d_1 showed a full retention of the deuterium label.

Another possible route for the formation of the two pyrroline metabolites is via N-hydroxynornicotine (101).



Secondary N-hydroxy compounds of similar structure have not been reported as metabolites but the compound 5',5'-dimethyl-N-hydroxynornicotine (102) has been synthesized and obtained in a stable crystalline form.[123]



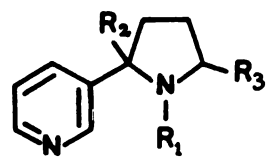
102

Thus, while the true route of formation of the nornicotine metabolites is as yet unknown, the formal insertion of oxygen into the C-H bonds α to the nitrogen is still the most generally applicable mechanism since it not only accounts for the metabolites demethylcotinine and γ-(3-pyridyl)-γ-aminobutyric acid (56) which were previously known but it also accounts for the Δ^5-pyrroline and Δ^1-pyrroline (myosmine) metabolites which were newly discovered in the present work.

2. Metabolism of nicotine in the presence of cyanide ion.

a. Historical.

The three carbon atoms of nicotine (1) directly attached to the nitrogen atom in the pyrrolidine moiety are in a prime position for enzymatic attack. This attack is thought to proceed by introduction of oxygen across the α-C-H bonds to form carbinolamines as initial intermediates. Thus three possible carbinolamines 45, 55 and 103 might be formed in the metabolism of nicotine.



1 : $R_1 = CH_3$, $R_2 = R_3 = H$

45 : $R_1 = CH_3$, $R_2 = H$, $R_3 = OH$

55 : $R_1 = CH_2OH$, $R_2 = R_3 = H$

103 : $R_1 = CH_3$, $R_2 = OH$, $R_3 = H$

The existence of the three carbinolamines 45, 55 and 103 has been previously postulated in a model system (the Fe^{III}-tartrate complex catalyzed decomposition of nicotine N-oxide) and verified by the isolation of N-methylmyosmine, nornicotine, myosmine and cotinine.[43a] 5'-Hydroxynicotine (45) has also been cited as a probable intermediate in the biotransformation of nicotine to cotinine.[7, 76] To date there is no evidence supporting the formation of 2'-hydroxynicotine (103) or of any secondary nicotine metabolite derived from this carbinolamine in

biological systems. N-Hydroxymethylnornicotine (55) is strongly believed to be an intermediate (see chapter II) in the enzymatic N-demethylation of tertiary amines (nicotine) to secondary amines (nornicotine).

Most of the minor metabolites of nicotine can also be explained by the subsequent hydroxylations at α-carbon atoms of the two principal metabolites, nornicotine (2) and cotinine (3).

With the aim of gaining a better understanding of the metabolic conversions involving the introduction of oxygen at carbon atoms α to nitrogen, we first sought to re-examine some of the early work on the metabolism of nicotine using rabbit liver preparations.

In 1960 Hucker, et al.[7] proposed that the biotransformation of nicotine to cotinine proceeds in two steps by the action of two distinct enzyme systems. The cytochrome P_{450} microsomal enzyme converts nicotine to 5'-hydroxynicotine which is then further converted, by the soluble enzyme "aldehyde oxidase", into cotinine. By blocking the action of the aldehyde oxidase system from rabbit liver homogenates with cyanide ion, these workers claimed to obtain 5'-hydroxynicotine. No structural proof was given except that both the metabolic intermediate and synthetic intermediate, which was obtained from partial reduction of cotinine with lithium aluminum hydride, gave positive aldehyde tests. Apparently, Hucker and his coworkers believed they had obtained 5'-hydroxynicotine

as an equilibrium mixture with the open ring amino aldehyde 47.

In 1973, Murphy[76] repeated Hucker's work and reported that gc-eims analyses showed 5'-cyanonicotine (104) was the major product from the incubation of nicotine with rabbit liver homogenate in the presence of cyanide ion. He also reported that a sodium borodeuteride reduction of this incubate mixture yielded nicotine-5'-d_1 (1d) and that the oxygen in cotinine came from water not from molecular oxygen. This information led him to extend Hucker's original hypothesis to include the equilibrium of 5'-hydroxynicotine (45) with nicotine $\Delta^{1'(5')}$-iminium ion (105, see Figure XXXIII). Thus the oxygen of 45 will exchange with water due to the rapid equilibration with the iminium ion 105. 5'-Cyanonicotine will form by the attack of cyanide ion on the $\Delta^{1'(5')}$-iminium ion 105.

Unfortunately, Murphy's metabolic studies provided inadequate proof for the position of the cyano group and the deuterium atom. The metabolic 5'-cyanonicotine was shown to have an identical mass spectrum to that for a synthetic cyano compound which was obtained from the oxidation of nicotine by mercuric acetate in the presence of EDTA followed by the addition of potassium cyanide solution. However the assignment of the cyano group to position $C_{5'}$ of this synthetic material was also in doubt.

Recently, Sanders, et al.[153] described briefly the eims of synthetic

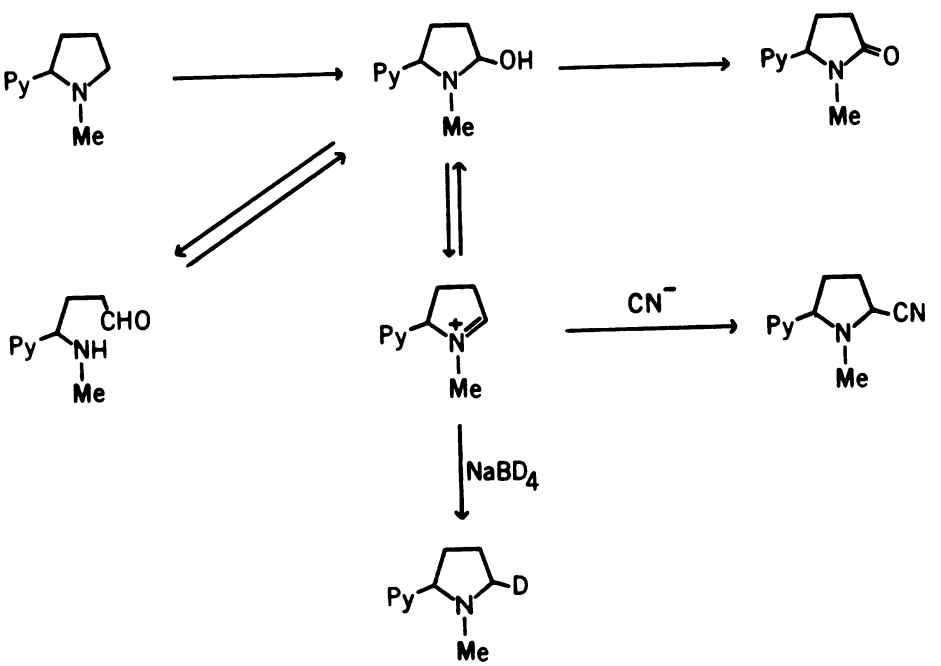


Figure XXXIII. Murphy's suggested pathway from nicotine to cotinine and to 5'-cyanonicotine.

5'- and 2'-cyanonicotine (104 and 106). 5'-Cyanonicotine was made by a sodium aluminum hydride reduction of cotinine, followed by treatment of the intermediate reduction product with cyanide ion. The intermediate reduction product was believed to be 5'-hydroxynicotine in equilibrium with its open ring aminoaldehyde form 47. The spectral data of the cyano product were reported to agree with the structure of a cyanonicotine product and the method of synthesis leaves little doubt as to the position of the cyano group.

These workers also reported that the oxidation of nicotine by mercuric acetate, which Murphy used to make synthetic 5'-cyanonicotine, yielded a 9 to 1 mixture of 2'-cyanonicotine (106) and 5'-cyanonicotine. The cyano group of the major product was assigned to position $C_{2'}$ of the pyrrolidine moiety because its proton nmr spectrum is different from that of the known compound 5'-cyanonicotine. The discrepancy between the two results was explained by the dependency of the 5'-cyanonicotine to 2'-cyanonicotine ratio on the exact manner by which the reactions were carried out.

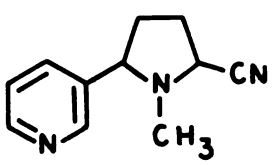
Table VII presents the electron impact mass spectra of cyanonicotine compounds reported by Murphy and Sanders, *et al*. Note that the two mass spectra of 5'-cyanonicotine do not agree. Furthermore, the report of m/e 159 as the base peak of 2'-cyanonicotine is surprising since the

Table VII

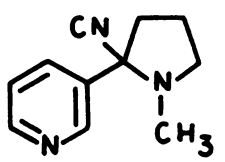
Reported electron impact mass spectra of cyanonicotine compounds.

	Murphy's		Sanders, et al.'s	
5'-Cyanonicotine	m/e	%	m/e	%
M^+	187	v.s.*	187	prom.*
M^+ - HCN	160	45	-	-
M^+ - Py.	109	20	109	100
(M^+ - HCN) - Py.	82	100	-	-
2'-Cyanonicotine				
M^+	-	-	187	v.s.
M^+ - 28	-	-	159	100

* v.s. = very small, prom. = prominent



104



106

expected loss of HCN from the molecular ion (M^+ - 27) would give a fragment of m/e 160 not m/e 159. Sanders, et al.[153] failed to explain either the mass spectrum of 2'-cyanonicotine (106) or why their mass spectrum of 5'-cyanonicotine (104) did not agree with Murphy's. However Sanders, et al. did confirm by a direct comparison of their

product with the material isolated by Murphy that Murphy had indeed obtained 5'-cyanonicotine.

Recently Hubert-Brierre, et al.[155] have repeated the oxidation of nicotine by mercuric acetate. They also reported that 5'-cyanonicotine and 2'-cyanonicotine are the products according to the spectral data. Unfortunately, their description of the mass spectra is too brief to settle the question raised above.

b. Identification of the cyanonicotine compounds.

Initially our intention was to use specifically deuterium labeled nicotines as substrates in in vitro metabolic studies to evaluate Murphy's assignment of 5'-cyanonicotine as the structure of the major product from the metabolism of nicotine in the presence of cyanide ion. Our in vitro experiments with rabbit liver preparations were carried out by employing similar incubation conditions to those used by Murphy. Incubations were conducted with 10,000 x g supernatant fractions or with 100,000 x g microsomal pellets made either with isotonic KCl solution or with pH 7.4 phosphate buffer solution in the presence of the required cofactors. The incubation mixtures were preincubated with cyanide ion at 0°C in air for 12 to 15 minutes to allow cyanide ion to interact with the aldehyde oxidases[156] before adding the substrates. The preincubation was omitted when the microsomal fractions were used since the soluble enzymes were not present. The incubations were carried out at 37°C in air for one hour. The incubates were worked up by extracting with either dichloromethane or diethyl ether and the extracts were analyzed by gc-eims.

The dichloromethane extracts obtained directly from the incubation mixtures were shown by tlc and gc-eims to contain a large amount of nicotinamide, steroids, esters of fatty acids and other nonbasic subs-

tances which interfered greatly with the analyses. When an acid-base extraction was applied these interfering substances were removed. A gc tracing of a typical experiment is shown in Figure XXXIV.

The extent of metabolism was estimated from the gc tracings to be generally from 30% to 50% although in some experiments the extent of metabolism was as low as 1%.

As expected, the gc-eims analyses showed the presence of unmetabolized nicotine as well as cotinine. However, instead of the single cyano product reported by Murphy,[76] two partially overlapping gc peaks were seen with retention times of about 9 minutes.

The eims of the first peak showed it to be identical to that of 5'-cyanonicotine (104) as reported by Murphy. The mass spectrum of the second peak showed it to have the same molecular ion as 5'-cyanonicotine and a base peak at m/e 109 (loss of the pyridine moiety). Therefore it was presumed to be structurally similar to 5'-cyanonicotine. The mass spectra of these two cyanonicotine compounds are shown in Figure XXXV.

When the base extracts of the post incubates were analyzed at a lower temperature of the mass spectrometer inlet lines and of the molecular separator a pronounced temperature dependence for the eims of 5'-cyanonicotine was observed (Figure XXXVI). The eims of 5'-cyanonicotine at the low temperature shows a base peak at m/e 109 and a prominent

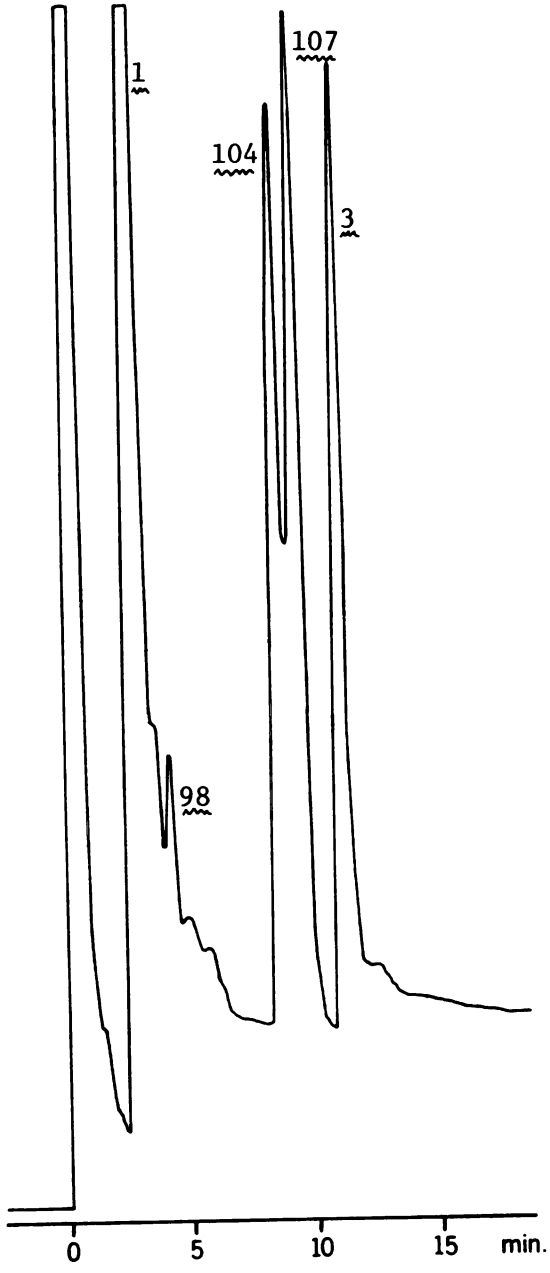


Figure XXXIV. The gc tracing (2% Dexyl, 160°C for one minute, then increasing 3.5°C per minute) of the CH_2Cl_2 extract obtained from the metabolism of nicotine in the presence of cyanide ion by the 10,000 xg rabbit liver supernatant fraction. 1, nicotine; 3, cotinine; 98, 2'-(3pyridyl)-$\Delta^{5'}$-pyrroline; 104, 5'-cyanonicotine; 107, N-cyanomethylnornicotine.

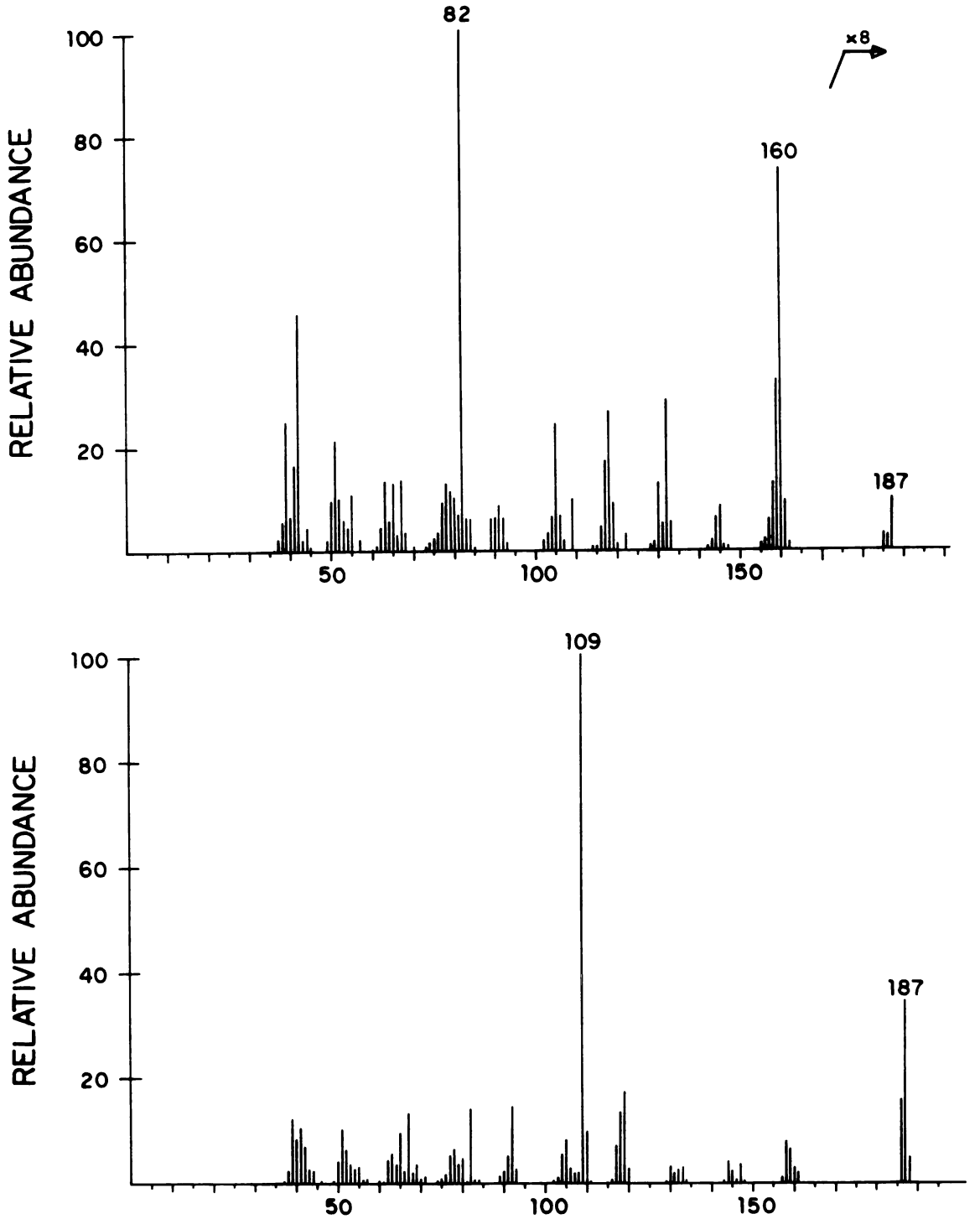


Figure XXXV. The ei mass spectra of 5'-cyanonicotine (top) and of N-cyanomethylnornicotine (bottom) from the metabolism of nicotine-d_0 in the presence of cyanide ion.

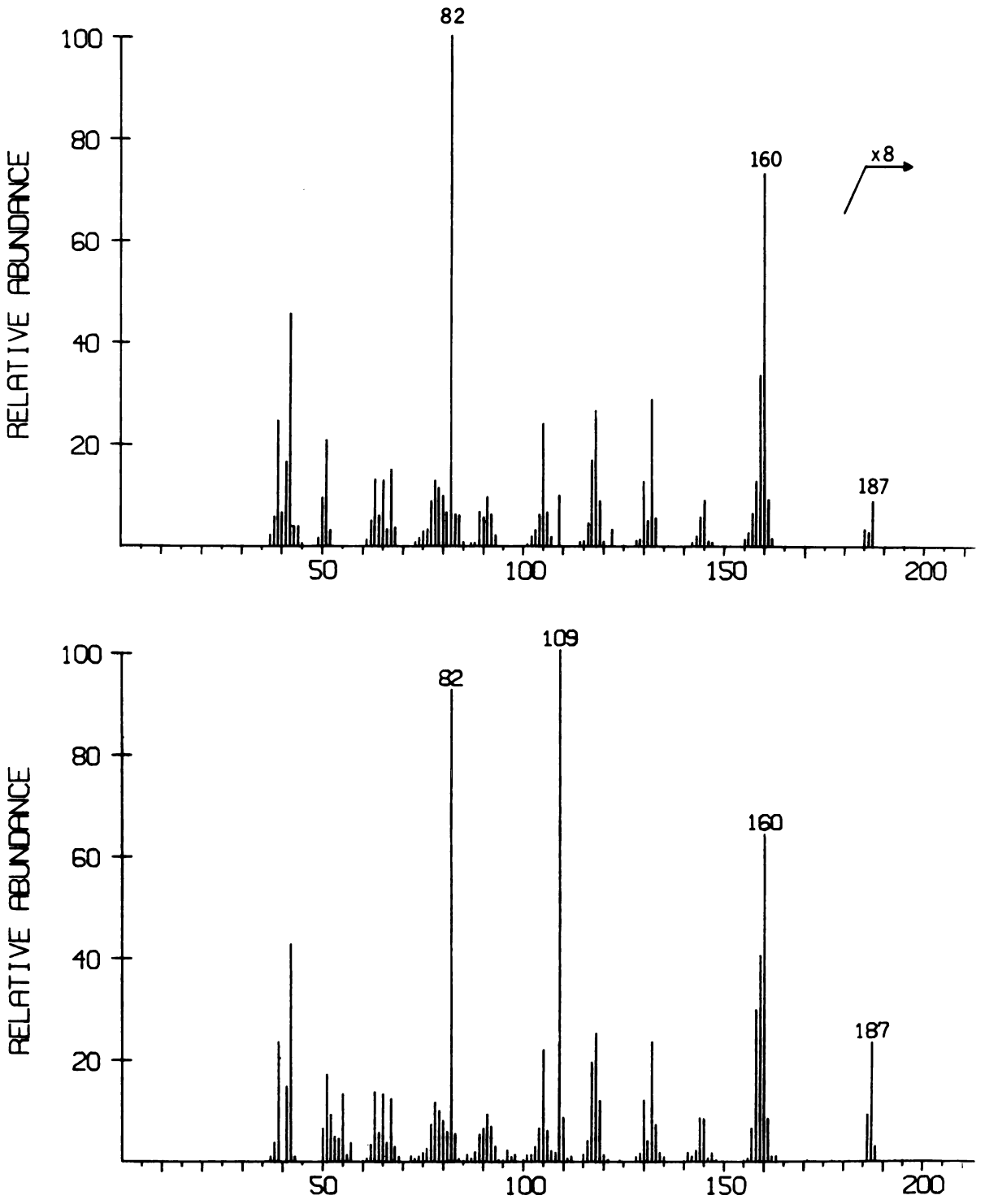
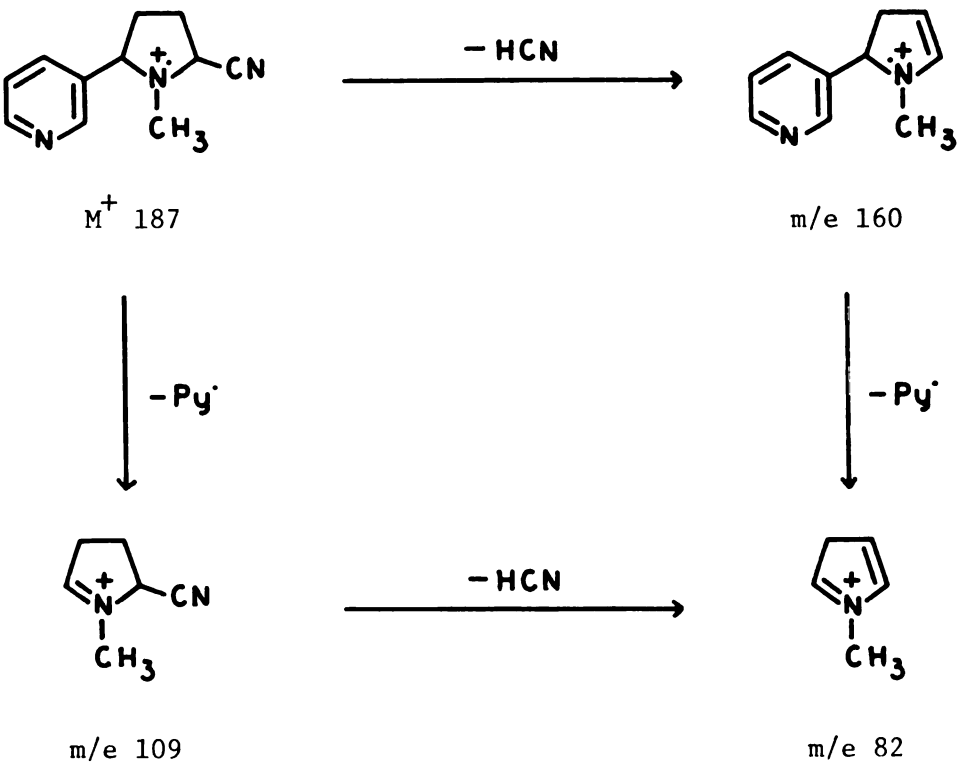


Figure XXXVI. The ei mass spectra of 5'-cyanonicotine at high inlet temperature (top) and at low inlet temperature (bottom).

molecular ion at m/e 187 as well as prominent fragments which are 27 mass units less at m/e 82 and m/e 160. On the other hand, the mass spectrum at the high temperature shows predominantly ions at m/e 82 and m/e 160. This marked temperature dependence of the mass spectrum is apparently due to a thermal elimination of hydrogen cyanide from 5'-cyanonicotine prior to entering the ion chamber. This thermal elimination may explain the differences in the mass spectra reported by Murphy and by Sanders. The fragmentation pattern at low inlet temperature fits well with the structure of 5'-cyanonicotine.



