Title

Stereochemical studies of the cytochrome P-450 catalyzed oxidation of (S)-nicotine to nicotine delta-1',5'-iminium ion

Author

Peterson, Lisa A.

Publication Date

1985

Peer reviewed|Thesis/dissertation

STEREOCHEMICAL STUDIES OF THE CYTOCHROME P-450 CATALYZED OXIDATION OF (S)-NICOTINE TO NICOTINE Δ-1',5'-IMINIUM ION

by

Lisa A. Peterson

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