M^+ 187 m/e 160

m/e 109 m/e 82

In order to determine the position of the cyano group in the two metabolic cyanonicotine compounds the following specifically deuterated nicotines were used as substrates: nicotine-5',5'-d_2 (1a), nicotine-2',-5',5'-d_3 (1b) and nicotine-N-methyl-d_3 (1c).

1a : $R_1 = CH_3$, $R_2 = H$, $R_3 = R_4 = D$

1b : $R_1 = CH_3$, $R_2 = R_3 = R_4 = D$

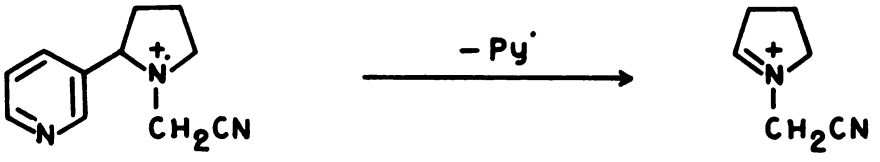
1c : $R_1 = CD_3$, $R_2 = R_3 = R_4 = H$

1d : $R_1 = CH_3$, $R_2 = D$, $R_3 = R_4 = H$

With nicotine-5',5'-d_2 as substrate, the major ions in the mass spectrum of 5'-cyanonicotine, as predicted, moved to one mass unit higher. The molecular ion and the base peak of the second cyanonicotine compound moved to two mass units higher. With nicotine-2',5',5'-d_3, a shift to 2 mass units higher was observed in the eims of 5'-cyanonicotine. The mass spectrum of the unknown compound showed a shift to 3 mass units higher. This indicates that the site of metabolism is neither at the 2' nor the 5' position. The only remaining site of the five membered ring where metabolism is likely to occur is at the N-methyl group. Thus the new cyanonicotine product was tentatively assigned the structure of N-cyanomethylnornicotine (107).

Figures XXXVII and XXXVIII show the eims of the two cyanonicotine compounds obtained from the incubations of nicotine-5',5'-d_2 and nicotine-2',5',5'-d_3.

The obvious experiment to carry out was to incubate nicotine-N-methyl-d_3 in order to confirm the position of the cyano group. The eims of 5'-cyanonicotine obtained from this experiment showed retention of all three deuterium atoms as expected. The eims of the tentatively assigned N-cyanomethylnornicotine showed that one deuterium atom is replaced in forming this product (Figure XXXIX). Thus the labeling experiments not only confirmed the assignment of the 5'-cyanonicotine structure (104) but also allowed an unambiguous assignment of the newly observed isomeric product as N-cyanomethylnornicotine (107).



M^+ 187 m/e 109

The mass spectral fragmentation pattern agrees with the assigned structure. The loss of hydrogen cyanide from 107 is unlikely since there are no protons β to the cyano group and thus the prominent fragments at m/e 160 and at m/e 82 are not seen as in the case of 104.

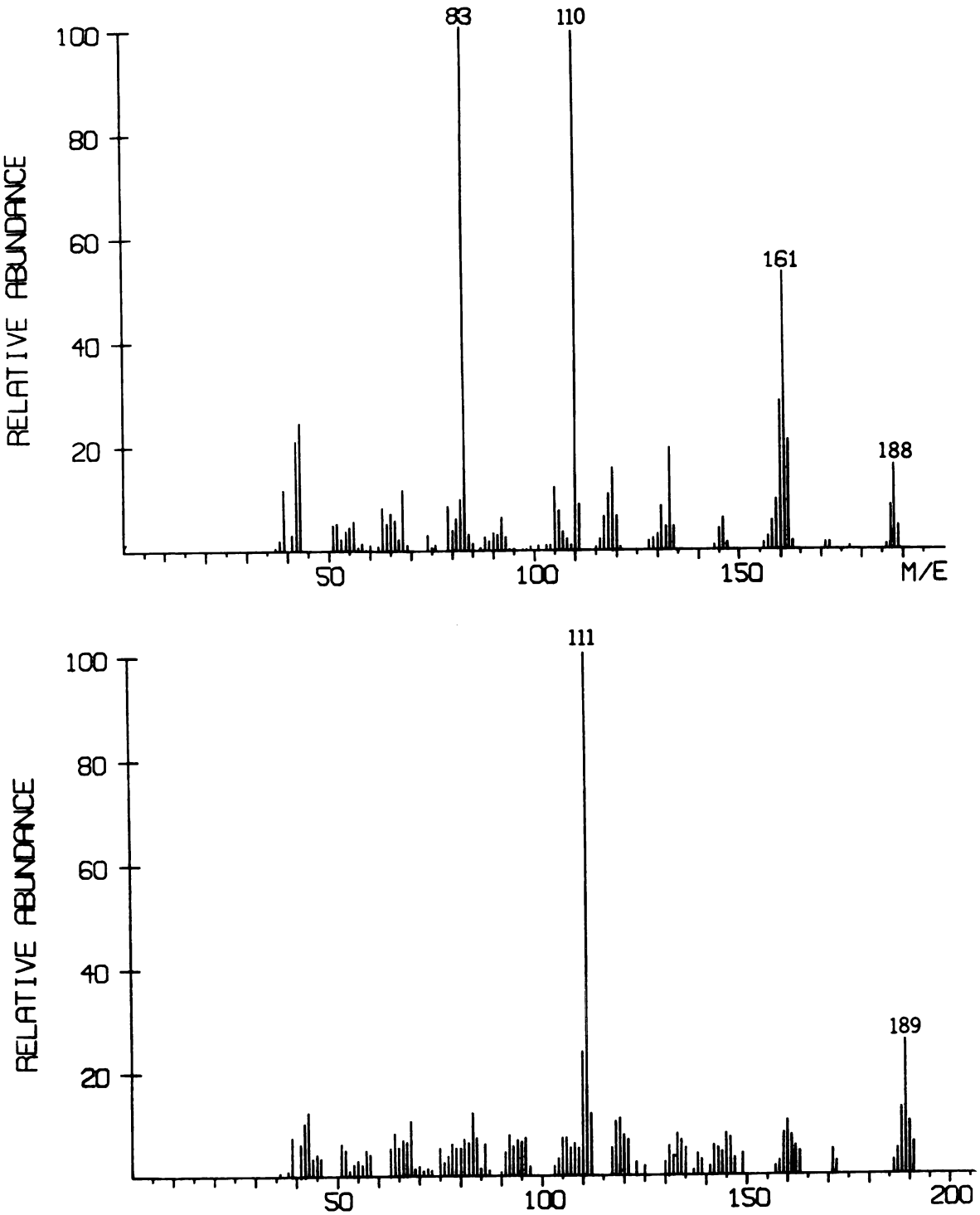


Figure XXXVII. The ei mass spectra of 5'-cyanonicotine-5'-d_1 (top) and of N-cyanomethylnornicotine-5',5'-d_2 (bottom) from the metabolism of nicotine-5',5'-d_2 in the presence of cyanide ion.

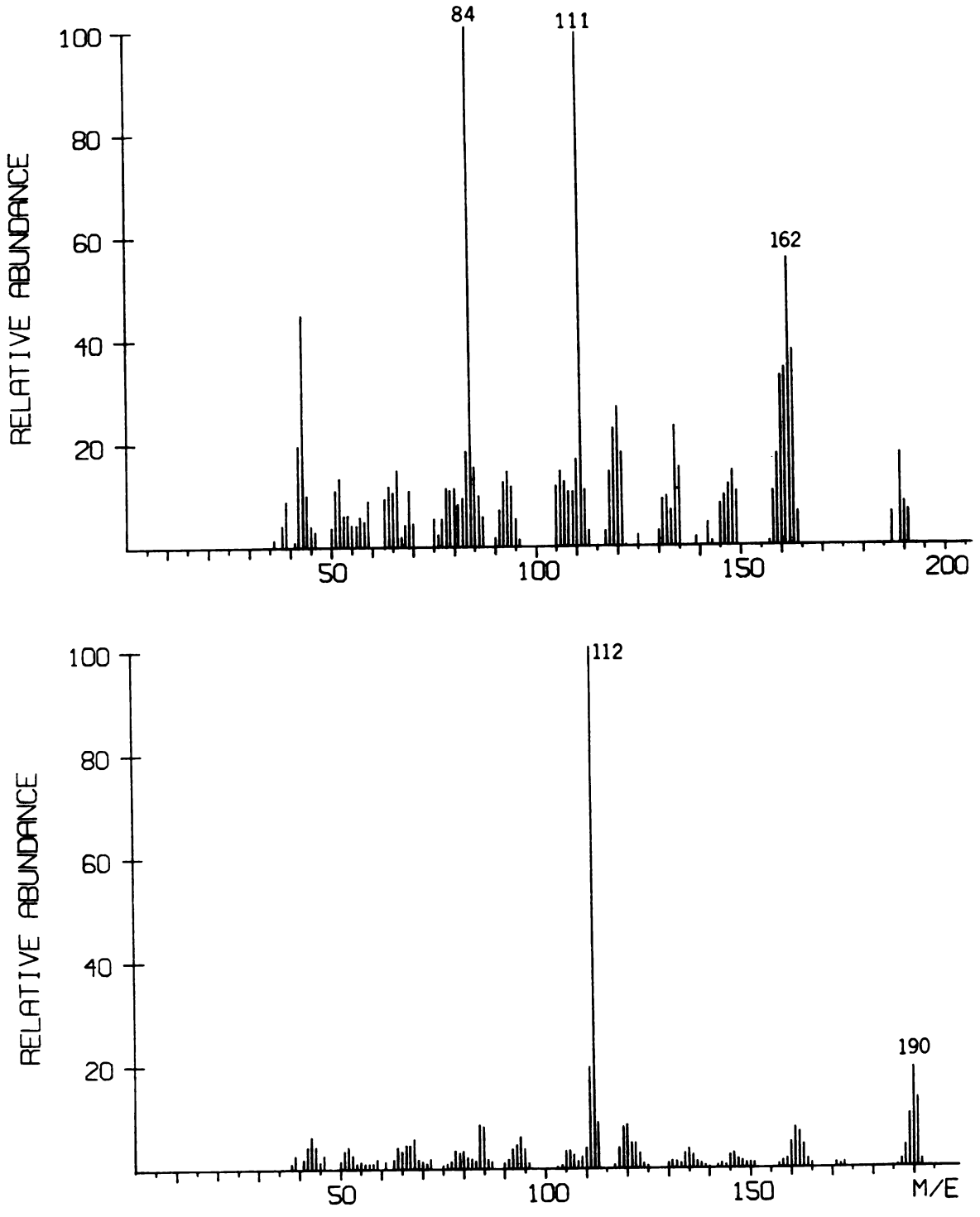


Figure XXXVIII. The ei mass spectra of 5'-cyanonicotine-2',5'-d_2 (top) and of N-cyanomethylnornicotine-2',5',5'-d_3 (bottom) from the metabolism of nicotine-2',5',5'-d_3 in the presence of cyanide ion.

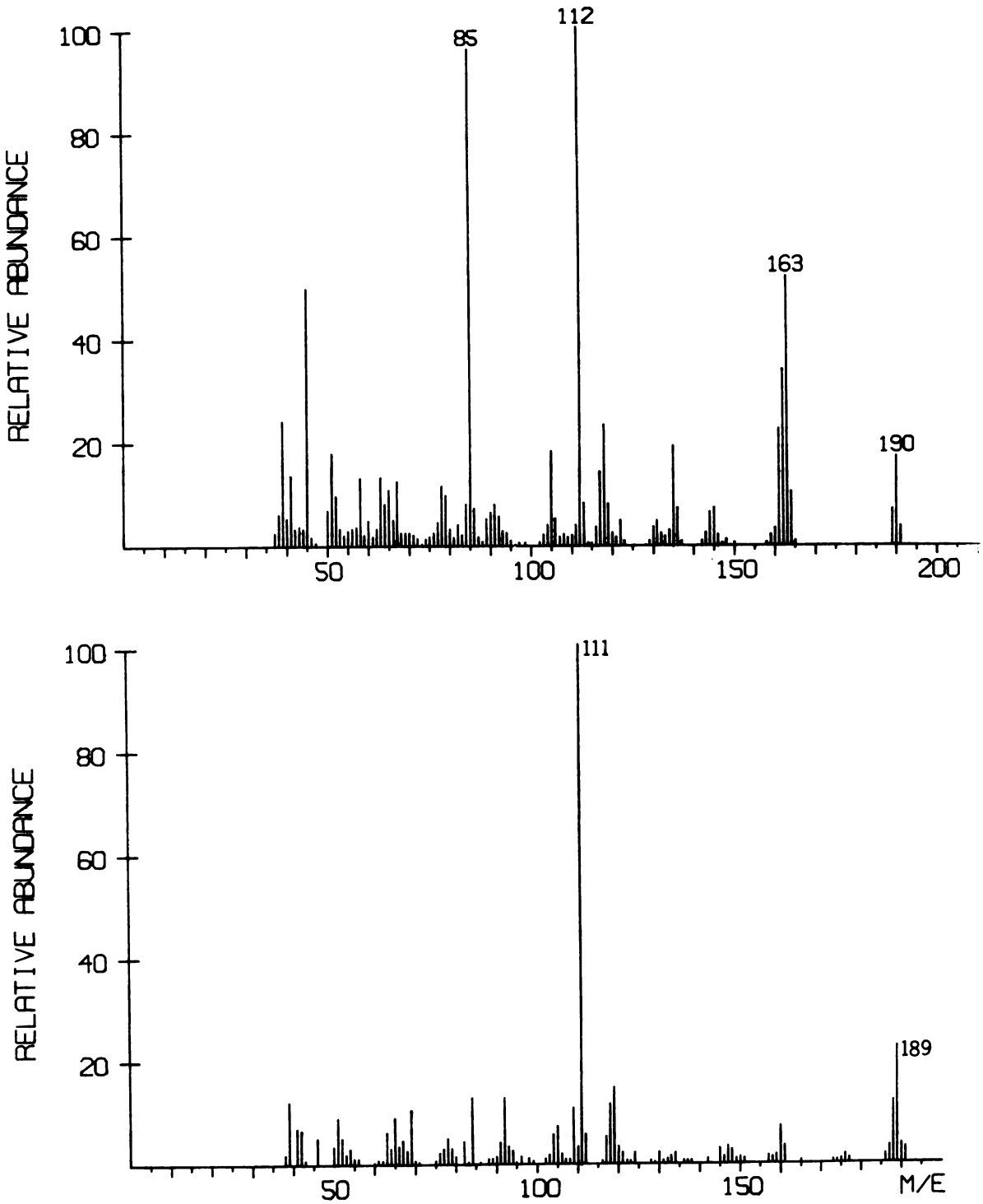


Figure XXXIX. The ei mass spectra of 5'-cyanonicotine-N-methyl-d_3 (top) and of N-cyanomethyl-d_2-nornicotine (bottom) from the metabolism of nicotine-N-methyl-d_3 in the presence of cyanide ion.

The structure of the metabolically formed product 107 was further confirmed by the synthesis of N-cyanomethylnornicotine. This was done simply by a Mannich base type of condensation. Nornicotine, cyanide ion and formaldehyde were allowed to react in an aqueous medium at room temperature for about 30 minutes. The spectral data of the product obtained agreed with the structure. The infrared spectrum showed a weak band at 2240 cm^{-1} for the C≡N stretching vibration. A cyano group usually displays strong absorption in this region. However, when there is an electronegative atom directly attached to the same carbon atom, the intensity of the C≡N stretching vibration absorption is reduced.[146] This is in agreement with the reported intensity for the C≡N bond in 5'-cyanonicotine[153] and in 2'-cyanonicotine.[155] The uv spectrum in ethanol is identical to that of nicotine.[157a]

The 60 MHz proton nmr spectrum (Figure XL) displays in addition to the characteristic signals of the protons of a 3-substituted pyridine from 7.0 to 9.0 ppm, a series of complex signals between 1.6 to 4.0 ppm integrating for 9 protons. Based on the known assignments for the pyrrolidinyl protons of nicotine,[158] these multiplets can be easily assigned. The multiplet at highest field between 1.6-2.5 ppm integrating for 4 protons can be assigned to the four protons at $C_{3'}$ and $C_{4'}$. A one proton triplet at 3.6 ppm (J = 7.5 Hz) is assigned to the $C_{2'}$ proton.

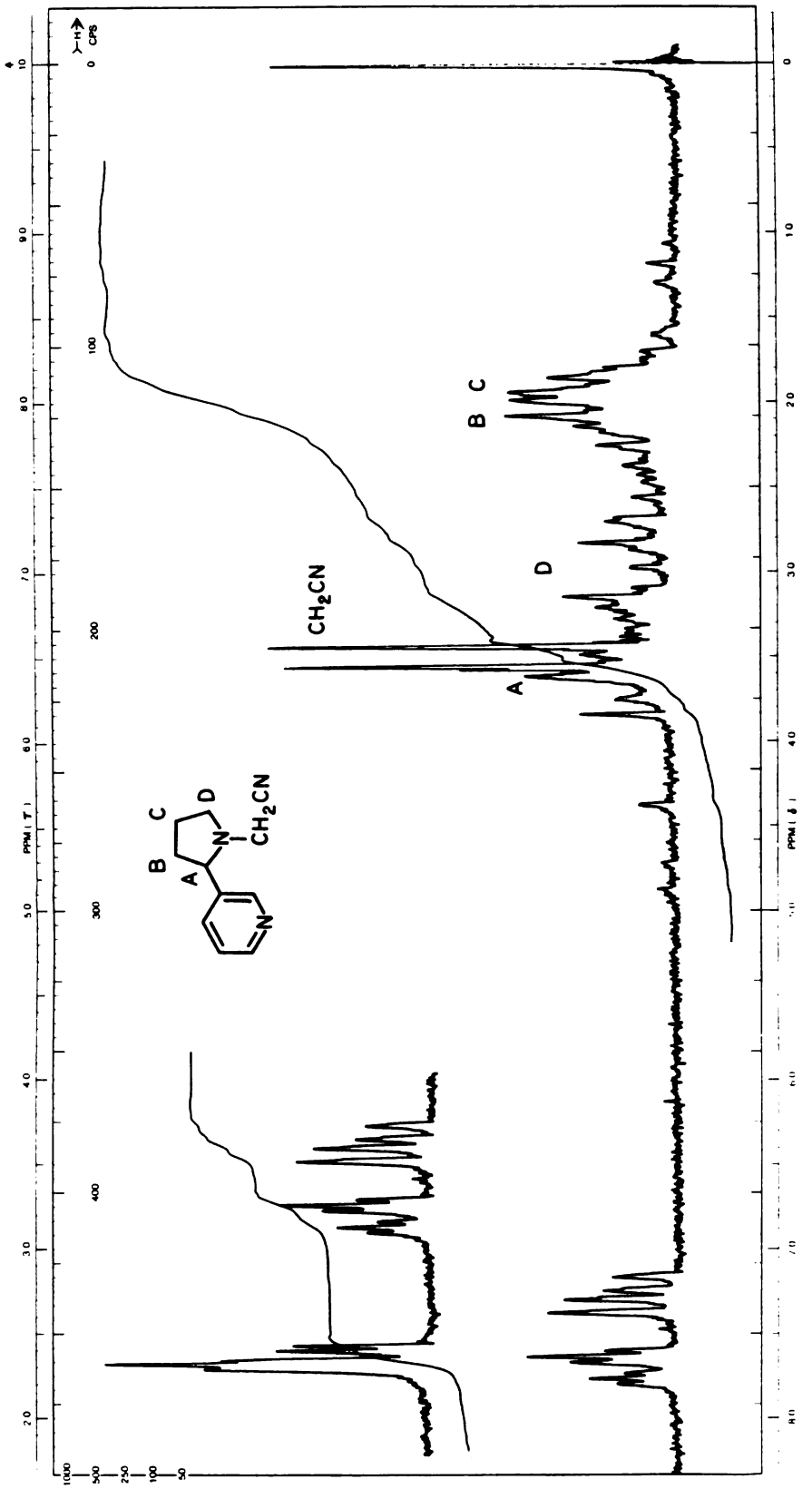


Figure XL. The nmr spectrum of synthetic N-cyanomethylnornicotine (60 MHz, $CDCl_3$, TMS).

The two multiplets from 2.5-3.1 ppm and from 3.1-3.5 ppm each integrating for one proton must be due to the two protons at $C_{5'}$. The nmr spectrum also displays an AB quartet centered at 3.48 ppm for the cyanomethylene protons with $\Delta\nu_{AB}$ = 17 Hz and J_{AB} = 17 Hz. This is consistent with the coupling constants observed in other substituted cyanomethyl compounds.[159] The gc and eims spectral characteristics of synthetic N-cyanomethylnornicotine were identical with those observed for the metabolic material from the nicotine incubation mixture.

c. Genesis of N-cyanomethylnornicotine (107).

It has been proposed that the metabolic conversion of nicotine to cotinine proceeds via 5'-hydroxynicotine (45) which, by loss of a hydroxyl group, is in equilibrium with nicotine $\Delta^{1'(5')}$-iminium ion (105). This iminium ion can be captured by cyanide ion to form 5'-cyanonicotine (104). Similarly we have shown that the metabolic N-demethylation of nicotine to nornicotine may be thought of as proceeding through an intermediate carbinolamine N-hydroxymethylnornicotine (55) with the analogous methyleniminium ion 108 being trapped by cyanide ion to form N-cyanomethylnornicotine (107, Figure XLI).

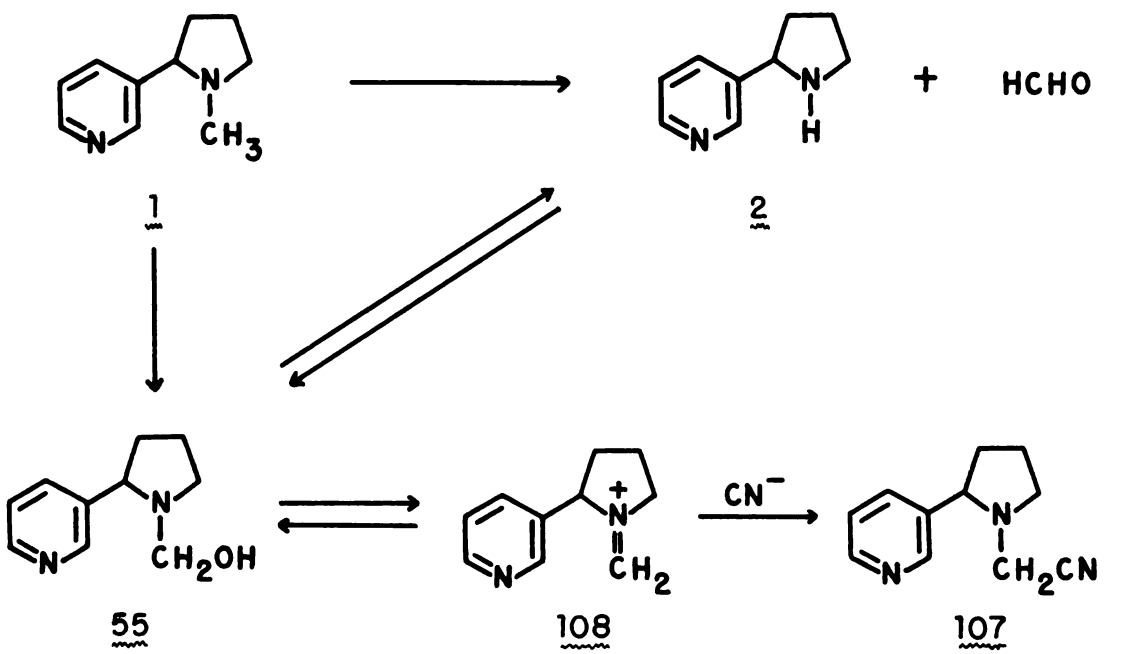
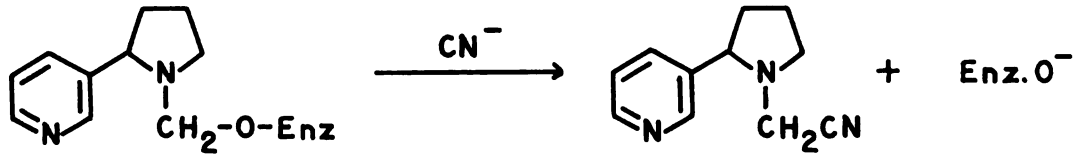


Figure XLI. Direct and indirect pathways for the formation of N-cyanomethylnornicotine via nicotine N-methyleniminium ion.

However there are other possibilities to be considered in discussing the origin of N-cyanomethylnornicotine (107). The cyanomethyl compound needs not be formed via the intermediate methyleniminium ion 108 but may be due to the reaction of cyanide ion with some other metabolic intermediate. One possibility which can be envisioned as an attack by cyanide ion on the enzyme-substrate complex 109, a possible intermediate in the metabolic oxidation.



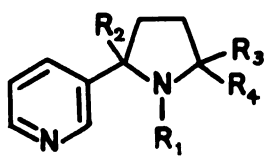
109 107

However mechanisms of this type, while they cannot be disproven, fail to provide as simple and as direct an explanation of both the synthetic and metabolic results as that shown in Figure XLI for the methyleniminium ion 108.

Another possibility to consider is that N-cyanomethylnornicotine might be the product of a nonenzymatic or enzymatic reaction between cyanide ion and one of the impurities in the starting nicotine samples. The gc analysis of synthetic nicotine-5',5'-d_2 showed four impurities which were estimated from gc peak heights to amount to 8% of the total

material. The major impurity (4%) has the same gc retention time as cotinine. This impurity may be a mixture of cotinine and another unidentified compound, since nicotine-5',5'-d_2 was obtained from a reduction of cotinine with lithium aluminum deuteride and the gc-eims of this peak showed other m/e peaks as well. The other three impurities each amounts to about 1% of the total material and are still unidentified.

Nicotine-2',5',5'-d_3 has four impurities which amount to 9% of the total material. One of these impurities (1% of the total) was identified as nornicotine-2',5',5'-d_3 (2d) by gc-eims. Possibly during the synthesis of nicotine-2',5',5'-d_3 (1b) some of the nornicotine-2'-d_1 (2a) was not converted into nicotine-2'-d_1 (1d) and underwent parallel oxidative bromination followed by dissolving zinc metal reduction to demethylcotinine-5-d_1 (49a) which finally was converted back to nornicotine-2',5',5'-d_3 by $LiAlD_4$.

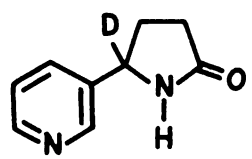


1b : $R_1 = CH_3$, $R_2 = R_3 = R_4 = D$

1d : $R_1 = CH_3$, $R_2 = D$, $R_3 = R_4 = H$

2a : $R_1 = R_3 = R_4 = H$, $R_2 = D$

2d : $R_1 = H$, $R_2 = R_3 = R_4 = D$



49a

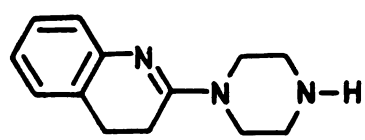
The other three impurities are the same as those found in the nicotine-5',5'-d_2 sample. That these impurities do not chemically give rise to N-cyanomethylnornicotine was shown by control incubations with these two nicotine samples using boiled enzymes. The control experiments except for the use of a boiled liver preparation, were carried out in exactly the same manner as the metabolic experiments including the use of cyanide ion, $MgCl_2$ and NADPH. It was found that the same impurities were still present in the recovered material and the relative percentage of the impurities were essentially unchanged. No N-cyanomethylnornicotine was detected by gc-eims from these control experiments.

The nicotine-d_0 and nicotine-N-methyl-d_3 samples were shown to contain no significant impurities (the total impurities were less than 0.5%) The corresponding control experiments also confirmed that there were no detectable impurities and no cyanomethyl compound was formed. Thus the possibility that N-cyanomethylnornicotine comes from one of the impurities by a chemical reaction can be ruled out.

That N-cyanomethylnornicotine comes from one of the impurities by an enzymatic action can also be ruled out for two reasons. For those nicotine substrates which were shown to be impure, the amount of the impurities was too small to account for the amount of N-cyanomethylnornicotine formed even if all of the impurities had been converted.

With nicotine-d_0 and nicotine-N-methyl-d_3 substrates, N-cyanomethylnornicotine was formed in substantial amounts from the incubations, even though it was shown that these samples contained insignificant amounts of impurities.

The easy synthesis of N-cyanomethylnornicotine by a Mannich base type of reaction suggests another way that the cyanomethyl compound recovered from the metabolic experiments might be formed as an artifact. Nornicotine, which is a known metabolic product of nicotine (see C.2.g.), will readily react with any source of formaldehyde in the presence of cyanide ion to form N-cyanomethylnornicotine. In a number of studies reported in the literature, potential metabolic products were revealed as artifacts arising from the reaction between a primary or especially a secondary amine with an aldehyde contaminating the extracting solvents. In the case of chlorinated hydrocarbon solvents, amines can also react slowly with the solvent itself. Leeling, et al.[160] reported that N-(2-quinolyl)piperazine (110) reacted slowly with chloroform, methylene chloride and ethylene chloride. On standing for 24 hours, four products were formed with $CHCl_3$ and ethylene chloride and one was seen with methylene chloride.

110 

Although a reaction between nornicotine and the extracting solvent methylene chloride on the time scale of our experiment seemed unlikely, the possibility that formaldehyde was present in methylene chloride as a contaminant seemed quite likely. This possibility was confirmed by control experiments.

When nornicotine was incubated under the same conditions as nicotine (in the presence of cyanide ion) and methylene chloride was used as the extracting solvent, N-cyanomethylnornicotine was identified by its gc-eims as the major component (60% of the recovered material, Figure XLII-A). Myosmine and 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline (98) were also detected by their ei mass spectra.

The incubation of nornicotine with boiled enzymes yielded only the cyanomethyl compound (14% of the extract) and one other product discussed below. Thus there is a substantial amount of a one carbon source at the oxidation level of formaldehyde which comes either from CH_2Cl_2 used as the extracting solvent for the experiment or from the incubation itself.

An interesting observation was that in the control experiments using boiled enzymes with nornicotine, 54% of the material recovered from the methylene chloride extract was tentatively identified from its mass spectrum (Figure XLIII-A) as N-carbomethoxynornicotine (111, cf.

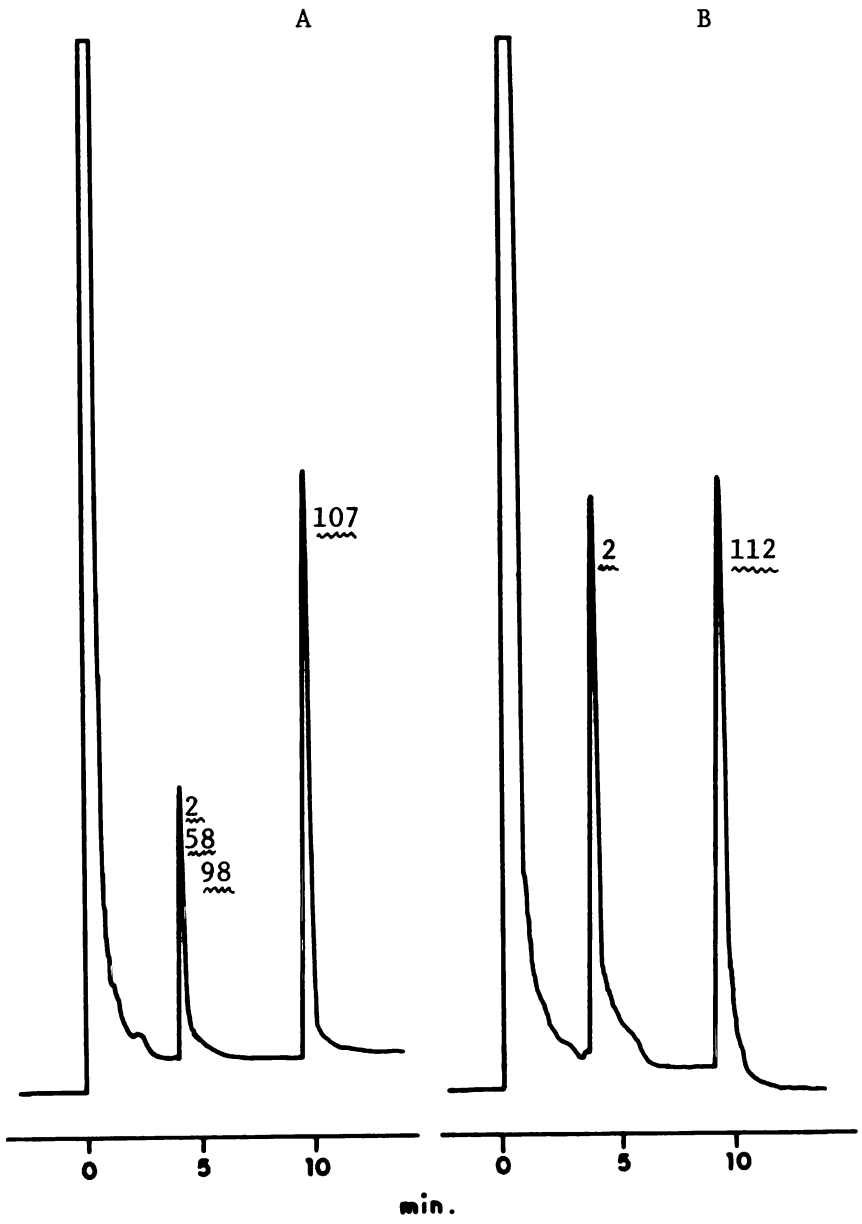


Figure XLII. The gc tracings (2% Dexyl, 160°C - 1 min, ↑ 3.5°C min^{-1}) (A) of the CH_2Cl_2 extract from the metabolism of nornicotine in the presence of CN^-; (B) of the diethyl ether extract from the incubation of nornicotine in the presence of CN^-. The identity of each gc peak as determined by gc retention time and gcms is indicated in the figure: 2, nornicotine; 58, myosmine; 98, 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline; 107, N-cyanomethylnornicotine; 112, N-(1-cyanoethyl)nornicotine.

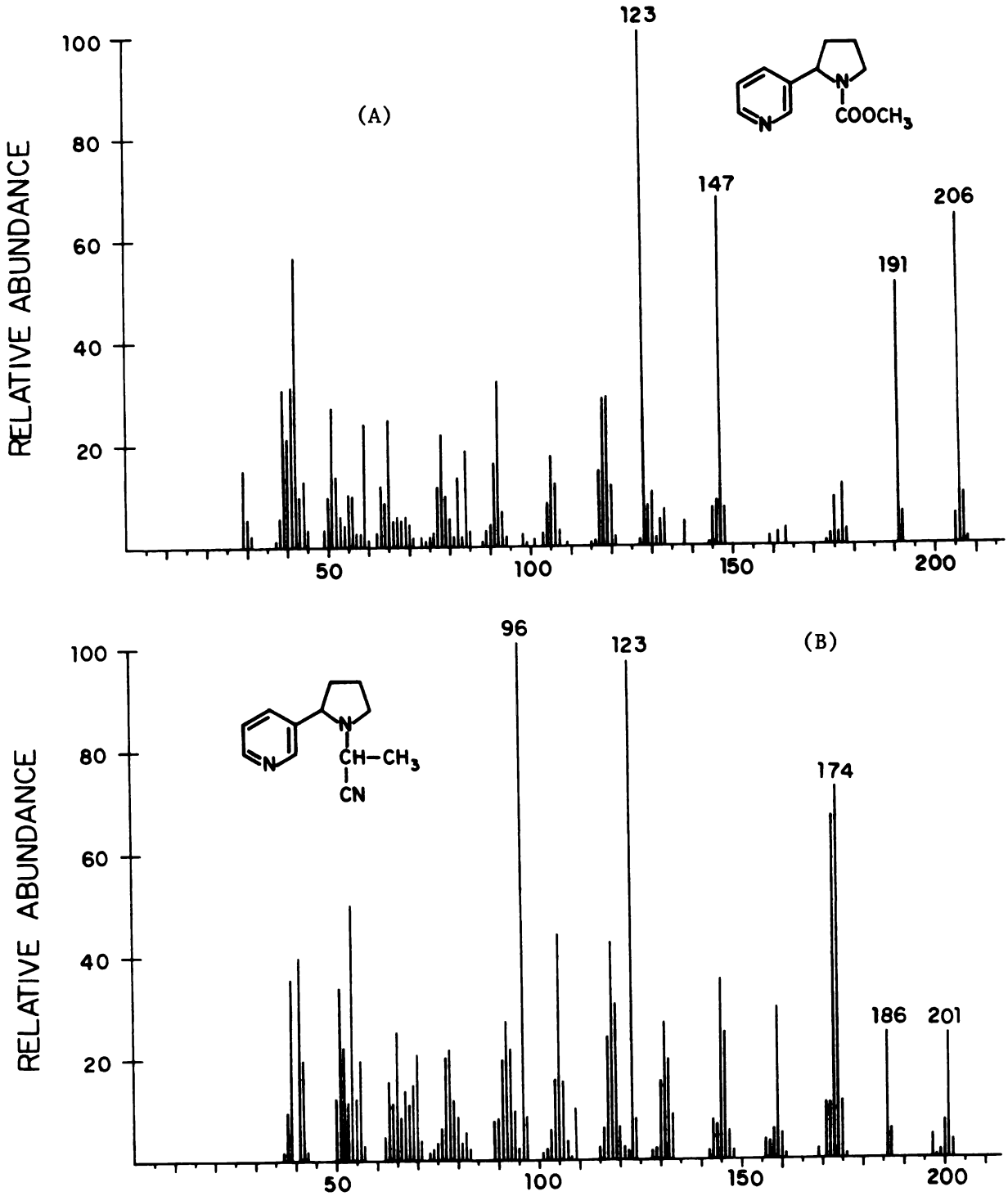
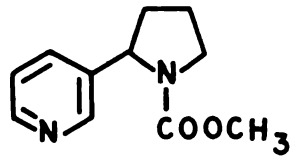
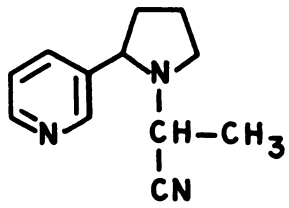


Figure XLIII. The ei mass spectra of artifacts seen in nornicotine control experiments: (A) the eims of N-carbomethoxynornicotine; (B) the eims of N-(1-cyanoethyl)nornicotine.

Figure XVII, the eims of N-carbethoxynornicotine).



111

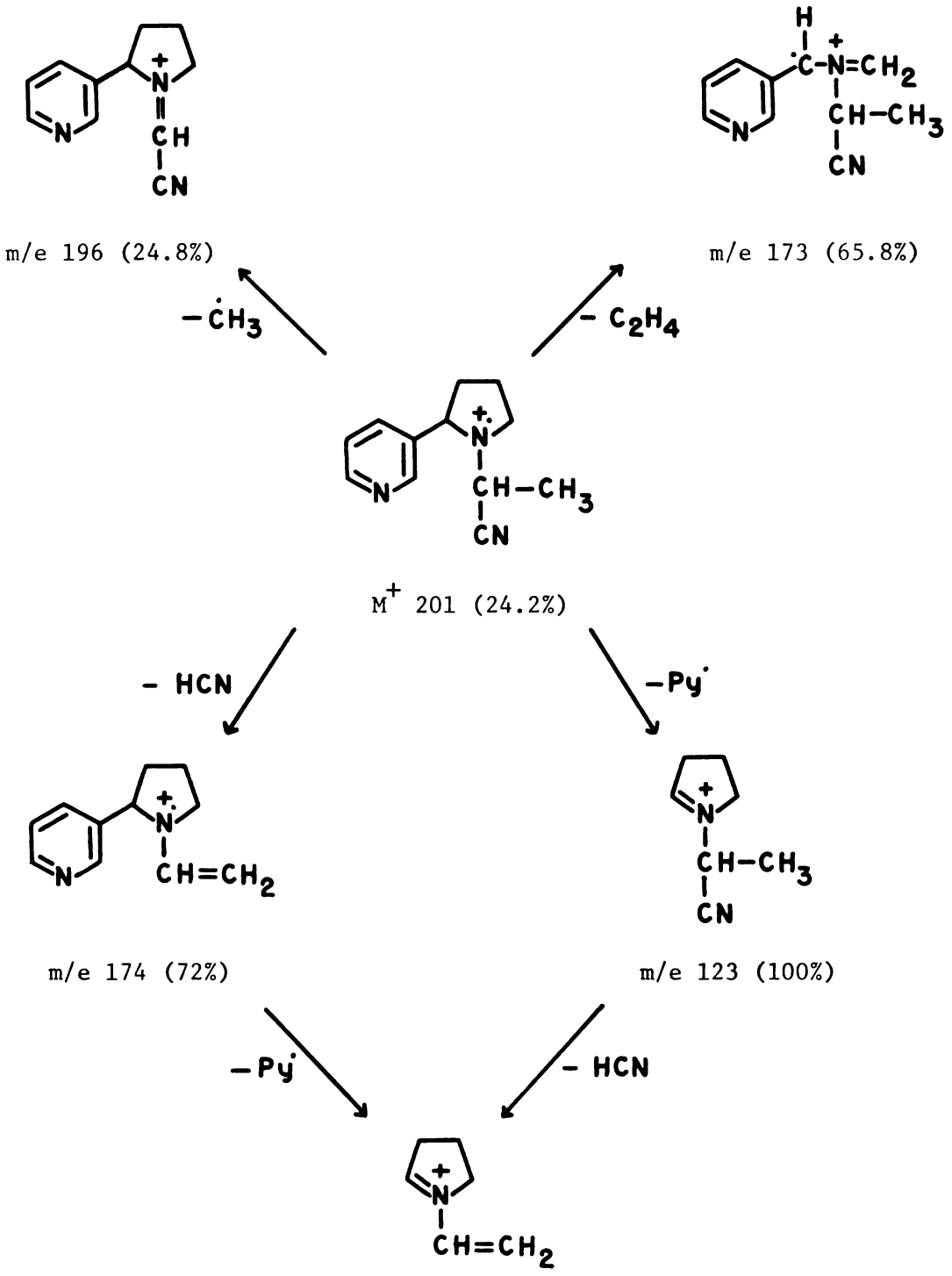


112

This same compound 111 was also the only product observed when nornicotine in CH_2Cl_2 was agitated at room temperature with an aqueous solution of sodium cyanide overnight. It is not yet clear how this carbamate 111 was formed but it seems likely to result from a reaction of nornicotine directly with a contaminant in methylene chloride without the intervention of cyanide ion.

When diethyl ether was used as the extracting solvent for the control experiments with nornicotine neither N-cyanomethylnornicotine nor the carbamate 111 was detected. However, of the material recovered, 44% was nornicotine and about 55% was a new artifact N-(1-cyanoethyl)-nornicotine (112), identified by its gc-eims (Figures XLII-B and XLIII-B). The ei-mass spectrum of 112 showed the general fragmentation pattern of a cyanonicotine derivative: a prominent molecular ion is seen at m/e 201 (24.2%), loss of the pyridine moiety from the molecular ion was seen to give the base peak at m/e 123 and loss of HCN (prominent

where there is a β proton) gave ions at m/e 174 (72%) and m/e 96 (100%).



That the control experiments with nornicotine using methylene chloride as the extracting solvent showed the formation of N-cyanomethylnornicotine while the control experiments using ether showed no cyanomethyl compound at all, indicates that the extra source of formaldehyde comes mainly from the extracting solvent methylene chloride and not from the liver fractions. Similarly, the formation of large amount of the cyanoethyl compound 112 when ether is used as the extracting solvent indicates that this solvent is contaminated with acetaldehyde. Since the formation of 112 does not interfere in the analysis of the cyanomethyl compound, incubates were subsequently extracted with freshly opened cans of ether rather than methylene chloride.

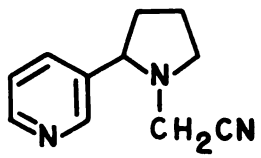
The analyses of the nicotine incubations using diethyl ether as the extracting solvent consistently showed that large amounts of N-cyanomethylnornicotine were still formed, proving that N-cyanomethylnornicotine is indeed the result of a metabolic process rather than an artifact of the work up conditions.

d. Proposed pathways for the formation of N-cyanomethylnornicotine.

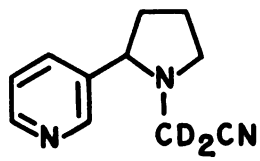
Although the control experiments demonstrated unequivocally that N-cyanomethylnornicotine (107) is formed from the metabolism of nicotine in the presence of cyanide ion, there still remained some ambiguity as to the pathway of its formation. As can be seen from figure XLI, there are at least two routes for the formation of N-cyanomethylnornicotine under biological conditions. One is the direct route where the intermediate N-hydroxymethylnornicotine (55) is formed and subsequently loses the hydroxyl group without any prior carbon-nitrogen bond breaking to form the methyleniminium ion 108 which is trapped by cyanide ion. The other route is an indirect pathway where nornicotine and formaldehyde (both are known metabolic products of nicotine metabolism) condense to form the carbinolamine 55 and subsequently the iminium ion 108 and the cyanomethyl compound 107. The indirect pathway involves the breaking of the N-C bond of nicotine at some step. This might occur by the elimination of formaldehyde from the intermediate hydroxymethylnornicotine but other mechanisms are also possible.

In order to determine whether the direct or indirect mechanism actually predominates, a 10 fold molar excess of formaldehyde was added to the incubation of nicotine-N-methyl-d_3. Thus the added formaldehyde is expected to dilute any metabolically formed DCDO at least 50 fold

and the amount of N-cyanomethyl-d_2-nornicotine (107a) formed should be negligible compared to the amount of N-cyanomethyl-d_0-nornicotine (107) formed if the indirect route is dominant.



107



107a

The gc-ei mass spectrum (Figure XLIV) of the cyanomethyl compound obtained from this experiment showed it to be a one to one mixture of N-cyanomethyl-d_0- and N-cyanomethyl-d_2-nornicotine (m/e 109 vs 111). Thus to a significant extent, N-cyanomethylnornicotine appears to be formed without prior N-C bond cleavage suggesting that the reactive methyleniminium ion 108 is generated at least in part by the direct pathway in the course of the *in vitro* metabolism of nicotine. However this conclusion is based on the assumptions that the added formaldehyde uniformly dilutes the metabolic formaldehyde pool and that there is a substantial amount of added formaldehyde in the incubation mixture throughout the experiment.

There does not seem to be any obvious way to verify whether or not the added formaldehyde was evenly distributed with respect to metabolic

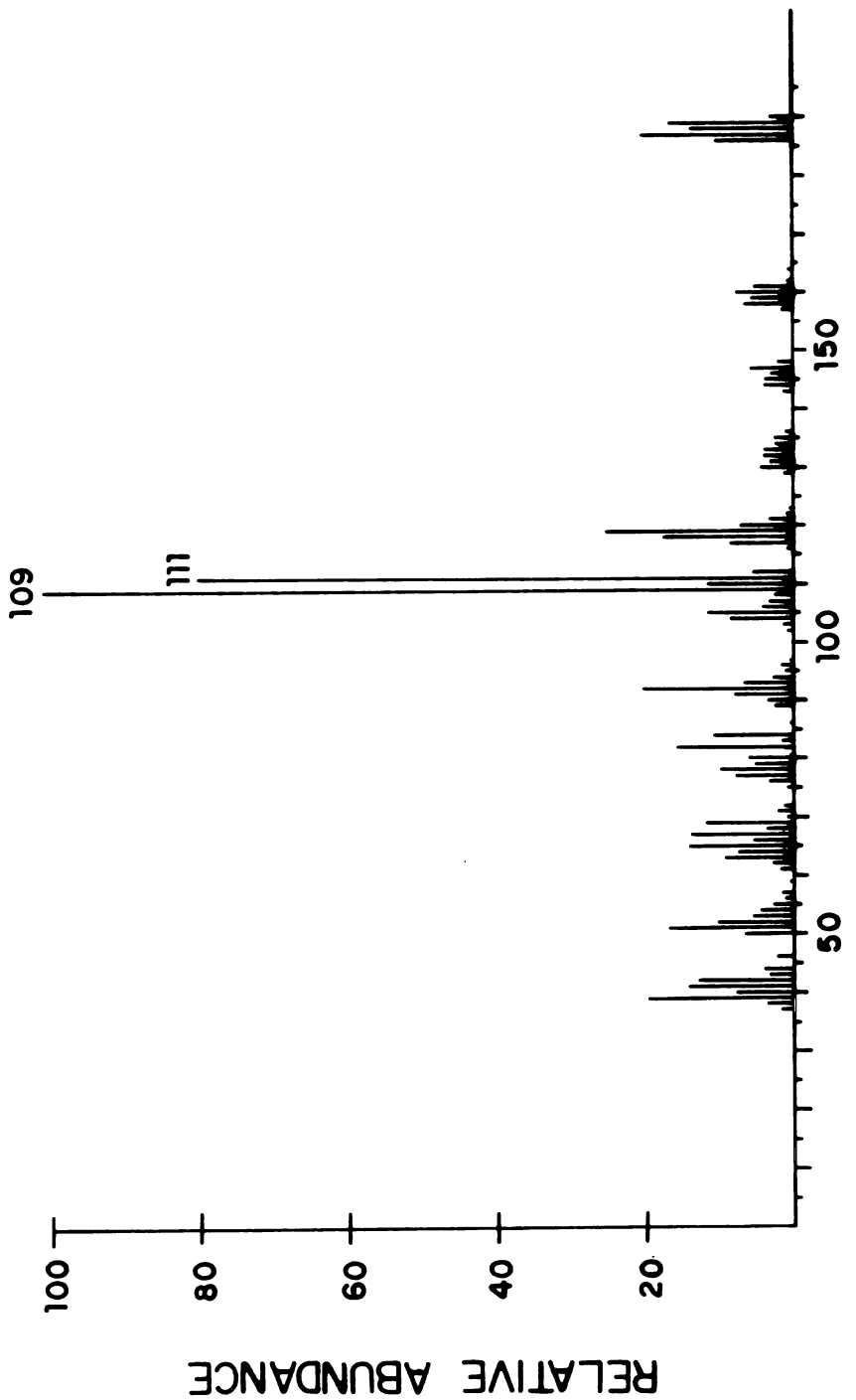


Figure XLIV. The ei mass spectrum of N-cyanomethyl-d_0- and N-cyanomethyl-d_2-nornicotine mixture from the metabolism of nicotine-N-methyl-d_3 in the presence of CN^- and added HCHO.

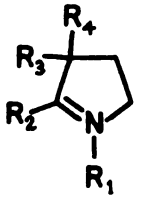
deuterated formaldehyde. However, according to a recent study[161] on *in vitro* N-demethylation by liver microsomes and endoplasmic reticulum segments in the presence of added mitochondria, formaldehyde produced by the mixed function oxidase is handled differently from formaldehyde added to the medium. The metabolic formaldehyde is oxidized by mitochondria much more quickly because the liver microsomes have the tendency to align themselves close to the mitochondria when present in the same medium. Consequently metabolic formaldehyde is released to the medium adjacent to the mitochondria.

While this example cannot be directly applied to the nicotine case, it does illustrate that it is dangerous to assume that metabolic oxidations can be treated as homogeneous reaction mixtures.

The question of whether there is still excess formaldehyde at the end of the incubation was answered more easily. Nicotine-N-methyl-d_3 was incubated with added formaldehyde and cyanide ion and nornicotine-2',3',3'-d_3 (2c) was added at the end of the incubation as a trap for formaldehyde. The ether extract obtained from this experiment was analyzed by the selected ion recording (SIR) technique (see Section C.2.f.) The results are summarized in Table VIII column A.

A large ion current recorded at mass 112 (ion 113b) was observed which indicates that cyanomethylnornicotine-2',3',3'-d_3 was the major

component of the cyanomethylnornicotine mixture as expected. Furthermore, from the gc tracing it was observed that all of the added nornicotine-2',3',3'-d_3 was consumed. This showed that a substantial amount of the formaldehyde added at the beginning of the incubation was still present. The ion currents recorded at mass 109 and mass 111 (ions 113 and 113a) are of about equal intensities indicating that cyanomethyl-d_0 and cyanomethyl-d_2-nornicotine were formed in approximately equal amounts. This confirms the earlier results with added formaldehyde where it was concluded that at least some of the cyanomethylnornicotine formed metabolically is produced by the direct mechanism which does not involve N-C bond cleavage.



113 : $R_1 = CH_2CN$, $R_2 = R_3 = R_4 = H$

113a : $R_1 = CD_2CN$, $R_2 = R_3 = R_4 = H$

113b : $R_1 = CH_2CN$, $R_2 = R_3 = R_4 = D$

113c : $R_1 = CD_2CN$, $R_2 = R_3 = R_4 = D$

The results from the experiments where excess formaldehyde was used to trap metabolically formed nornicotine were confirmed by an inverse experiment where excess nornicotine-2',3',3'-d_3 was used to trap metabolically formed formaldehyde-d_2.

The results from the SIR analysis of the co-incubation of nicotine-

Table VIII

The selected ion current record of the cyanomethylnornicotine gc peak from the incubation of nicotine-N-methyl-d_3 in the presence of cyanide ion and

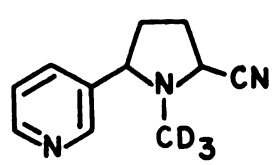
A. added HCHO and nornicotine-2',3',3'-d_3 added at the end of the incubation.

B. equal molar nornicotine-2',3',3'-d_3 as cosubstrate.

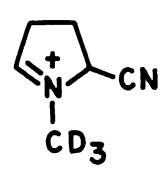
		Ratio %	
m/e	ion	A	B
109	113	33	5
111	113a	30	100
112	113b	100	39
114	113c	5	53

N-methyl-d_3 and nornicotine-2',3',3'-d_3 are shown in column B of Table VIII.

The ion current seen at mass 112 from the cyanomethylnornicotine gc peak is largely due to the base peak (ion 114) of 5'-cyanonicotine-N-methyl-d_3 (104a).



104a



114, m/e 112

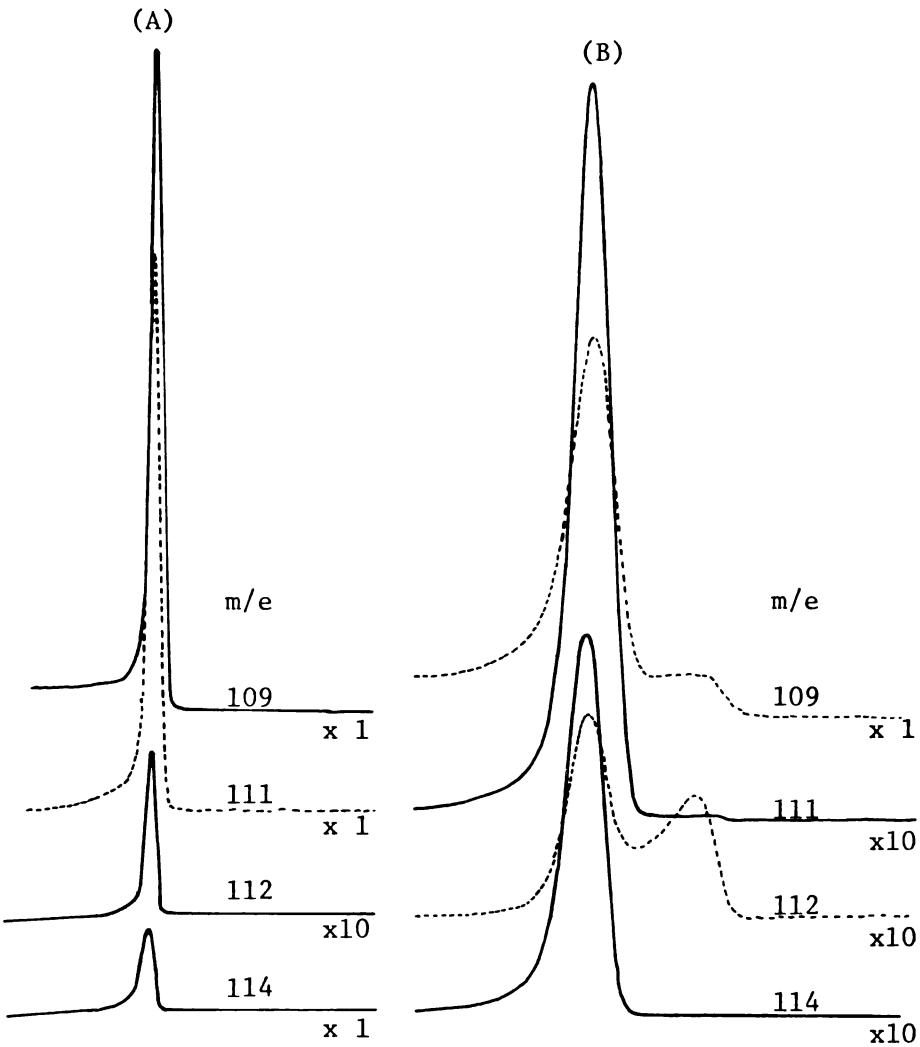


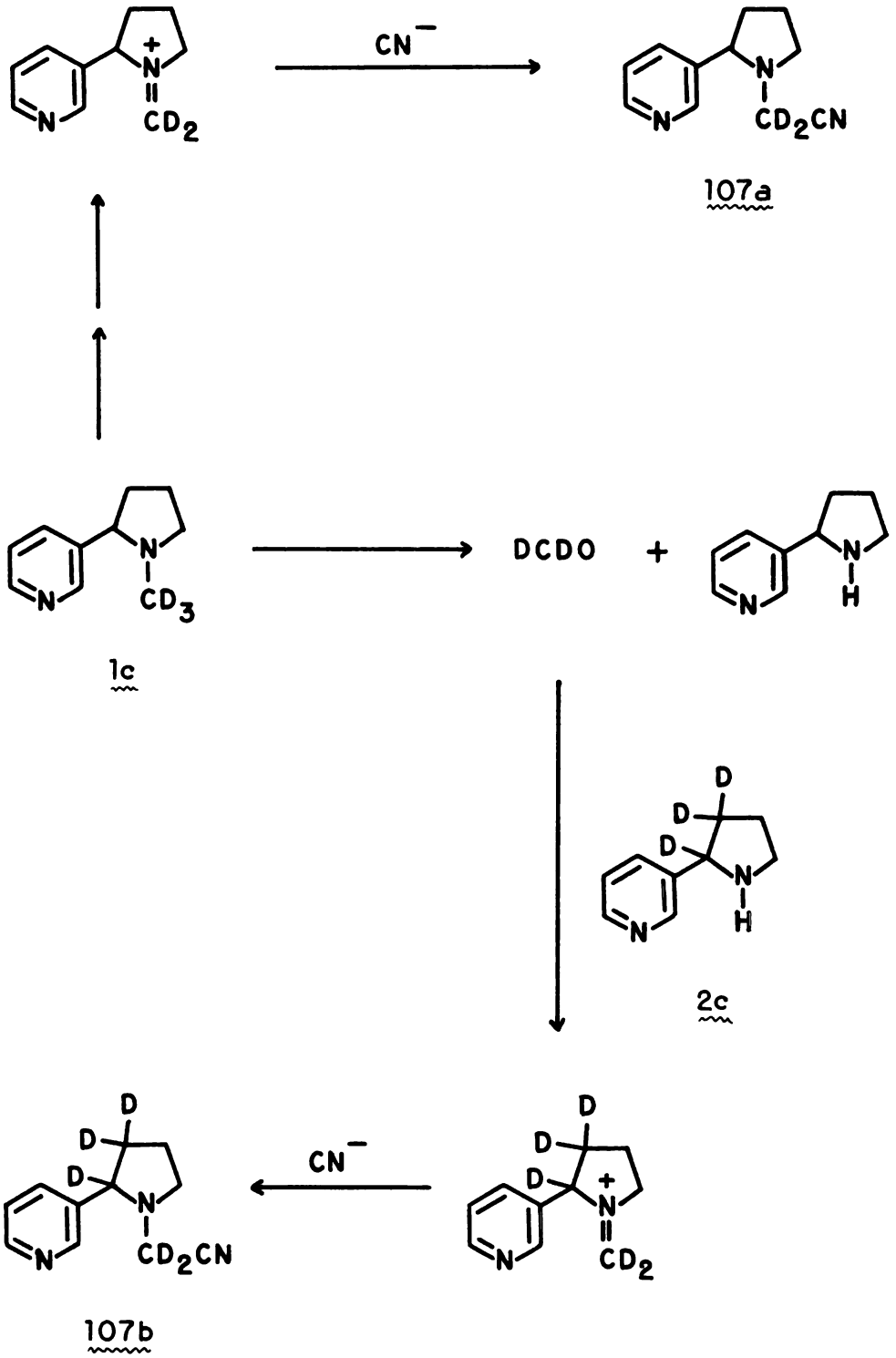
Figure XLV. The selected ion current records of the N-cyanomethyl-nornicotine gc peak from the incubation of nicotine-N-methyl-d_3 in the presence of cyanide ion and

(A) added HCHO with nornicotine-2',3',3'-d_3 added at the end of the incubation.

(B) equal molar nornicotine-2',3',3'-d_3 as cosubstrate.

This was confirmed by a gc-eims which shows that other major fragment ions expected for 104a were also present in the proper ratios. The gc peak for 5'-cyanonicotine has a pronounced tendency to tail. Apparently the 104a gc peak adsorbs on the dexyl column and is subsequently released and carried along by the elution of the cyanomethylnornicotine peak. This phenomenon was observed in virtually every case where substantial amounts of 5'-cyanonicotine were present and the dexyl column was used.

The ion currents recorded at mass 111 and mass 114 are a measure of the relative amounts of cyanomethylnornicotine which are formed by the direct and indirect pathway respectively. The amount of nornicotine-d_0 produced by metabolic N-demethylation of nicotine-N-methyl-d_3 should be too small to compete with the added nornicotine-2',3',3'-d_3 for metabolic formaldehyde-d_2. Thus only cyanomethylnornicotine-d_5 (107b) should be formed by the indirect pathway while cyanomethyl-d_2-nornicotine (107a) is produced by the direct pathway (see the scheme shown on the next page). The results of the experiment shown on column B of Table VIII agree with those from the experiments with added formaldehyde. It was found that a substantial amount of the cyanomethyl compound is formed via the direct pathway thus suggesting that the methyleniminium ion 108 is a true intermediate in the in vitro metabolism of nicotine by


CN⁻
CD2
CD2CN
107a
CD3
1c
DCDO
+
D
D
D
2c
CN⁻
CD2CN
107b
CD2

rabbit liver preparations. As in the earlier experiments this conclusion is based on two assumptions: (i) the added trap, nornicotine-2',3',3'-d_3, is mixed into the metabolic nornicotine-d_0 pool and (ii) there is still excess nornicotine-2',3',3'-d_3 left at the end of the incubation. As was the case in the experiments with added formaldehyde, the first assumption is difficult to confirm. However, the second assumption was shown to be correct by gcms. Although there was only a small amount of nornicotine left at the end of the incubation, a SIR analysis of the nornicotine peak showed it to be an approximately 6 to 1 mixture of nornicotine-2',3',3'-d_3 and nornicotine-d_0.

e. Factors governing the amounts of metabolites formed.

Estimation from the gc tracing of the incubate extracts showed that the extent of metabolism was generally from 30% to 50%. Although there was a considerable variability in both the extent of metabolism and the relative amounts of metabolites formed from one experiment to another, the overall results were remarkably consistent.

The relative amount of cotinine formed varied from one experiment to another. As expected, incubations with microsomal fractions gave no cotinine at all. This is consistent with the fact that the soluble enzyme "aldehyde oxidase" (which may in fact be an alcohol dehydrogenase) is not present in the 100,000 x g pellets. The variation among the results from 10,000 x g supernatant fractions may be in part due to variance in the extent of inhibition of the aldehyde oxidases by cyanide ion. According to Hurwitz,[156] the extent of inhibition is linear with the preincubation time up to 20 minutes and reached 100% at 45 minutes. A preincubation time of 12 to 15 minutes would give from 60% to 80% inhibition. However the extent of inhibition in our experiments is probably lower because the cited values of inhibition are obtained from studies with partially purified enzymes.

The ratio of 5'-cyanonicotine to N-cyanomethylnornicotine was approximately 1 to 1 by peak height measurements. This ratio however is

larger by peak area since the 5'-cyanonicotine peak tails and overlaps partially with the cyanomethylnornicotine peak. The peak overlapping makes the extent of correction unknown.

Cyanomethylnornicotine sometimes was formed only in a very small amount as compared to that of 5'-cyanonicotine. As a result, only one broad peak or a large peak with a slight shoulder was observed. When the substrate was one of the various deuterium labeled nicotines, the detection of the corresponding cyanomethyl compound was not difficult since the two cyanonicotine isomers have different base peaks in their mass spectra (see Figures XXXVII-XXXIX). However when the substrate was nicotine-d_0, the detection of cyanomethylnornicotine by gc-eims on the 2% dexyl column was very difficult. In such cases, a 2% carbowax KOH column was used to confirm the formation of N-cyanomethylnornicotine. This gc column causes 5'-cyanonicotine to decompose by elimination of HCN but leaves cyanomethylnornicotine intact. Unfortunately, this basic column cannot be used in the analysis of cyanomethyl-d_2-nornicotine because deuterium exchange of the two methylene deuterons occurs.

There was no systematic difference in the ratio of the two isomeric cyanonicotine compounds or in the extent of metabolisn in going from the 10,000 x g preparations to the 100,000 x g preparations. However more variability was seen in the 100,000 x g preparations.

In those experiments where formaldehyde and nornicotine-2',3',3'-d_3 were added to trap the metabolically formed nornicotine and formaldehyde-d_2 respectively, the amount of the cyanomethyl compound was consistently much greater than that of 5'-cyanonicotine. The extent of metabolism remained approximately the same.

No systematic changes were observed in going from incubations carried out with racemic nicotine-2',5',5'-d_3 and racemic nicotine-N-CD_3 to incubations carried out with samples having the natural configuration (S)-nicotine-d_0 and (S)-nicotine-5',5'-d_2.

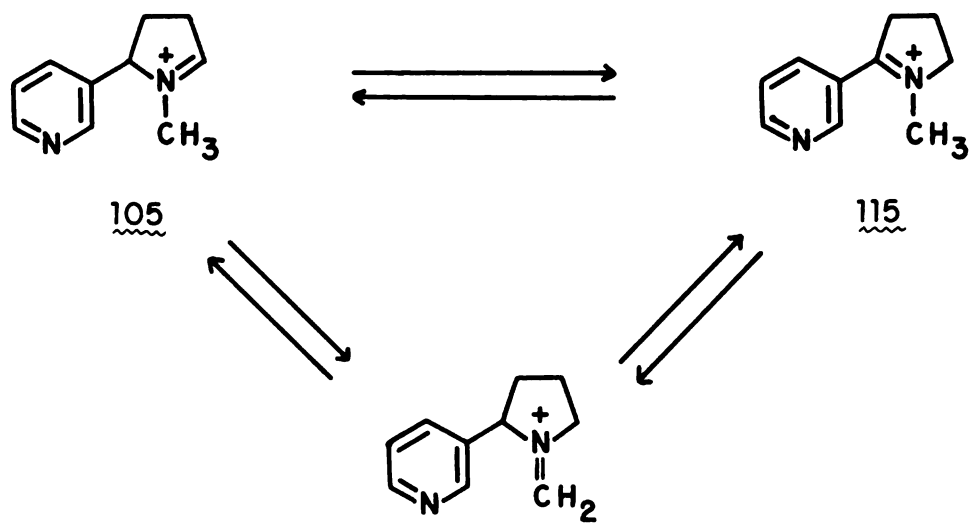
Our in vitro metabolic studies of nicotine were carried out with both the homogenate fraction and the microsomal fraction. Each was used for incubations carried out at two different pH values, at 7.4 and at 9. Since our studies deal with the capture of the methyleniminium ion 108 by cyanide ion, a change in pH of the incubation mixture would certainly be expected to affect the results. Since the pKa of HCN is 9.3, a pH change from 9 to 7.4 would decrease the concentration of cyanide ion forty fold. Thus the trapping of the iminium ions 105 and 108 is probably not as efficient at lower pH as it is at higher pH.

It is also expected that enzyme activity will change with pH. Since the pKa's of the functional groups at the binding site and at the catalytic site of an enzyme are among the factors governing the activity

of the enzyme, often an enzyme has a characteristic pH at which its activity is maximal. For example Das and Ziegler[162] reported that the pH optima for N-oxidation and N-demethylation of N,N-dimethylaminopyrine are 8.4 and 7.4, respectively. If the metabolic conversion does not involve a neutral substrate it seems reasonable that the optimum pH for an enzyme varies depending upon the pKa of the functional group of the substrate. In fact, for the metabolic N-demethylation process, several optimal pH values have been reported. Axelrod[163] found an optimum pH from 7.0 to 7.5 for morphine, ℓ-methadone and meperidine while Thompson and Holtzman[164] observed an optimum pH at 7.85 for the N-demethylation of ethylmorphine. According to Takemori and Mannering[165] the optimum pH is between 7.9 and 8.1 for the N-demethylation of narcotic drugs. Mao and Tardrew[166] reported that the maximum N-demethylation of Erythromycine A occurs from 7.8 to at least 8.2 and of 2'-propionylerythromycine A at pH 7.4. In spite of these reasons for expecting changes in metabolic activity with changes in pH, the in vitro nicotine trapping experiments did not show any observable differences between those carried out at pH 7.4 and those carried out at pH 9. However it should be noted that careful quantitation of the extent of metabolism was not done and small changes may have gone unnoticed.

In explaining the two products 5'-cyanonicotine (104) and 2'-cyano-

nicotine (106) obtained from the oxidation of nicotine by mercuric acetate and EDTA followed by the quenching of the intermediate mixture with cyanide ion, Sanders, et al.[153] suggested that the ratio of 5'-cyanonicotine to 2'-cyanonicotine is dependent on the pH of the reaction mixture to which cyanide ion is added. These workers seemed to implicate a pH dependent equilibrium between the $\Delta^{1'(5')}$-iminium ion and $\Delta^{1'(2')}$-iminium ion (105 and 115). If this implication is correct, then it is plausible that the methyleniminium ion 108 is included in this pH dependent equilibrium.



105 115

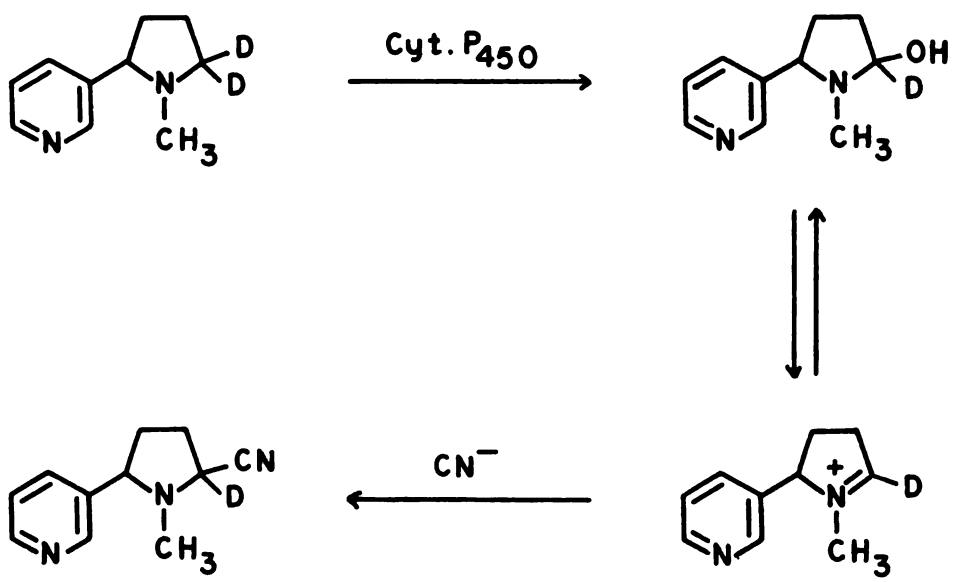
108

However, a close examination of the eims of various deuterated 5'-cyanonicotines and deuterated cyanomethylnornicotine obtained from incubation mixtures (Figures XXXVII-XXXIX) has revealed only the

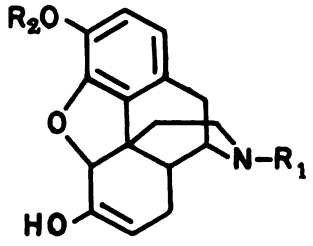
expected numbers of deuterium atoms in each case. This is not consistent with the equilibria shown since this would cause an exchange of the α-C deuterons with the protons from the medium. Furthermore we did not see any change in the relative amounts of 5'-cyanonicotine and N-cyanomethylnornicotine formed metabolically with pH, nor did we see any evidence for the metabolic formation of 2'-cyanonicotine.

f. Determination of the isotope effect in the metabolism at $C_{5'}$ of nicotine.

The first step in the metabolic transformation of nicotine (1) to cotinine (3) is believed to be an α-C-hydroxylation.[7, 76] In the presence of cyanide ion the 5'-hydroxynicotine (45) formed can be trapped as 5'-cyanonicotine (104) due to its equilibrium with the iminium ion 105. If a mixture of 5',5'-d_2 labeled and unlabeled nicotine is used in the incubation the ratio of the amount of deuterated and undeuterated 5'-cyanonicotine formed should reflect the deuterium isotope effect of the initial α-C-hydroxylation. The isotope effect determined from this experiment can be compared to those determined for metabolic N-dealkylations since an initial α-C-hydroxylation is believed to be the first step in this type of metabolic transformation too.



There are several studies reported in the literature of deuterium isotope effects in microsomal oxidative demethylations when α-C-H bonds are replaced by α-C-D bonds. The isotope effects (k_H/k_D) reported are usually less than 2. By measuring the amount of formaldehyde evolved Elison, et al.[167] observed that morphine (46) underwent N-demethylation 1.4 times as fast as morphine-N-methyl-d_3 (46a), while Thompson and Holtzman[164] determined the average isotope effects in the N-demethylation of ethylmorphine-N-methyl-d_3 (11b) to be about 1.25.



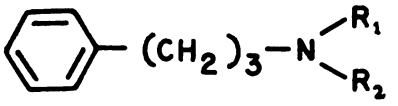
46 : $R_1 = CH_3$, $R_2 = H$

46a : $R_1 = CD_3$, $R_2 = H$

11b : $R_1 = CD_3$, $R_2 = -CH_2CH_3$

By replacing only one of the methyl groups of N,N-dimethyl-3-phenylpropane (116a) with a trideuteriomethyl moiety, a deuterium isotope effect was determined from the ratio of deuterio to protio products (116b and 116c) formed from the same compound. In this way any isotope effects due to changes in the basicity of nitrogen upon substituting a CH_3 group with a CD_3 group were avoided. Abdel-Monem[168] reported the k_H/k_D value in this case to be 1.45 when mouse microsomes were used and

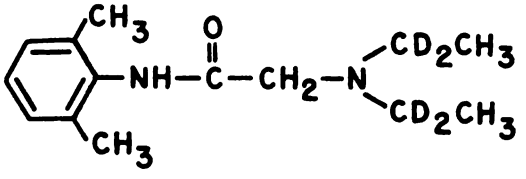
1.32 when rat microsomes were used. Nelson et al.[169] reported that the isotope effect in the N-de-ethylation of lidocaine-d_4 (117a) was 1.49 which is similar to those found for N-demethylations.



116a : $R_1 = R_2 = CH_3$

116b : $R_1 = H$, $R_2 = CD_3$

116c : $R_1 = CH_3$, $R_2 = H$



117a

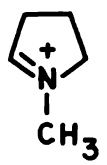
These k_H/k_D values are small as compared to the normal primary deuterium isotope effects (6-10) observed for most chemical reactions. The minimum theoretical k_H/k_D value is equal to the square root of the mass ratio m_D/m_H which is 1.4.[170] This has led many investigators to conclude that primary deuterium isotope effects are involved in enzymatic oxidative N-dealkylations and that the breaking of the α-C-H bond is the rate determining step.[164,167-169]

Our isotope effect studies were carried out with the microsomal fraction from rabbit liver. By completely removing the soluble enzymes the loss of 5'-hydroxynicotine (45) via oxidation to cotinine is minimized and thus it should eventually be trapped quantitatively as

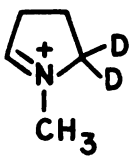
5'-cyanonicotine (104). The presence of cyanide ion in the system is known not to affect the activity of the microsomal cytochrome P_{450} enzymes[176] and thus the isotope effect should not be affected either.[62]

Approximately 1:1 and 2:1 mixtures of nicotine and deuterated nicotines were incubated and the 5'-cyanonicotine formed was analyzed by gc-eims selected ion recording (SIR).[171] In this method of analysis the mass spectrometer is used as a mass specific detector at a limited number of masses. This is accomplished by holding the mass spectrometer's magnetic field constant while switching the accelerating voltage between the values necessary to bring the desired ions into focus. If the switching rate is fast enough one obtains an essentially continuous record of ion current intensities at a few selected masses vs time. This method of isotope ratio determination is superior to magnetic scanning because of its greater sensitivity and because there is normally a slight difference in gc retention time between deuterated and undeuterated compounds. This difference while not usually enough to separate the gas chromatogram into two peaks is enough to cause the instantaneous ratio of deuterated to undeuterated compound being eluted from the gc column to change continuously throughout the width of the gc peak. Thus magnetic scanning which gives an essentially instantaneous sampling of the gc peak is not likely to give an accurate measurement of the isotope

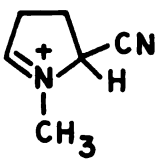
ratio. However by using the technique of SIR the total ion current record at each mass is available and the isotope ratio can be determined from the peak areas at each mass. Unfortunately by changing the accelerating voltage the sensitivity of the mass spectrometer is also changed and if accurate quantitation is desired a standard curve must be constructed.



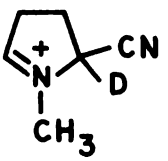
m/e 84



m/e 86



m/e 109



m/e 110

In an initial experiment, the selected ion recording method was used to analyze the results from the incubation of an approximate 1:1 ratio of (S)-nicotine (1) and (S)-nicotine-5',5'-d_2 (1a). By monitoring m/e 84 which is the base peak in the eims of 1 and m/e 86 which is the base peak in the eims of 1a, an ion current ratio of 0.83 (m/e 84 to m/e 86) was obtained (see Figure XLVI-A). This value was taken as the ratio of the amount of nicotine-d_0 to the amount of nicotine-d_2 present. By monitoring m/e 109 which is the base peak in the eims of 5'-cyanonicotine (104) and m/e 110 which is the base peak in the eims of 5'-cyanonicotine-5'-d_1 (104b), a ratio of 0.74 (m/e 109 to m/e 110) was found (see Figure XLVI-B). Before this value can be taken as the ratio of the

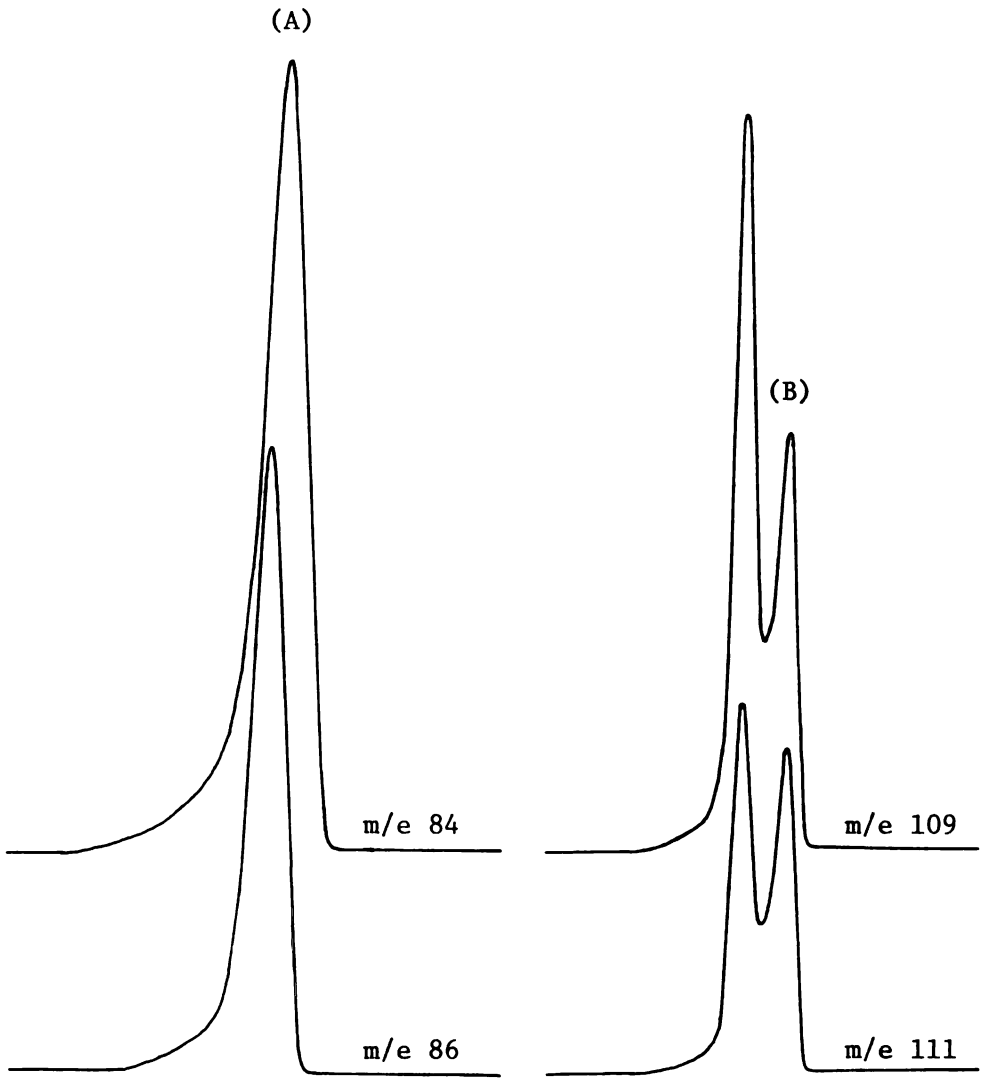


Figure XLVI. The selected ion current records of the (A) nicotine gc peak and (B) 5'-cyanonicotine gc peak from the incubation of an approximately 2:1 mixture of nicotine-d_0 and nicotine-2',5',5'-d_3 in the presence of $CN^{-\cdot}$.

amount of 5'-cyanonicotine to the amount of 5'-cyanonicotine-5'-d_1 present it must be corrected for the natural ^{13}C abundance at m/e 110 in the eims of 5'-cyanonicotine. This was done by obtaining the selected ion record for 5'-cyanonicotine; the ratio of m/e 110 to m/e 109 was found to be 0.074 (theoretical for C_6N_2, 0.074). After making this correction, the isotope effect was calculated to be 0.96. Since the pure 5'-cyanonicotines were not available in adequate amounts for the construction of a standard curve this calculation may be as much as 10% in error.

In a second series of experiments the problem of having to correct for the ^{13}C was avoided by using nicotine-2',5',5'-d_3 (1b) as the labeled substrate. This experiment also allows us to observe the effect of a change in asymmetry at $C_{2'}$ since the nicotine-d_0 used was the pure (S) form while the nicotine-2',5',5'-d_3 used was racemic. This experiment was carried out using two different ratios (m/e 84 to m/e 87 = 0.86 and 1.32) of natural (S)-nicotine to racemic nicotine-2',5',5'-d_3. A selected ion analysis showed a ratio of 0.86 and 1.33 respectively (m/e 109 to m/e 111) for the relative amounts of 5'-cyanonicotine-d_0 to 5'-cyanonicotine-2',5'-d_2 formed in each experiment. This indicates an isotope effect of 1.0 ± .1 for both ratios.

The results from these experiments show a surprising lack of specificity; neither the presence of deuterium at the site of bond breaking

nor a change in stereochemistry of the molecule appears to have had any influence on the rate of nicotine metabolism at the $C_{5'}$ position. The reason for this unexpected result is as yet unknown.

g. Confirmation of nornicotine as a nicotine metabolite.

Early investigators concerned with the metabolism of nicotine did not find any evidence that nicotine underwent enzymatic N-demethylation to form nornicotine. Larson and Haag[172] reported that no demethylated products were detected in urine of dogs given nicotine methiodide, nicotine isomethiodide or nicotine dimethiodide. Furthermore, a number of studies with randomly ^{14}C labeled nicotine showed no radioactivity in the respiratory carbon dioxide from rats, mice and dogs.[173] On the basis of these early studies it was concluded that no N-demethylation of nicotine occurred in biological systems. However, in later experiments with nicotine specifically labeled at the N-methyl group with ^{14}C, approximately 5% to 15% of the original activity was reported to be in the expired carbon dioxide.[81,91,174] Further studies both *in vivo* and *in vitro* with nicotine-N-$^{14}CH_3$ and nicotine-2'-^{14}C as substrates have demonstrated not only the formation of up to 20% of $^{14}CO_2$ or $H^{14}CHO$ but also the presence of nornicotine among the metabolites of nicotine.[91-93] The negative results in studies with randomly labeled nicotine-^{14}C may have been due to the very low activity located at the N-methyl carbon. However this issue even now is not considered to be settled. There have been a number of *in vivo* and *in vitro* studies with nicotine-N-$^{14}CH_3$ in which neither nornicotine nor a significant amount of $^{14}CO_2$ was

detected.[7, 175, 68]

Gorrod and his coworkers[74] have also failed to detect nornicotine in in vitro studies by glc even though the method is capable of detecting submicrogram levels. Very recently, Gorrod and Jenner in an extensive review of the metabolism of tobacco alkaloids[17] again raised the question as to whether nornicotine is truly a hepatic metabolite of nicotine. These authors reasoned that since demethylcotinine (49) is a metabolite of nicotine and radioactive expired carbon dioxide was detected only after 30 minutes of incubation,[175] it was the N-demethylation of cotinine to demethylcotinine which was responsible for the $^{14}CO_2$ detected. These authors concluded that if the N-demethylation of nicotine to nornicotine indeed occurs, it must occur in the kidneys.

A careful examination of the experimental details of all the above cited studies reveals that either chloroform, methanol or a mixture of the two solvents was used in the isolation of the metabolites. In those studies where successful detection of nornicotine was reported, milligram levels of nicotine were used while in those studies where nornicotine was not seen, only microgram levels of nicotine were used. In the studies by Gorrod, Jenner and their coworkers, 20 ml of methanol was used to stop the incubation of 5 μmoles of nicotine.

In our own studies we have observed that small amounts of nornico-

tine are difficult to recover by extraction, apparently because of the propensity of nornicotine to react with the trace impurities present in the commonly employed solvents. In our synthesis of nornicotine by a sodium borohydride reduction of myosmine in methanol-water, it was observed that a small amount of nicotine is also produced. Since formaldehyde is known to be a trace impurity in methanol, presumably the nicotine was formed by the reaction of nornicotine with formaldehyde to form the methyleniminium ion 108 which is then reduced with sodium borohydride. What the fate of the methyleniminium ion is in the absence of $NaBH_4$ and in the presence of a biological medium or other solvent impurities is unknown. A further example of nornicotine by-products being formed from solvent contaminants is discussed in the "genesis of N-cyanomethylnornicotine" section of this dissertation. It should also be noted that chloroform has been shown to react slowly with secondary amines.[160]

Another reason why nornicotine may be difficult to detect in small scale biological studies is that much of the nornicotine formed may undergo further metabolism. Our own work clearly illustrates some of the difficulties in recovering nornicotine from the biological medium. In a typical study where a relatively large amount of a 1:1 mixture of nicotine-N-CD_3 (1c) and nornicotine-2',3',3'-d_3 (2c) were co-incubated in

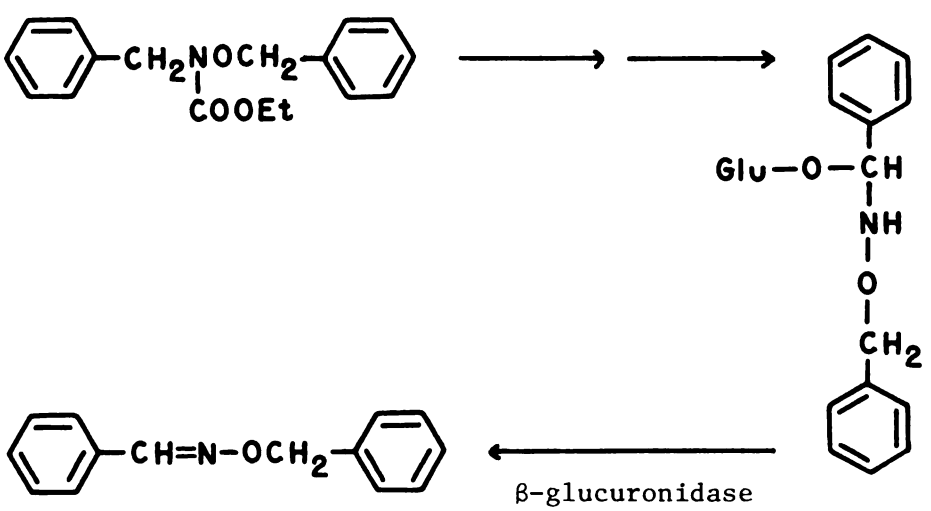
the presence of cyanide ion, the ratio of the substrates recovered at the end of the incubation period was more than 20:1. At least 10% of the missing nornicotine was accounted for by the metabolites myosmine and 2'-(3-pyridyl)-$\Delta^{5'}$-pyrroline (98). Another 6% was accounted for as a cyanopropylnornicotine which presumably arises from a three carbon aldehyde present as an impurity in the work-up solvent diethyl ether. The remaining 84% of the missing nornicotine was unaccounted for and presumably was lost as other metabolites or due to reactions with other solvent impurities.

In experiments where nicotine was incubated alone in the presence of cyanide ion we were rarely able to detect the formation of nornicotine itself. However we were able to consistently detect the presence of the Δ^5-pyrroline 98 which as we have shown (Section C.1.b.) is a nornicotine metabolite.

It is believed that the presence of cyanide ion does not affect the biotransformations mediated by the cytochrome P_{450} enzyme systems.[176] If this is true those experiments discussed in the previous sections where formaldehyde was added to trap metabolically formed nornicotine and where nornicotine-2',3',3'-d_3 was used to trap metabolically formed formaldehyde have shown that nornicotine is a metabolite of nicotine and that enzymatic N-demethylation of nicotine does take place in the liver.

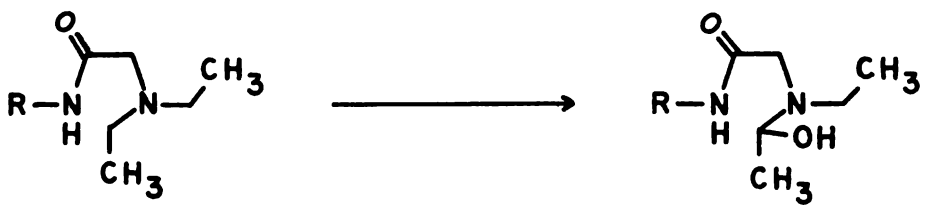
Conclusion.

The bulk of evidence in the literature indicates that the metabolic hydroxylation at carbon atoms α to a nitrogen atom is a very common biotransformation in drug metabolism. The product of this biotransformation pathway in the case of cyclic amines may be a lactam, resulting from further metabolism of the α-hydroxylated intermediate or a dealkylated product (together with an aldehyde or ketone) which results from the breakdown of the inital intermediate. There are now a number of examples where the product obtained is the result of a simple dehydration of the initially formed intermediate. The nornicotine metabolites myosmine and the isomeric Δ^5-pyrroline compound are examples which are first reported in this dissertation. The formation of an oxime in the metabolism of benzyl N-benzylcarbethoxamate[177] may be another example.



In addition to the elimination of water from the hydroxylated intermediate the reversible elimination of OH^- to form an iminium ion may occur. Literature evidence for iminium ions is based on the formation of two types of products.

(1) When a built-in nucleophile is available, a stable cyclic product is formed spontaneously, such as in the metabolism of lidocaine (117)[178] and in the metabolism of Bisolvon (118).[179]



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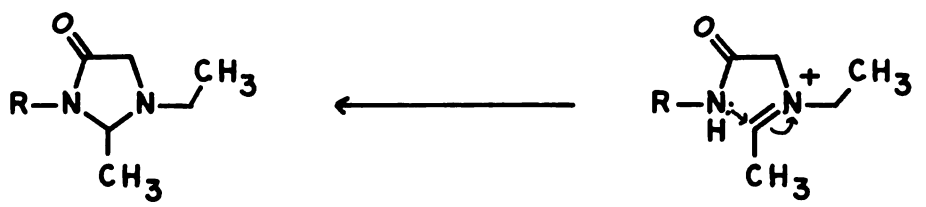

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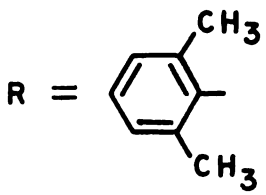
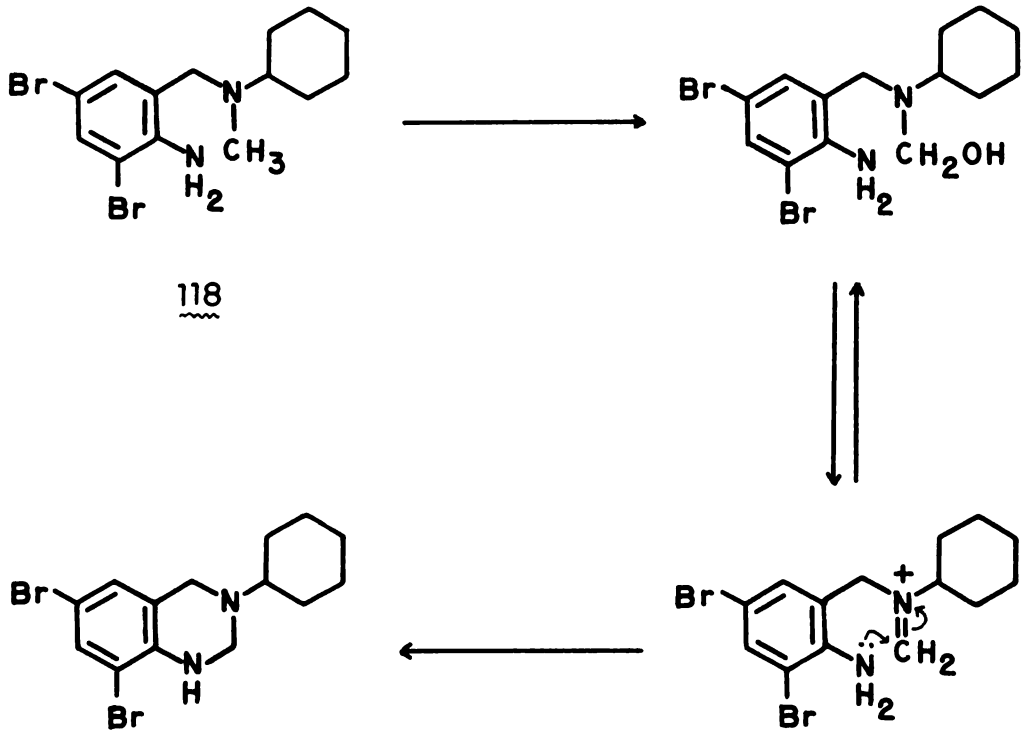


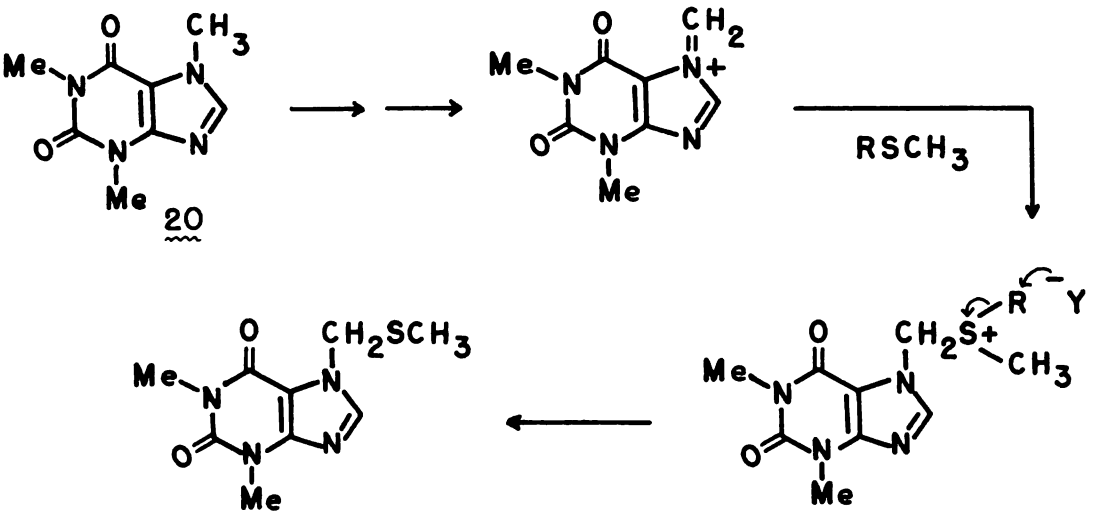


118

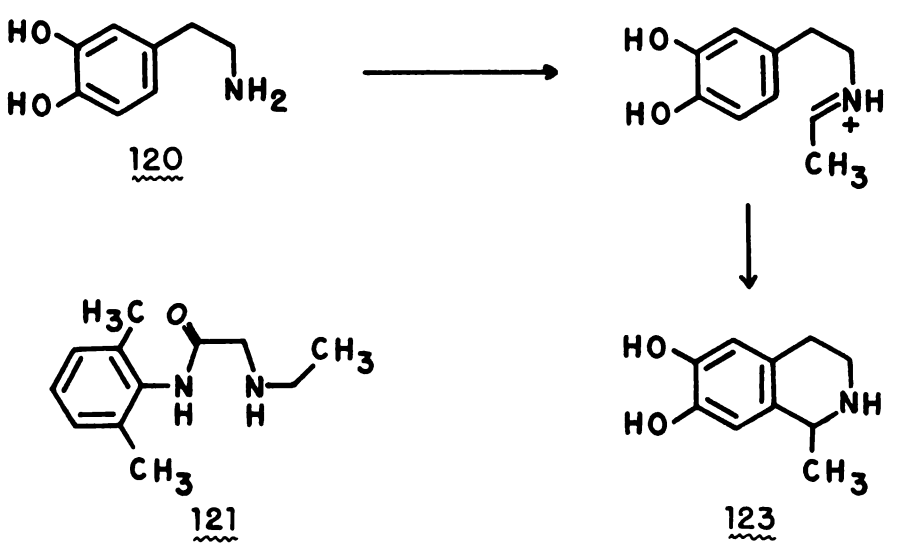
(2) When there is no built-in nucleophile available, any nucleophile present in the medium may trap the iminium ion.

The trapping of nicotine $\Delta^{1'(5')}$-iminium ion with cyanide ion has already been discussed as have the results of the work from this dissertation on the trapping of the nicotine N-methyleniminium ion.

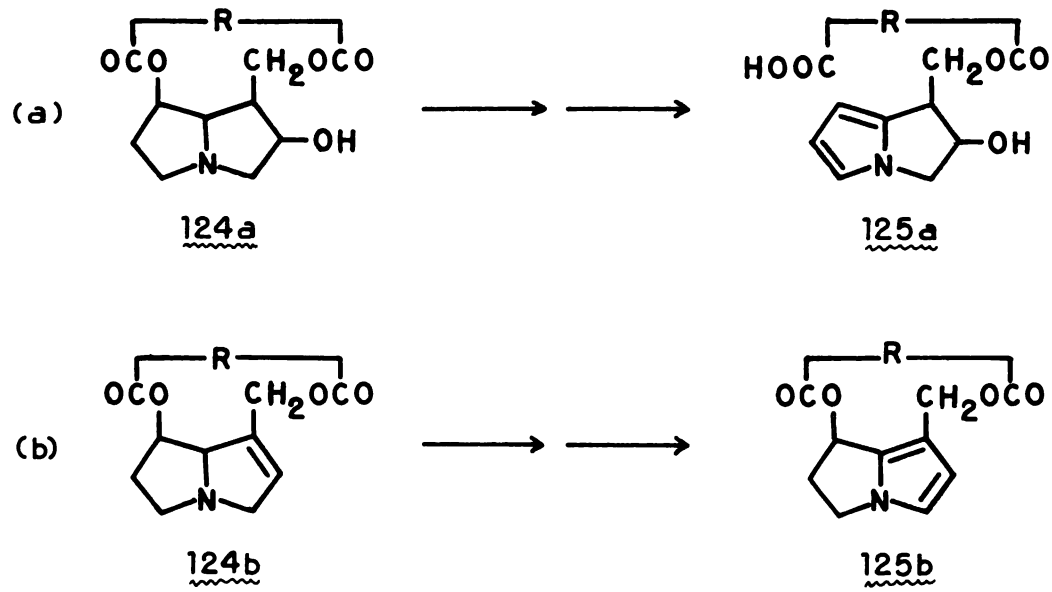
Another example may be the identification of 119 as a metabolite of caffeine (20) in the horse.[180] The authors of this work were unable to explain how this metabolite is formed or what the source is of the S-methyl group. Although the source of the S-methyl group is not clear, the mechanism shown below may account for formation of the metabolite.



Besides the direct metabolic formation of iminium ions there is also evidence that they can be formed *in vivo* by the reaction of a secondary amine with an aldehyde. This has been found to occur when large amounts of alcohol are ingested. The compound 123 is formed from L-dopa (120) and the compound 122 is formed from a metabolite (121) of lidocaine by the condensation of these compounds with acetaldehyde formed from the metabolism of ethanol.[178,181]

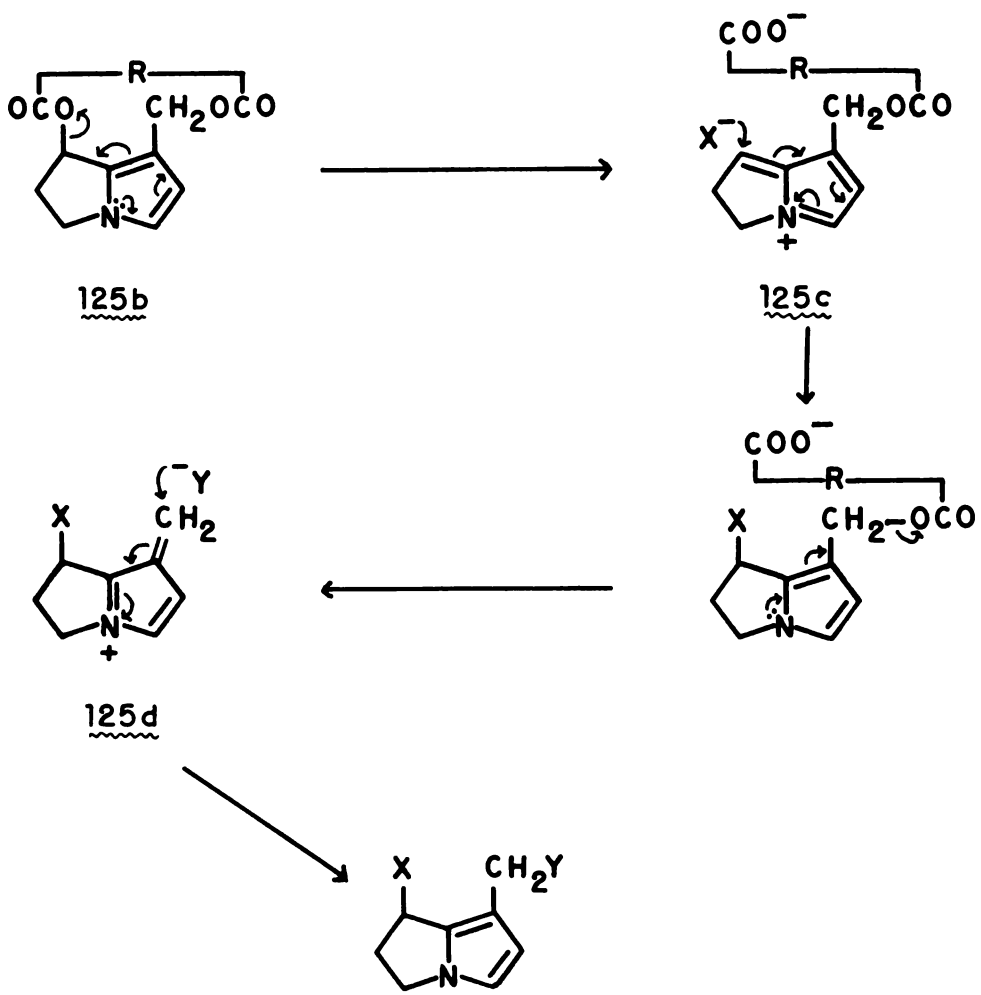


The iminium ions generated from the pyrrolic metabolites of the pyrrolizidine alkaloids have been attributed to be the cause of the hepatotoxicity of these compounds.[182] Mattocks and White[183] studied the metabolism of two types of pyrrolizidine alkaloids 124a and 124b in rats both *in vivo* and *in vitro*. The main products are the pyrroles 125a and 125b.



It was observed that only the formation of the type b metabolite was associated with liver damage; a definite relationship was found between the amount of type b pyrrolic metabolite formed *in vitro* and the hepatotoxicity to rats *in vivo*. It was also shown that the acute toxicity of retrorsine decreased in rats fed on a protein free or sucrose free diet. Microsomes made from rats fed with this diet were shown in *in vitro* experiments to produce lesser amounts of metabolites than

microsomes prepared from rats receiving a normal diet. Thus it was concluded that the formation of the type b pyrrolic metabolite is related to the toxicity of the parent drug. This was explained as being due to the ability of the pyrrolic metabolites formed from unsaturated precursors to form highly electrophilic iminium ions c and d which are capable of binding to cell protein. Metabolism of the saturated precursors gives the pyrrolic metabolite 125a which can not give similar iminium ions.



The lidocaine iminium ion 122a and the nicotine $\Delta^{1'(5')}$-iminium ion 105 mentioned above have been cited as species which may bind to macromolecules.[67,178] Thus a study of iminium ions formed by metabolic alteration of xenobiotics not only provides us with clues as to the nature of the mechanism of metabolic oxidations by the cytochrome P_{450} enzyme system but also may lead us to an understanding of how certain of these compounds cause cellular damage.

Chapter V

EXPERIMENTAL

General

All reactions were carried under a nitrogen atmosphere unless otherwise stated. Melting points and boiling points are uncorrected. All chemicals used were reagent grade unless otherwise specified.

Infrared (ir) spectra were taken on a Perkin Elmer 337 grating spectrophotometer. Intensities of infrared absorption bands are given as (sh) sharp, (s) strong, (m) medium, (w) weak, and (b) broad. A Varian A 60 instrument was used to obtain nuclear magnetic resonance (nmr) spectra. Chemical shifts are given in parts per million downfield from internal tetramethylsilane (TMS) in all solvents except D_2O where the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (SDSS) was used. Spin multiplicity is given as (s) singlet, (d) doublet, (t) triplet, (q) quartet, (p) pentet or (m) multiplet. Many of the compounds reported here contain a 3-substituted pyridine moiety. The pattern seen in the nmr for 3-substituted pyridines is discussed for nicotine in Section A.5. of Chapter IV.

Ultraviolet (uv) spectra were obtained on a Cary 15 spectrophotometer.

Electron impact mass spectra were obtained either by direct inser-

tion or by gas chromatography mass spectrometry (gcms) on an AEI MS-12 mass spectrometer which is interfaced to a PDP 8/I computer using the DS-30 software. The AEI MS-12 is a medium resolution single focusing magnetic sector instrument. An Infotronics 2400 gas chromatograph using helium as the carrier gas is interfaced to the mass spectrometer via a Biemann-Watson molecular separator. Unless otherwise stated, mass spectra were taken with an 8 kV accelerating voltage, a trap current of 500 μA, an electron beam energy of 70 ev, a source temperature of 200°C and a resolving power of 1,200.

Optical rotation was measured on a Perkin Elmer 141 polarimeter at a concentration of 1 g of compound in 100 ml of methanol.

N-Nicotinoyl-2-pyrrolidinone (60a).

In a 1000 ml 3-necked round bottom flask equipped with a mechanical stirrer, a dropping funnel, a condenser and a nitrogen bubbler, was placed nicotinic acid (10 g, 0.0813 moles). Thionyl chloride ($SOCl_2$, 30 g, 0.25 moles) was added dropwise with stirring. The resulting mixture was heated under reflux for 2.5 hours. Excess $SOCl_2$ was removed by distillation at atmospheric pressure. The remaining traces of $SOCl_2$ were removed by co-distillation with absolute benzene. After the solid in the reaction flask had cooled, pyridine (10 g) was added in one portion and the reaction mixture was stirred for 1 hour at room temperature following which 40 ml of dry dichloromethane was added to facilitate stirring. To this solution 2-pyrrolidinone (63, 30 g, 0.31 moles) in 60 ml of dichloromethane was added dropwise over a period of 1/2 hour. The reaction mixture turned dark brown when about 1/3 of the total amount of 2-pyrrolidinone had been added. When the addition was complete the reaction mixture was heated to 50-60°C to help break up the large chunks of solid and then stirred at room temperature for 15 hours. The resulting mixture was made to pH 4-5 with dilute HCl and extracted seven times with 350 ml portions of dichloromethane. Both the aqueous and the organic layers were dark. The extracts were combined and dried over sodium sulfate and the solvent was removed on the rotary evaporater.

The brown residue was analyzed by gc on a 3% OV-17 column (75°C for 1 min then increasing 10°C per minute) and was found to contain the desired compound 60a, pyridine and 2-pyrrolidinone. On standing the residue partially solidified and most of the pyridine and 2-pyrrolidinone were poured off. The crude solid after several alternate recrystallizations from ethyl acetate and benzene yielded white needle like crystals (8.45 g, 54% based on nicotinic acid), mp 103-105°C (lit.[100] mp 103-105°C); nmr ($CDCl_3$, TMS, Figure V) δ 1.8-2.3 (distorted p, 2H, C_4), 2.2-2.9 (q, 2H, C_3), 3.9 (t, J = 6.5 Hz, 2H, C_5), 7.0-9.0 (3m, 4H, a typical 3-substituted pyridine pattern); eims m/e (relative intensity) M^+ 190 (35%), 162 (24.7%), 106 (100%), 78 (74%).

N-($\Delta^{2'}$-Pyrrolinyl)-2-pyrrolidinone (66d).

In experiments where thionyl chloride was not exhaustively removed the above procedure yielded a dark brown oil which solidified upon standing to give 647 mg of a crystalline material. Upon sublimation (70°C, 0.1 mm) a white solid , mp 92-93°C, was obtained; eims m/e (relative intensity) M^+ 152 (93.7%), 151 (100%), 123 (22%), 97 (87.5%), 68 (53%); nmr ($CDCl_3$, TMS, Figure IV) δ 1.7-3.3 (m, 6H, C_4, C_3 and $C_{3'}$), 3.55 (s, 1H, N-H), 3.7 (t, 2H, J = 8 Hz, $C_{5'}$), 4.1 (t, 2H, C_5, J = 9 Hz), 5.05 (t, 1H, $C_{2'}$, J = 3 Hz); ir ($CHCl_3$) ν 1720 cm^{-1} (s, C=O), 1630 cm^{-1}

(s, C=C), 1400 cm^{-1} (s, =C-H).

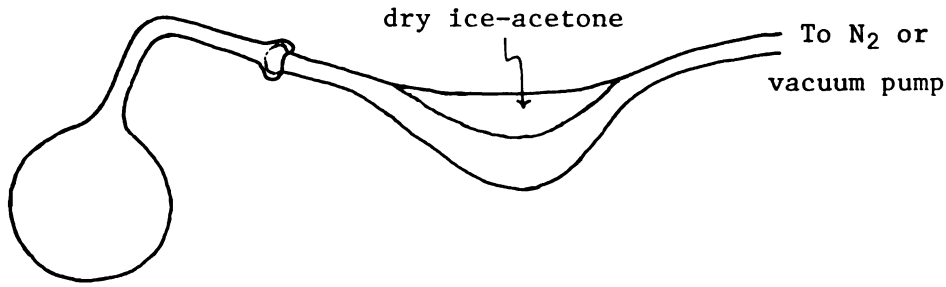
The optimum pH for the extraction of N-nicotinoyl-2-pyrrolidinone (60a).

Pure N-nicotinoyl-2-pyrrolidinone (100 mg) was dissolved in 20 ml of water. The solution was made to the desired pH with dilute HCl (0.2 M) and concentrated potassium carbonate solution. The pH was measured to the nearest 1/2 pH unit by using non-bleeding pH strips. The resulting solution was extracted four times with 20 ml portions of dichloromethane. The organic extracts were dried over sodium sulfate. Thin layer chromatography (ethanol, precoated silica gel GF, 250 microns, Uniplate, Analtech Inc.) of the extract showed in every case only a single spot corresponding to 60a. Upon removal of the solvent the residue was weighed; the percentage of recovery is shown in Table II (see Chapter IV Section A.1).

Pyrolysis of N-nicotinoyl-2-pyrrolidinone (60a) to myosmine (58).

Equal weights of powdered N-nicotinoyl-2-pyrrolidinone (1.5 g, 7.36 mmoles) and powdered calcium oxide (ultrapure grade, 1.5 g, 26.78 mmoles) were placed in a special 10 ml round bottom flask fitted with a special dry ice acetone condenser-receiver (see drawing below). The pyrrolyses were carried out either with a free flame or a salt bath (an

equal molar mixture of $NaNO_2$ and KNO_3) under atmospheric pressure or reduced pressure (0.3 mm) in air or in nitrogen, gradually or quickly heated, with crude or with highly purified starting materials. In all, 40 different variations in technique were attempted. All of these experiments gave similar results. The pyrolysates obtained were between 0.6 g and 0.7 g. The gc tracings of the pyrolysates (on OV-17 3%, 70°C for one minute then increasing 10°C per minute) were very similar. Pyridine, 2-pyrrolidinone, myosmine, and at least eight other major pyrolitic products were seen by gc. The estimated yield of myosmine as determined from the gc tracings was 8-10%.



3-Nicotinoyl-2-pyrrolidinone (68).

The procedure used is essentially that of Korte and Steinen[99] except for the workup. All glassware was baked in the oven for at least one hour before use.

A solution of N-nicotinoyl-2-pyrrolidinone (60a, 19.0 g, 0.10 moles) in 400 ml of absolute benzene (dried over metallic sodium) was warmed to 50°C and introduced slowly through a dropping funnel to a well stirred suspension of NaH (57% in oil, 7.2 g, 0.17 moles) in 10 ml of absolute benzene contained in a 3-necked round bottom flask equipped with a condenser. The greenish yellow reaction mixture was stirred in a water bath at 50-60°C for three days. After cooling in an ice bath, ice water was added to decompose the excess hydride. This mixture separated into two layers. The top benzene layer was yellow and the bottom aqueous layer was milky white. Cold concentrated HCl was added with constant stirring to achieve a pH of about 1. The aqueous layer after a pre-extraction with chloroform (4 x 80 ml) was adjusted to pH 9 and extracted again with chloroform (4 x 80 ml). The chloroform extracts were combined and dried over sodium sulfate. After solvent removal by rotary evaporation, 15 g of a clear brown syrupy oil was obtained. This oil solidified upon standing at room temperature for two days to give a dirty white solid. After recrystallization from toluene or upon sublimation (90°C, 0.1 mm)

a white crystalline solid (13 g, 69%) was obtained: mp 100-101°C; nmr ($CDCl_3$, TMS, Figure VI) δ 2.0-3.7 (2m, 4H, C_4 and C_5), 4.45 (2d, 1H, C_3), 7.68 (broad s, 1H, N-H), 7.3-9.3 (4H, a typical 3-substituted pyridine pattern); ir ($CHCl_3$) ν 3440 cm^{-1} (sh, m), 3325 cm^{-1} (b, m), 1720 cm^{-1} (s), 1700 cm^{-1} (s); uv (EtOH) λ 300 nm (ε 1100), 267 (ε 3750), 225 nm (ε 9800); cims (isobutane, on an AEI MS-9 with a specially modified source for chemical ionization) MH^+ 191.

Anal. Calc'd for $C_{10}H_{10}N_2O_2$: C, 63.14; H, 5.30; N, 18.73. Found: C, 63.33; H, 5.30; N, 18.84.

2'-(3-Pyridyl)-$\Delta^{1'}$-pyrroline (myosmine, 58).

This compound was synthesized according to the method of Korte and Steinen.[99]

In a 100 ml round bottom flask were placed 13 g (0.069 moles) of 3-nicotinoyl-2-pyrrolidinone (68) and 20 ml of concentrated HCl (37-38%). The reaction mixture was heated under reflux in air for five hours. After cooling to room temperature, the pH was adjusted to 9.5-10 with saturated aqueous potassium carbonate. The resulting solution was extracted with 4 x 80 ml portions of chloroform. The combined chloroform extracts were dried over anhydrous sodium sulfate and evaporated under reduced pressure to give 6.3 g of a crude solid (63% yield): mp 39-41°C

(lit.[98] 45°C). Myosmine was recrystallized from diethyl ether to yield yellowish white crystals; nmr ($CDCl_3$, TMS, Figure VII) δ 2.1 (distorted p, 2H, $C_{4'}$), 3.0 (distorted t of t, 2H, $C_{3'}$), 4.15 (t of t, 2H, $C_{5'}$), 7.2-9.0 (4H, a typical 3-substituted pyridine pattern); ir ($CHCl_3$) identical to the reported spectrum,[98] ν 1621 cm^{-1} (sh, s, C=N); eims (Figure XII) identical to the reported spectrum,[111] m/e (relative intensity) M^+ 146 (83%), 145 (43.8%), 118 (100%), 91 (7.7%).

Myosmine-3',3'-d_2 (58a).

All glassware was baked over night in the oven. Potassium carbonate was placed in a test tube and heated by a free flame to expel adsorbed moisture and was then baked in the oven overnight before use.

In a 25 ml round bottom flask fitted with a condenser and a magnetic stirrer were placed myosmine (730 mg, 5 mmoles), potassium carbonate (305 mg, 2.2 mmoles) and D_2O (5 ml, 99.8% D, 275 mmoles). The reaction flask was heated in an oil bath at 80-90°C for five days. The solution was cooled to room temperature and extracted with dry chloroform (4 x 10 ml). The extracts were combined and dried over sodium sulfate. The extent of deuterium incorporation was analyzed by nmr and eims. The nmr spectra of myosmine-d_0 and myosmine-3',3'-d_2 in D_2O are shown in Figures X and XI. The distorted triplet at δ 2.9 ppm

assigned to the two protons at $C_{3'}$ of myosmine-d_0 disappeared in the spectrum of myosmine-3',3'-d_2; the pentet at 2.1 ppm became a triplet (2H, J = 8 Hz, $C_{4'}$); the triplet at 4.1 ppm became more defined (2H, J = 8 Hz, $C_{5'}$). The small signal from 2.9-3.2 ppm was believed to be an impurity since the nmr of myosmine-3',3'-d_2 after a second exchange showed the same signal. The eims of myosmine-3',3'-d_2 is shown in Figure XII: m/e (relative intensity) M^+ 148 (64.6%), 147 (43%), 118 (100%). The isotopic composition of myosmine-3',3'-d_2 could not be determined directly due to the interference of the $[M-1]^+$ and $[M-2]^+$ peaks in the molecular ion region. The minimum deuterium incorporation as determined indirectly by reduction of myosmine-3',3'-d_2 to nornicotine-3',3'-d_2 (see below) was 96% d_2 and 4% d_1.

Asymmetric reduction of myosmine with (-) di-isopinocampheylborane (73).

In a 3-necked round bottom flask equipped with a condenser, magnetic stirrer, addition funnel, and nitrogen bubbler,was placed (+) α-pinene (680 mg, 5 mmoles) in 1.25 ml of diglyme. To this was added sodium borohydride (69 mg, 1.8 mmoles) in 2 ml of diglyme. The reaction vessel was cooled in an ice bath and boron trifluoride etherate (0.63 ml, 2.5 mmoles) in 1.3 ml of diglyme was added rapidly dropwise with stirring under nitrogen. The resulting mixture was stirred at 0°C for four hours.

During this time a white precipitate was formed. Myosmine (146 mg, 1.0 mmoles) in 0.5 ml of diglyme was added dropwise and the reaction mixture immediately turned orange. This was stirred at room temperature for four days. Water (2 ml) followed by acetic acid (2 ml) was added to stop the reaction. The resulting mixture was stirred overnight and then was dumped into 20 ml of water. This was made to pH 1 with hydrochloric acid and was then extracted with ethyl acetate (4 x 20 ml) to remove diglyme and α-pinene. The aqueous layer was made to pH 10 with potassium carbonate and was extracted with chloroform (4 x 10 ml). The chloroform extracts were dried over anhydrous sodium sulfate and the solvent was removed to give 0.5 g of a liquid residue. This was taken up in 10 ml of diethyl ether and the ether solution was filtered. GC analysis (3% OV-17, 100°C for one minute then increasing 10°C per minute) showed two peaks in an approximate 10:1 ratio. The major component corresponded in retention time to diglyme and the minor component corresponded to nornicotine. An eims obtained by direct insertion showed mainly the m/e peaks expected for nornicotine: m/e (relative intensity) 148 (58%), 147 (56%), 119 (100%), 70 (93%).

An analysis of the enantiomeric composition of this product was done using PFBPI (see below); the gc tracing showed a 1.4:1 ratio of diastereomers.

Asymmetric reduction of myosmine with lithium methyl-(hydro)-(-)-dipinan-3α-ylborate (77).

A solution of borane in THF (1 M, 40 ml, 0.04 moles) was added dropwise over a period of five hours to a solution of (+) α-pinene (8.14 g, 0.06 moles) in 25 ml of diglyme. The reaction mixture was magnetically stirred under a nitrogen atmosphere and cooled with an ice bath. When the addition was complete methyl lithium (20 ml, 1.5 M ether solution, 0.03 moles) was added dropwise with stirring to the reaction mixture over a period of 3 hours. Myosmine (146 mg, 0.0010 moles) in 5 ml of diglyme was then introduced. The resulting reaction mixture was allowed to warm to room temperature. Half of the reaction mixture was removed and worked up after 45 hours and the other half was worked up after four days according to the following procedure: the mixture was made acid with 4 N HCl and was then evaporated to dryness under reduced pressure. The residue was taken up in 20 ml of water. This was extracted three times with 20 ml portions of ether. The aqueous layer was made alkaline (pH 9) with concentrated aqueous potassium carbonate and was extracted four times with 20 ml portions of ether. The ether extracts were dried over sodium sulfate. Upon removal of the solvent each fraction gave about 50 mg of residue. A gc analysis (2% carbowax KOH, 185°C) showed diglyme, nornicotine and myosmine in a 7:1:5 ratio for the 45 hour fraction and a 7:2:2

ratio for the four day fraction.

Nornicotine [2'-(3-pyridyl)pyrrolidine, 2].

In a 100 ml round bottom flask fitted with a magnetic stirrer were placed 760 mg of sodium borohydride (20 mmoles) and 120 ml of an ethanol water mixture (1:3). Myosmine (1.46 g, 10 mmoles) dissolved in 10 ml of the same solvent mixture was added in one portion to the reaction flask. The reaction mixture stirred at room temperature for 5-6 days. It was then extracted five times with chloroform (140 ml portions). The combined extracts were dried over sodium sulfate. Solvent removal by rotary evaporation gave a pale yellow liquid residue which weighed 1.37 g. A short path distillation (94°C at 0.5 mm) yielded a colorless liquid (1.30 g, 90%); nmr ($CDCl_3$, Figure VIII) δ 1.2-2.4 (m, 4H, $C_{3'}$ and $C_{4'}$), 2.65 (s, 1H, NH), 3.1 (m, 2H, $C_{5'}$), 4.15 (t, 1H, $C_{2'}$), 7.0-9.0 (4H, a typical 3-substituted pyridine pattern); the ir and uv agreed with the reported spectra;[157] eims (Figure XIII) m/e (relative intensity) M^+ 148 (23%), 147 (32%), 120 (34%), 119 (100%), 70 (90%).

Nornicotine-2-d_1 (2a).

This compound was synthesized according to the procedure described above except that sodium borodeuteride (96% D) was used instead of sodium borohydride. The nmr spectrum of the product in $CDCl_3$ (Figure IX) showed by the disappearance of the signal at δ 4.25 which was assigned to the proton at $C_{2'}$ that there was a deuterium incorporation of about 95%. An analysis of the ei mass spectrum gave an isotopic composition of 96% d_1 and 4% d_0 (see Figure XIV): m/e (relative intensity) M^+ 149 (30%), 120 (100%), 71 (99%), 70 (7.7%).

Nornicotine-3',3'-d_2 (2b).

This compound was synthesized in the same manner described for the synthesis of nornicotine-d_0 but starting with myosmine-3',3'-d_2. The eims (Figure XIII) showed a deuterium incorporation of at least 96% d_2 and 4% d_1: m/e (relative intensity) M^+ 150 (28%), 119 (100%), 72 (91%), 71 (3.9%).

Nornicotine-2',3',3'-d_3 (2c).

This compound was synthesized in the same manner described above for nornicotine-d_0 except that myosmine-3',3'-d_2 was used instead of myosmine-d_0 and sodium borodeuteride was used instead of sodium boro-

hydride. The eims (Figure XIV) showed an isotopic composition of 94% d_3 and 6% d_2 with most of the deuterium loss at $C_{2'}$: m/e (relative intensity) M^+ 151 (35%), 120 (100%), 73 (99%), 72 (11.6%); nmr ($CDCl_3$, TMS, Figure XV) δ 1.8 (broad t, 2H, $C_{4'}$), 2.65 (s, 1H, NH), 2.8-3.4 (m, 2H, $C_{5'}$), 4.1 (small broad singlet, 8% of one proton, $C_{2'}$), 7.0-9.0 (a typical 3-substituted pyridine pattern, 4H).

N-Ethoxycarbonylnornicotine (87).

To a well stirred solution under nitrogen of nornicotine (222 mg, 1.5 mmoles) and triethylamine (251 mg, 2.5 mmoles) in 10 ml of freshly distilled ether (or THF) was added slowly dropwise ethyl chloroformate (267 mg, 2.5 mmoles) in 3 ml ether (or THF). When the addition was complete, the mixture was allowed to stir for 5 minutes. The resulting mixture was filtered through paper and the solvent was removed on the rotary evaporator. The crude product was distilled (bulb to bulb, oven temp. 133°C, at 65 microns) to yield a colorless liquid (265 mg, 80%); ir ($CHCl_3$) ν 1710 cm^{-1} (s, C=O); nmr ($CDCl_3$, TMS) δ 1.1 (t, 3H, J = 6.5 Hz, O-CH_2-CH_3), 1.6-2.5 (m, 4H, $C_{3'}$ and $C_{4'}$), 3.6 (t, 2H, $C_{5'}$), 4.0 (q, 2H, J = 6.5 Hz, O-CH_2-CH_3), 4.9 (2d, 1H, $C_{2'}$), 7.0-9.0 (4H, a typical 3-substituted pyridine pattern); eims (Figure XVII) m/e (relative intensity) M^+ 220 (34.8%), 191 (100%), 147 (60%), 142 (51.5%).

Anal. Calc'd for $C_{12}H_{16}N_2O_2$: C, 65.43; H, 7.32; N, 12.71. Found: C, 64.17; H, 7.26; N, 12.52.

Nicotine (1).

Crude N-ethoxycarbonylnornicotine (87, 210 mg), prepared according to the procedure described above, was dissolved in 10 ml of freshly distilled THF and was added dropwise to a well stirred suspension of lithium aluminum hydride (76 mg, 2 mmoles) in 5 ml of freshly distilled THF. The resulting mixture turned yellow immediately. The reaction was stirred at room temperature for 1.5 days before it was stopped by chilling the reaction vessel in an ice water bath and adding water (1/4 ml) dropwise followed by 15% NaOH (1/4 ml), and finally an additional 1.2 ml of water. The resulting mixture was stirred until a white salt was formed. After filtering the liquid was dried over sodium sulfate and the solvent was removed by rotary evaporation. A short path distillation (84°C, 0.6 mm) gave 118 mg (73%) of a colorless liquid. All the spectral data (ir, nmr, eims) were identical to those reported for nicotine.[157,184]

(S)-Cotinine (3).

This compound was synthesized according to the method of Bowman and McKennis.[97] A 70% yield of a colorless liquid was obtained. This material solidified upon storage at 0°C; nmr ($CDCl_3$, TMS, Figure XIX) δ 1.8-2.8 (complex pattern, 4H, C_3 and C_4), 2.75 (s, 3H, NCH_3), 4.75 (distorted t, 1H, C_5), 7.0-9.0 (4H, a typical 3-substituted pyridine pattern); eims (Figure XVIII) m/e (relative intensity) M^+ 176 (40%), 175 (12%), 98 (100%).

(S)-3,3-Dibromocotinine hydrobromide perbromide (88·HBr·Br_2).

The procedure of Bowman and McKennis was used.[97] Reddish orange crystals in a yield of 71% were obtained: mp 144-145°C (lit.[97] mp 140-150°C). These crude crystals were used directly in the Zn reduction to cotinine.

(S)-3,3-Dibromocotinine (88).

The crude reaction mixture obtained by the Bowman and McKennis[97] procedure for the preparation of (S)-3,3-dibromocotinine hydrobromide perbromide was cooled in an ice bath. Sodium sulfite was added with stirring until all of the orange color disappeared. The inorganic salts were filtered off and the resulting solution was made alkaline with

aqueous potassium carbonate. The resulting solution was extracted with chloroform. The combined extracts were dried with sodium sulfate. Removal of the solvent gave white crystals which were recrystallized from aqueous ethanol to give a 50% yield of the desired product: mp 119-120°C; nmr ($CDCl_3$, TMS, Figure XXI) δ 3.25 (2d, 1H, C_4), 3.38 (2d, 1H, C_4), 4.75 (2d, 1H, C_4), 7.0-9.0 (a typical 3-substituted pyridine pattern). The three upfield protons showed a typical ABX splitting pattern: J_{AB} = 14 Hz, $\Delta\nu_{AB}$ = 13.5 Hz, J_{AX} = 8 Hz, J_{BX} = 6 Hz.

(S)-Nicotine-5',5'-d_2 (1a).

All glassware was baked in the oven at least one hour before use. In a 100 ml 3-necked round bottom flask fitted with a magnetic stirrer a condenser and a dropping funnel, was placed lithium aluminum deuteride (210 mg, 5 mmoles) in 10 ml of freshly distilled THF. The reaction flask was cooled in a water bath while (S)-cotinine (880 mg, 5 mmoles) in 2 ml of THF was introduced slowly dropwise. When the addition was complete the reaction mixture was stirred under reflux overnight. The reaction mixture was chilled in an ice water bath then 0.2 ml of water was added followed by 0.2 ml of 15% NaOH and finally 1 ml of water was added slowly. The resulting mixture was allowed to stir until white salts were formed. It was then filtered and dried over anhydrous sodium sulfate.

The solvent was removed under reduced pressure to yield a colorless liquid residue. A short path distillation yielded 445 mg (55%) of a colorless liquid; eims (Figure XXIII) m/e (relative intensity) M^+ 164 (30.8%), 135 (20.7%), 86 (100%), 85 (4.6%); 96% d_2 and 4% d_1.

Nicotine-2',5',5'-d_3 (1b).

Nicotine-2'-d_1 was obtained by the same procedure described above for the synthesis of nicotine-d_0 but starting with nornicotine-2'-d_1 instead of nornicotine-d_0. Nicotine-2'-d_1 was not characterized; the crude product was used directly for the synthesis of nicotine-2',5',5'-d_3.

Nicotine-2',5',5'-d_3 was synthesized from nicotine-2'-d_1 via cotinine-5-d_1 in the same manner that nicotine-5',5'-d_2 was synthesized from nicotine-d_0 (see above): eims (Figure XXIV) m/e (relative intensity) M^+ 165 (20.7%), 136 (20%), 87 (100%), 86 (13.8%), 85 (2.7%), 84 (3.0%); 86% d_3 and 13% d_2.

Nicotine-N-methyl-d_3 (1c).

This compound was synthesized according to the procedure described above for the synthesis of nicotine-d_0 but using lithium aluminum deuteride instead of lithium aluminum hydride for the reduction of

N-ethoxycarbonylnornicotine (87). The nmr spectrum in $CDCl_3$ is identical to that of nicotine-d_0 except for the absence of the singlet at 2.17 ppm assigned to the N-methyl protons; eims (Figure XXIV) m/e (relative intensity) M^+ 165 (33.8%), 136 (36%), 87 (100%), 86 (6.7%), 85 (9.0%), 84 (1.6%); 96% d_3 and 4% d_2.

5-Hydroxycotinine (52).

This compound was synthesized according to the procedure of Dagne:[143]

(i) 5-(3'-pyridyl)-5-methoxy-2-pyrrolinone (97): eims m/e (relative intensity) M^+ 204 (39.5%), 173 (100%), 126 (95.9%); nmr ($CDCl_3$, TMS) δ 2.62 (s, 3H, NCH_3), 3.25 (s, 3H, OCH_3), 6.67 (AB quartet, J_{AB} = 6 Hz, $\Delta\nu$ = 33 Hz, 2H, C_3 and C_4), 7.0-9.0 (4H, a typical 3-substituted pyridine pattern); ir ($CHCl_3$) ν 1700 cm^{-1} (s, C=O).

(ii) 5-(3'-pyridyl)-5-hydroxy-N-methyl-2-pyrrolinone (98): eims m/e (relative intensity) M^+ 190 (72.8%), 112 (93.8%), 106 (39.5%), 79 (100%), 78 (73.6%); ir ($CHCl_3$) ν 1700 cm^{-1} (s, C=O); nmr ($CDCl_3$, TMS) δ 2.72 (s, 3H, NCH_3), 6.60 (AB quartet, J_{AB} = 6 Hz, 2H, $\Delta\nu_{AB}$ = 33 Hz, 2H, C_3 and C_4), 7.0-9.0 (4H, a typical 3-substituted pyridine pattern).

(iii) 5-(3'-pyridyl)-5-hydroxy-N-methyl-2-pyrrolidinone (5-hydroxycotinine, 52): eims m/e (relative intensity) M^+ 192 (2%), 174 (29.1%), 163 (38.5%), 114 (25%), 106 (100%), 79 (50.4%), 78 (100%); ir ($CHCl_3$)

ν 1685 cm^{-1} (s, C=O); nmr ($CDCl_3$, TMS) δ 2.3-3.0 (an intense singlet and a complex multiplet, 7H, NCH_3 C_3 and C_4), 7.0 (broad s, 1H, O-H), 7.3-9.0 (4H, a typical 3-substituted pyridine pattern); uv (ethanol) λ 300 nm (ε 385), 265 nm (ε 960), 258 (ε 1270), 253 (ε 1150).

Incubation of nicotine with lithium aluminum deuteride.

To a well stirred suspension under nitrogen of lithium aluminum deuteride (168 mg, 4 mmoles) in 10 ml of freshly distilled THF was added slowly (S)-nicotine-d_0 (648 mg, 4 mmoles). The reaction mixture turned yellow as soon as the addition was begun. The mixture was stirred at room temperature for 10 days and was then worked up by adding 0.2 ml of water to the ice cold reaction mixture followed by 0.2 ml of 15% NaOH and finally 0.6 ml of water very slowly. The resulting mixture was stirred until a white salt like solid was formed. After filtering, the THF was removed by rotary evaporation under reduced pressure. About 9 ml of water was added to the wet residue and the nicotine was recovered by extracting four times with 10 ml portions of chloroform. After being dried over sodium sulfate, the solvent was removed under reduced pressure and the residue was distilled (81°C, 0.2 mm) to give 358 mg of a colorless liquid. The nmr spectrum (between 7.0-9.0 ppm) of this material in $CDCl_3$ is shown in Figure XXII.

N-Cyanomethylnornicotine (107).

To a solution of nornicotine (740 mg, 5 mmoles) and NaCN (490 mg, 10 mmoles) in 166 ml of water was added dropwise 84 ml of 1.76% aqueous formaldehyde (1.5 g of H_2CO, 50 mmoles) at room temperature with stirring and under nitrogen. After stirring an additional 10 minutes at room temperature, the reaction mixture was extracted with five 120 ml portions of diethyl ether. The combined extracts were dried (sodium sulfate), and the residue (750 mg, 78%) obtained after removing the solvent was distilled (bp 118-119°C, 0.3 mm) to give a colorless liquid: nmr ($CDCl_3$, TMS) δ 1.6-2.5 (m, 4H, $C_{3'}$ and $C_{4'}$), 2.5-3.1 (m, 1H, $C_{5'}$), 3.1-3.5 (m, 1H, $C_{5'}$), 3.6 (t, 1H, J = 7.5 Hz, $C_{2'}$), 3.48 (center of AB quartet, 2H, J = 17 Hz, $\Delta\nu_{AB}$ = 17 Hz, CH_2CN), 7.0-9.0 (4H, a typical 3-substituted pyridine pattern); ir (neat) ν 2240 cm^{-1} (w, C≡N); uv (EtOH) λ 260 nm (ε 1880); eims m/e (relative intensity) M^+ 187 (22.5%), 186 (12.4%), 109 (100%).

Anal. Calc'd for $C_{11}H_{13}N_3$: C, 70.56%; H, 6.99%; N, 22.44%. Found: C, 70.49%; H, 7.01%; N, 21.96%.

Dipicrate salt: A large excess of a saturated solution of picric acid in ethanol was added to a solution of cyanomethylnornicotine in ethanol. A gummy solid appeared when the sides of the reaction flask were scratched. This mixture was heated on a steam cone for 1/2 min

and a yellow crystalline solid was obtained: mp 149-151.5°C. This was recrystallized twice from 2-propanol: mp 149.5-151.5°C.

Anal. Calc'd for $C_{23}H_{19}N_9O_{14}$: C, 42.80%; H, 2.96%; N, 19.53%. Found: C, 42.65%; H, 3.03%; N, 19.23%.

6,6'-Dinitro-2,2'-diphenic acid (86).

This acid was synthesized according to the procedure of Kenner and Stublings:[132] mp 252-254°C (lit.[132] 258-259°C); nmr (DMSO-d_6, TMS) δ 7.82 (t, 2H, J = 8 Hz, $C_{4'}$ and C_4), 8.33 (2d, 2H, J = 8 Hz and J = 1.5 Hz, $C_{3'}$ and C_3), 8.43 (2d, 2H, J = 8 Hz and J = 1.5 Hz, $C_{5'}$ and C_5), 8.60 (broad s, 2H, COOH); eims m/e (relative intensity) M^+ 332 (0.6%), 286 (100%), 240 (53%), 196 (28%).

Resolution of 6,6'-dinitro-2,2'-diphenic acid (86).

Racemic diphenic acid 86 (830 mg, 2.5 mmoles) in 23 ml of ethanol was combined with a solution of (-)-quinine (1.95 g, 6.0 mmoles) in 17 ml of ethanol. The resulting solution was placed in the refrigerator overnight. The diastereomeric salt obtained (960 mg, 1.46 mmoles) gave a mp of 220-223°C. After three recrystallizations from a 2-propanol-ethanol mixture, 765 mg (1.16 mmoles) of the salt was obtained: mp 226-229°C (lit.[131] mp 231-233°C).

The free acid was recovered from the salt (700 mg) by treating it with 5 ml of conc HCl and extracting with ether. Upon removal of the ether 272 mg (0.82 mmoles) of free acid was obtained: mp 227-229° (lit. mp 229°C); $[\alpha]_D$ = -125° (lit.[133] $[\alpha]_D^{27}$ = -126°).

Resolution of nornicotine with (-)-6,6'-dinitro-2,2'-diphenic acid.

Racemic nornicotine (148 mg, 1 mmole) in 2 ml of methanol was mixed with (-)-6,6'-dinitro-2,2'-diphenic acid (333 mg, 1 mmole) in 4 ml of methanol. After three days in the refrigerator, 326 mg (from 480 mg total salt) of yellow crystalline salt was obtained: mp 202-204°C. A portion of this material (300 mg, 0.625 mmoles) was dissolved in saturated potassium carbonate solution and nornicotine was recovered by extraction with dichloromethane. The extracts were dried over anhydrous sodium sulfate and the solvent was then removed by rotary evaporation under reduced pressure to give 30 mg of nornicotine (40.5%): $[\alpha]_D$ = -49° in methanol (lit.[130] $[\alpha]_D$ = -88.8°C).

Derivatization of nornicotine with trifluoroacetyl (S)- prolyl chloride: Formation of a diastereomeric glc derivative of nornicotine.

Nornicotine (5 mg, 0.03 mmoles) in 1 ml of benzene was placed in a culture tube with a teflon lined screw cap. To this was added trifluoro-

(S)-prolyl chloride (TPC, 1 ml, 0.1 M in $CHCl_3$, contains about 4% of the (R) isomer, Regis Co.), followed by 10 drops of triethylamine. The tube was capped and heated in an oil bath at 90°C for five min. Upon cooling 1 ml of 6 N HCl was added and the tube was shaken. The organic layer was removed and dried over anhydrous sodium sulfate. The volume was reduced under a stream of nitrogen to 0.1 ml and the resulting solution was analysed on a 3% OV-17 column at 260°C.

Metabolic studies.

Chemicals.

Tobacco alkaloids and their deuterated analogs were synthesized as previously described. NADPH was purchased from Sigma Chemical Co. (St. Louis, Mo.). All other chemicals used were reagent grade or better. Glassware was cleaned in a nitric acid bath before use in metabolic studies.

Liver preparations.

Male Dutch black rabbits 6 months to 1 year old were used. The animals were stunned by a blow to the neck followed by decapitation. Their livers were quickly removed and rinsed in ice cold 1.15% KCl. Liver tissue (12.5 g) was minced in 25 ml of cold isotonic KCl solution or pH 7.4 phosphate buffer solution and homogenized using a Potter-Elvehjem teflon pestle homogenizer. The homogenates were centrifuged at 10,000 x g for 30 min in a Sorvall RCZ-B refrigerated centrifuge at 0-4°C. The supernatant fractions were recentrifuged at 100,000 x g for 1 hour in a refrigerated Spinco Model L centrifuge at 0-4°C. The supernatant (the soluble fraction) contains the soluble enzymes. The sediment (the microsomal pellet) was resuspended in the amount of ice cold pH 7.4 phosphate buffer needed to make 1 ml of solution correspond to 0.2 g of liver for

the nornicotine metabolism studies and 1 ml of solution correspond to 0.5 g of liver for the studies on the metabolism of nicotine in the presence of cyanide ion.

Incubations.

Incubations were done at 37°C, in air for 1 hour using the metabolic shaker.

(i) Nornicotine metabolism:

Each incubate (3 ml total volume) contained 2.5 ml of 10,000 x g supernatant or microsomal fraction (0.2 g of liver/ml), magnesium chloride (275 µg, 0.996 mM), nornicotine (544 µg, 0.1 mM) and NADPH (4 mg added every 20 min). The incubation was stopped by chilling the incubate in an ice bath and extracting with dichloromethane. The extracts were dried over anhydrous sodium sulfate and the volume was reduced under a stream of nitrogen to 0.1 ml. Thin layer chromatography (TLC) was carried out on preprepared silica gel plates (3 cm x 6 cm, silica gel GF, 250 microns, Uniplate, Analtech Inc.) using an EtOH : acetone : benzene : conc aqueous NH_4OH (5:40:50:5) solvent system. Each band from the TLC plate was analysed by gcms using a 2% carbowax KOH column (140°C for 1 min then increasing 5°C per min).

(ii) Nicotine metabolism in the presence of cyanide ion.

Each incubate (10 ml total volume) contained 8 ml of either 10,000 x g supernatant or microsomal fraction (0.5 g of liver/ml), magnesium chloride (1.43 mg, 1.5 mM), sodium cyanide (5 mg, 10 mM), NADPH (added at 20 min intervals in 8 mg portions) and nicotine (810 µg, 0.5 mM). When the 10,000 x g supernatant was used as the enzyme source, the incubate mixture was preincubated at 0°C for 15 min before the substrate was added and incubated at 37°C.

The incubation was stopped by chilling in an ice bath followed by a direct extraction with dichloromethane or diethyl ether at 0°C. An acid-base (0.1 M HCl, conc K_2CO_3) extraction was then done to remove non-basic substances. The base extracts were dried over anhydrous sodium sulfate and the volume was reduced to 0.1 ml under a stream of nitrogen. This solution was analysed by gcms on a 2% Dexyl (160°C for 1 min then increasing 3.5°C per min) or on a 2% carbowax KOH column (140°C for 1 min then increasing 4°C per min).

(iii) Control experiments.

Several control incubations were done for both the nornicotine and nicotine studies using exactly the reagents and procedures described above except that the liver preparation was heated for 10 min on a steam cone before the cofactors and substrate were added.

REFERENCES

1. A. Claude in "Microsomes and Drug Oxidations," Gillette, Conney, Cosmides, Estabrook, Fouts, and Mannering, eds., Academic Press, New York, 1969.

2. A. Y. H. Lu, K. W. Junk, and M. J. Coon, J. Biol. Chem., 244, 3714 (1969).

3. D. M. Ziegler, E. M. McKee, and L. L. Poulsen, Drug Metab. Disp., 1, 314 (1973).

4. R. W. Estabrook in "Concepts in Biochemical Pharmacology," Part II, Brodie and Gillette, eds., Springer-Verlag, Berlin, 1971.

5. J. E. Tomaszewski, D. M. Jerina, ans J. W. Daly, Ann. Rpts. Med. Chem., vol. 9, 290 (1974).

6. (a) B. B. Brodie, J. R. Gillette,and B. N. La Du, Ann. Rev. Biochem., 27, 427 (1958).
   (b) J. R. Gillette, Progr. Drug Res., 6, 13 (1963).
   (c) J. R. Gillette, Advan. Pharmacol., 4, 219 (1966).

7. H. B. Hucker, J. R. Gillette, and B. B. Brodie, J. Pharmacol. Exp. Ther., 129, 94 (1960).

8. A. K. Cho, W. L. Haslett, and D. J. Jenden, Biochem. Biophys. Res. Comm., 5, 276 (1961).

9. H. A. Dugger, R. A. Coombs, H. J.Schwarz, B. H. Migdalof, and B. A. Orwig, Drug Metab. Disp., 4, 262 (1976).

10. P. N. Giraldi, G. Tosolini, E. Dradi, G. Mannini, R. Longo, G. Meinardi, G. Monti, and I. de Carneri, Biochem. Pharmacol., 20, 339 (1971).

11. M. A. Schwartz and S. J. Kolis, J. Pharmacol. Exp. Ther., 180, 180 (1972).

12. (a) D. L. Hill, W. R. Laster, Jr., and R. F. Struck, Cancer Res., 32, 658 (1972).

(b) M. Jarman, Experentia, 29, 812 (1973).

13. J. Werringloer and R. W. Estabrook, Arch. Biochem. Biophys., 167, 270 (1975).

14. T. Butler and M. Bush, J. Pharmacol. Exp. Ther., 65, 205 (1939).

15. G. C. Mueller and J. A. Miller, J. Biol. Chem., 202, 579 (1953).

16. D. Y. Cooper, S. Levin, S. Narasimhulu, O. Rosenthal, and R. W. Estabrook, Science, 147, 400 (1965).

17. J. W. Gorrod and P. Jenner, The Metabolism of Tobacco Alkaloids, in "Assays in Toxicology," vol. 6, Academic Press, 1975.

18. N. E. Sladek and G. J. Mannering, Molec. Pharmacol., 5, 174 (1969).

19. A. P. Alvares, G. Schilling, W. Levin, and R. Kuntzman, Biochem. Biophys. Res. Comm., 29, 521 (1967).

20. N. E. Sladek and G. J. Mannering, Molec. Pharmacol., 5, 186 (1969).

21. R. McMahon, J. Pharm. Sci., 55, 457 (1966).

22. T. E. Gram, ref. 4, page 334.

23. J. Axelrod, J. Pharmacol. Exp. Ther., 114, 430 (1955).

24. R. McMahon and H. Sullivan, Biochem. Pharmacol., 14,1085 (1965).

25. H. Keberle, W. Riess, K. Schmid und K. Hoffmann, Arch. int. Pharmacodyn., 142, 125 (1963).

26. H. W. Dorough and J. E. Casida, J. Agric. Fd. Chem., 12, 294 (1964).

27. T. C. Butler, J. Pharmacol. Exp. Ther., 106, 235 (1952).

28. D. L. Smith, H. H. Keasling and A. A. Forist, J. Med. Pharm. Chem., 8, 520 (1965).

29. H. H. Cornish and A. A. Christman, J. Biol. Chem., 228, 315 (1957).

30. A. R. Jones and H. Jackson, Biochem. Pharmacol., 17, 2247 (1968).

31. R. McMahon, J. Med. Pharm. Chem., 4, 67 (1961).

32. C. Hansch, A. Ruth Steward,and J. Iwasa, J. Med. Chem., 8,868

(1965).

33. B. N. La Du, L. Gaudette, N. Trousof, and B. Brodie, J. Biol. Chem., 214, 741 (1955).

34. R. McMahon, H. Culp, and F. Marshall, J. Pharmacol. Exp. Ther., 149, 436 (1965).

35. L. G. Dring, R. L. Smith, and R. T. Williams, Biochem. J., 116, 425 (1970).

36. D. S. Hewick and J. R. Fouts, Biochem. J., 117, 833 (1970).

37. P. Jenner and B. Testa, Drug Metab. Rev., 2, 117 (1973).

38. R. Locke and V. Mayer, Biochem. Pharmacol., 23, 1979 (1974).

39. W. Sadee, W. Garland and N. Castagnoli, Jr., J. Med. Chem., 14, 643 (1971).

40. R. McMahon, H.Culp, and J. Occolowitz, J. Amer. Chem. Soc., 91, 3389 (1969).

41. R. T. Williams, ref. 4, page 229.

42. D. M. Ziegler and F. H. Pettit, Biochem., 5, 2932 (1966).

43. (a) J. C. Craig, N. Mary, N. Goldman, and L. Wolff, J. Amer. Chem. Soc., 86, 3866 (1964).
(b) M. S. Fish, N. M. Johnson, E. P. Lawrence, and E. C. Horning, Biochim. Biophys. Acta, 18, 564 (1955).

44. N. Castagnoli, Jr. and W. Sadee, J. Med. Chem., 15, 1076 (1972).

45. D. F. Heath, D. W. J. Lane, and P. O. Park, Phil. Trans., B239, 191 (1955).

46. E. Hodgson and J. E. Casida, Biochem. Pharmacol., 8, 179 (1961).

47. H. B. Hucker, Drug Metab. Rev., 2, 33 (1973).

48. J. W. Gorrod and D. Temple, Xenobiotica, 6, 265 (1976).

49. S. I. Omelchenko, Z. V. Pushkaveva, and S. G. Bogomolov, J. Gen. Chem., 27, 3257 (1957).

50. M. H. Bickel, Pharmacol. Rev., 21, 325 (1967).

51. A. H. Beckett, Xenobiotica, 1, 365 (1971).

52. P. Jenner, J. W. Gorrod and A. H. Beckett, Xenobiotica, 3, 341 (1973).

53. R. M. Dajani, J. W. Gorrod, and A. H. Beckett, Biochem. Pharmacol., 24, 109 (1975).

54. M. Kiese, G. Renner and R. Schlaeger, Arch. Pharmak., 268, 247 (1971).

55. (a) M. H. Bickel, H. J. Weder and H. Aebi, Biochem. Biophys. Res. Comm., 33, 1012 (1968).
(b) G. Stöhrer and G. B. Brown, J. Biol. Chem., 244, 2498 (1969).

56. R. Kuntzman, A. Phillips, I. Tsai, A. Klutch, and J. Burns, J. Pharmacol. Exp. Ther., 155, 337 (1967).

57. J. J. Kamm, Fed. Proc., 30, 225 (1971).

58. M. H. Bickel, Xenobiotica, 1, 313 (1971).

59. A. H. Beckett, J. M. Van Dyk, H. H. Chissick, and J. W. Gorrod, J. Pharm. Pharmac., 23, 809 (1971).

60. J. W. Gorrod, D. Temple, and A. H. Beckett, Xenobiotica, 5, 465 (1975).

61. M. S. Gold and D. M. Ziegler, Xenobiotica, 3, 179 (1973).

62. J. W. Gorrod, P. Jenner, G. Keysell, and A. H. Beckett, Chem. Biol. Interactions, 3, 269 (1971).

63. M. Pailer in "Tobacco Alkaloids and Related Compounds", Von Euler, ed., Pergamon Press, New York, 1964.

64. (a) F. E. Guthrie, R. L. Ringer, and T. G. Bowery, J. Econ. Entom., 50, 827 (1957).
(b) L. S. Self, F. E. Guthrie, and E. Hodgson, Nature, 204, 300 (1964).

65. (a) M. Biebl, H. Essex, and F. Mann, Am. J. Physiol., 100, 167 (1932).

(b) H. B. Haag, P. S. Larson, and J. F. Finnegan, J. Pharmacol. Exp. Ther., 85, 356 (1945).

66. E. Hansson and C. Schmitterlöw, ref. 63, page 87.

67. D. M. Turner, A. K. Armitage, R. H. Briant, and C.T. Dollery, Xenobiotica, 5, 539 (1975).

68. E. Hansson, P. Hoffman, and C. Schmitterlöw, Acta Physiol. Scand., 61, 380 (1964).

69. J. Booth and E. Boyland, Biochem. Pharmacol., 20, 407 (1971).

70. P. Jenner, J. W. Gorrod, and A. H. Beckett, Xenobiotica, 3, 563 (1973).

71. N. Papadopoulos and J. Kintzios, J. Pharmacol. Exp. Ther., 140, 269 (1963).

72. N. M. Papadopoulos, Arch. Biochem. Biophys., 106, 182 (1964).

73. (a) A. Beckett, P. Jenner, and J. Gorrod, Xenobiotica, 3, 557 (1973).
(b) B. Testa, P.Jenner, and A. Beckett, Xenobiotica, 6, 553 (1976).

74. P. Jenner, J. W. Gorrod, And A. Beckett, Xenobiotica, 3, 573 (1973).

75. H. McKennis, Jr., L. Turnbull, and E. Bowman, J. Amer. Chem. Soc., 79, 6342 (1957).

76. P. Murphy, J. Biol. Chem., 248, 2797 (1973).

77. H. McKennis, Jr., L. B. Turnbull, and E.R. Bowman, J. Amer. Chem. Soc., 80, 6597 (1958).

78. H. McKennis, Jr., Ann. N. Y. Acad. Sci., 90, 36 (1960).

79. E. R. Bowman, L. B. Turnbull, and H. McKennis, J. Pharmacol. Exp. Ther., 127, 92 (1959).

80. H. McKennis, Jr., L. B. Turnbull, E. R. Bowman, and E. Wada, J. Amer. Chem. Soc., 81, 3951 (1959).

81. H. McKennis, Jr., L. Turnbull, S. Schwartz, E. Tamaki, and E.

Bowman, J. Biol. Chem., 237, 541 (1962).

82. E. R. Bowman, E. Hansson, L. Turnbull, H. McKennis, Jr., and C. Schmitterlöw, J. Pharmacol. Exp. Ther., 143, 301 (1964).

83. P. Morselli, H. Ong, E. Bowman, and H. McKennis, Jr., J. Med. Chem., 10, 1033 (1967).

84. D. M. Turner, Biochem. J., 115, 889 (1969).

85. H. McKennis, Jr., L. Turnbull, E. Bowman, and E. Tamaki, J. Org. Chem., 28, 383 (1962).

86. E. Dagne and N. Castagnoli, Jr., J. Med. Chem., 15, 356 (1972).

87. H. McKennis, Jr., E. Bowman, and L. Turnbull, J. Amer. Chem. Soc., 82, 3974 (1960).

88. H. McKennis, Jr., L. Turnbull, E. Bowman, and S. Schwartz, J. Amer. Chem. Soc., 84, 4598 (1962).

89. H. McKennis, Jr., L. Turnbull, and E. Bowman, J. Biol. Chem., 238, 719 (1963).

90. E. Dagne and N. Castagnoli, Jr., J. Med. Chem., 15, 840 (1972).

91. H. P. Harke, B. Frahm, Ch. Schultz, und W. Dotenwill, Biochem. Pharmacol., 19, 495 (1970).

92. N. Papadopoulos, Can. J. Biochem., 42, 435 (1964).

93. K. Decker und R. Sammeck, Biochem. Z., 340, 326 (1964).

94. E. Wada, E. Bowman, L. Turnbull, and H. McKennis, Jr., J. Med. Pharm. Chem., 4, 21 (1961).

95. H. Kuhn, ref. 63, page 37.

96. T. Kisaki and E. Tamaki, Phytochem., 5, 293 (1966).

97. E. Bowman and H. McKennis, Jr., Biochem. Prep., 10, 36 (1963).

98. B. Witkop, J. Amer. Chem. Soc., 76, 5597 (1954).

99. F. Korte und H. J. Schulze-Steinen, Chem. Ber., 95, 2444 (1962).

100. B. P. Mundy, B. R. Larsen, L. F. McKenzie, and G. Braden, J. Org.

Chem., 37, 1635 (1972).

101. C. P. Haines, A. Eisner, and C. F. Woodward, J. Amer. Chem. Soc., 67, 1258 (1945).

102. (a) E. Leete, M. Chedekel, and G. Bodem, J. Org. Chem., 37, 4465 (1972).
(b) M. L. Stein and A. Burger, J. Amer. Chem. Soc., 79, 154 (1957).

103. B. P. Mundy, K. B. Lipkowitz, M. Lee, and B. Larsen, J. Org. Chem., 39, 1963 (1974).

104. A. W. Titherly and T. H. Holden, J. Chem. Soc., 101, 1871 (1912).

105. B. C. Challis and J. A. Challis in "The Chemistry of Amides," Zabicky, ed., Interscience Publishers, 1970, page 759.

106. N. Castagnoli, Jr., Ph.D. Thesis, University of California, Berkeley, 1964.

107. H. Brederick and K. Brederick, Chem. Ber., 94, 2278 (1961).

108. H. Eilingsfeld, M. Seefelder, and H. Weidinger, Angew. Chem., 72, 836 (1960).

109. J. Dyer in "Applications of Absorption Spectroscopy of Organic Compounds," Prentice Hall Inc., Englewood Cliffs, N.J., 1965.

110. S. Brandänge and L. Lindblom, Acta Chem. Scand., B 30, 93 (1976).

111. A. Duffield, H. Budzikiewcz, and C. Djerassi, J. Amer. Chem. Soc., 87, 2926 (1965).

112. M. Polonovski and M. Polonovski, Compt. rend., 184, 1333 (1927).

113. J. Braun and K. Weissbach, Ber., 63, 2018 (1930).

114. E. Späth, L. Marion, and E. Zajic, Ber., 69, 251 (1936).

115. E. Wynder and D. Hoffman in "Tobacco and Tobacco Smoke: Studies in Experimental Carcinogenesis," Academic Press, New York, 1967.

116. L. C. Craig, J. Amer. Chem. Soc., 55, 2854 (1933).

117. H. C. Brown, N. Ayyangar, and G. Zweifel, J. Amer. Chem. Soc., 86,

397 (1964).

118. H. C. Brown, N. Ayyangar, and G. Zweifel, J. Amer. Chem. Soc., 86, 1071 (1964).

119. G. Zweifel, N. Ayyangar, and H. C. Brown, J. Amer. Chem. Soc., 85, 2075 (1963).

120. J. F. Archer, D. R. Boyd, W. R. Jackson, M. F. Grundon, and W. A. Khan, J. Chem. Soc., (C), 2560 (1971).

121. K. Harada in "The Chemistry of The Carbon-Nitrogen Double Bond," Patai, ed., Interscience Publishers, 1970, page 255.

122. (a) J. H. Billman and A. C. Diesing, J. Org. Chem., 22, 1068 (1957).
(b) Z. Horii, T. Sakai, and T. Inoi, J. Pharm. Soc. Jap., 75, 1161 (1955).

123. N. Castagnoli, Jr., A. Melikian, and V. Rosnati, J. Pharm. Sci., 58, 860 (1969).

124. J. C. Craig and S. Y. C. Lee, J. Org. Chem., 40, 663 (1969).

125. N. C. Gaylord in "Reduction with Complex Metal Hydrides," Interscience Publishers, New York, 1956.

126. (a) P. R. Girardot and R. W. Parry, J. Amer. Chem. Soc., 73, 2368 (1951).
(b) W. Jolly and R. E. Mesmer, J. Amer. Chem. Soc., 83, 4470 (1961).

127. J. D. Roberts and M. C. Caserio in "Basic Principles of Organic Chemistry," W. A. Benjamin, Inc., New York, 1965, page 475.

128. (a) S. B. Matin, P. S. Callery, J. S. Zweig, A. O'Brien, H. Rapoport, and N. Castagnoli, Jr., J. Med. Chem., 17, 877 (1974).
(b) M. Ames, Ph.D. Thesis, University of California, San Francisco, 1975.

129. A. Pictet and A. Rotschy, Ber., 37, 1225 (1904).

130. E. Späth and E. Zajic, Ber., 68, 1667 (1935).

131. E. Späth and F. Kesztler, Ber., 69, 2725 (1936).

132. J. Kenner and W. Stublings, J. Chem. Soc. London, 119, 593 (1921).

133. A. W. Ingersoll and J. R. Little, J. Amer. Chem. Soc., 56, 2123 (1934).

134. J. March in "Advanced Organic Chemistry: Reactions, Mechanisms, and Structure," McGraw-Hill, New York, 1968, page 668 and refs. cited therein.

135. L. C. Craig, J. Amer. Chem. Soc., 55, 295 (1933).

136. Ref. 125, page 636 and refs. cited therein.

137. F. Marshall and R. McMahon, J. Labelled Cpds., 6, 261 (1970).

138. E. Hansson and C. Schmitterlöw, J. Pharmacol. Exp. Ther., 137, 91 (1962).

139. E. Dagne, L. Gruenke, and N. Castagnoli, Jr., J. Med. Chem., 17, 1330 (1974).

140. E. W. Garbisch, Jr., J. Chem. Ed., 45, 402 (1968).

141. P. T. Lansburry and J. O. Peterson, Am. Soc., 83, 3537 (1961); 85, 2236 (1963).

142. P. S. Larson and H. Silvette in "Tobacco, Experimental and Clinical Studies," Williams and Wilkins Co., Baltimore, 1968, p. 9.

143. E. Dagne, Ph.D. Thesis, University of California, San Francisco, 1972.

144. (a) N. Cromwell and K. Cook, J. Amer. Chem. Soc., 80, 4573 (1958). (b) R. Lukes and Z. Linhartova, Coll. Czech. Chem. Commun., 25, 502 (1960).

145. L. D. Gruenke and N. Castagnoli, Jr., unpublished results.

146. R. Silverstein and G. Bassler in "Spectrometric Identification of Organic Compounds," 2nd Ed., John Wiley and Sons Inc., N.Y. 1967.

147. C. N. R. Rao in "Ultraviolet and Visible Spectroscopy," 2nd Ed., London Butterworths, 1967.

148. P. S. Larson and H. B. Haag, Ind. Eng. Chem. Anal. Ed., 16, 86 (1944).

149. Mayor, Arch. Sci. Phy. Nat., 17 418 (1904).

150. (a) M. Clark, M. Rand, and S. Vanov, Arch. Int. Pharmacodyn., 156, 363 (1965).
(b) A. K. Armitage, et al., J. Pharmacol. and Chemo., 27, 33 (1966).

151. P. S. Larson, H. B. Haag, and J. K. Finnegan, Proc. Soc. Exp. Biol. and Med., 58, 231 (1945).

152. H. B. Hucker and P. S. Larson, J. Pharmacol., 123, 259 (1958).

153. E. Sanders, J. DeBardeleben, and T. Osdene, J. Org. Chem., 40, 2848 (1975).

154. H. McKennis, Jr., ref 63, page 53.

155. Y. Hubert-Brierre, D. Herlem, and F. Khuong-Huu, Tetrahedron, 31, 3049 (1975).

156. J. Hurwitz, J. Biol. Chem., 212, 757 (1955).

157. (a) M. Swain, A. Eisner, C. Woodward, and B. Brice, J. Amer. Chem. Soc., 71, 1341 (1949).
(b) C. R. Eddy and A. Eisner, Anal. Chem., 26, 1428 (1954).

158. M. Cushman and N. Castagnoli, Jr., J. Org. Chem., 37, 1268 (1972).

159. (a) E. Snyder, J. Amer. Chem. Soc., 85, 2624, (1963).
(b) J. Pople, J. Chem. Phys., 42, 1339 (1965).

160. J. Leeling, B. Phillips, R. Schut, and O. Fancher, J. Pharm. Sci., 54, 1736 (1965).

161. H. Denk, P. Moldeus, R. Schultz, J. Schenkman, S. Keyes, and D. Cinti, J. Cell Biol., 69, 589 (1976).

162. M. L. Das and D. M. Ziegler, Arch. Biochem. Biophys., 140, 300 (1970).

163. J. Axelrod, J. Pharmacol. Exp. Ther., 117, 322 (1956).

164. J. A. Thompson and J. L. Holtzman, Drug Metab. and Disp., 2, 577 (1974).

165. A. E. Takemori and G. J. Mannering, J. Pharmacol. Exp. Ther., 123, 171 (1958).

166. J. Mao and P. Tardrew, Biochem. Pharmacol., 14, 1049 (1965).

167. C. Elison, H. Elliott, M. Look, and H. Rapoport, J. Med. Chem., 6, 237 (1963).

168. M. Abdel-Monem, J. Med. Chem., 18, 427 (1975).

169. S. Nelson, L. Pohl, and W. Trager, J. Med. Chem., 18, 1062 (1975).

170. K. E. Wiberg, Chem. Rev., 55, 713 (1955).

171. L. D. Gruenke, presented at the Twenty-Fourth Annual Conference on Mass Spectrometry and Allied Topics, 1976.

172. P. S. Larson and H. B. Haag, J. Pharmacol. Exp. Ther., 77, 343 (1943).

173. (a) A. Ganz, F. Kelsey, and E. Geiling, J. Pharmacol. Exp. Ther., 103, 209 (1951).
(b) D. R. Bennett, R. E. Tedeschi, and P. S. Larson, Arch. int. Pharmacodyn., 98, 221 (1954).
(c) F. B. Owen, Jr., P.S. Larson, Arch. int. Pharmacodyn., 115, 402 (1958).

174. H. McKennis, Jr., E. Wada, E. Bowman, and L. Turnbull, Nature, 190, 910 (1961).

175. T. Stalhanske, Acta Physiol. Scand., 78, 236 (1970).

176. G. J. Mannering in "Fundamentals of Drug Metabolism and Drug Disposition," La Du, Mandel, Leong Way, eds., Williams and Wilkins Co., Baltimore, 1972, page 213, and refs. cited therein.

177. J. Edelson, A. Schlosser, and J. Douglas, Biochem. Pharmacol., 17, 779 (1968).

178. S. Nelson, Ph.D. Thesis, University of California, San Francisco, (1974).

179. E. Schraven, F. Koss, J. Keck, and Beisenherz, Eur. J. Pharmacol., 1, 445 (1967).

180. K. Kamei, M. Matsuda, and A. Momose, Chem. Pharm. Bull., 23, 683 (1975).

181. M. Sandler, S. B. Carter, K. R. Hunter, and G. M. Stern, Nature, 241, 439 (1973).

182. A. R. Mattocks, Nature, 217, 723 (1968).

183. A. R. Mattocks and I. N. H. White, Chem. Biol. Interac., 3, 383 (1971).

184. Varian NMR Catalog, Vol. 1, 1962, Spectrum No. 269.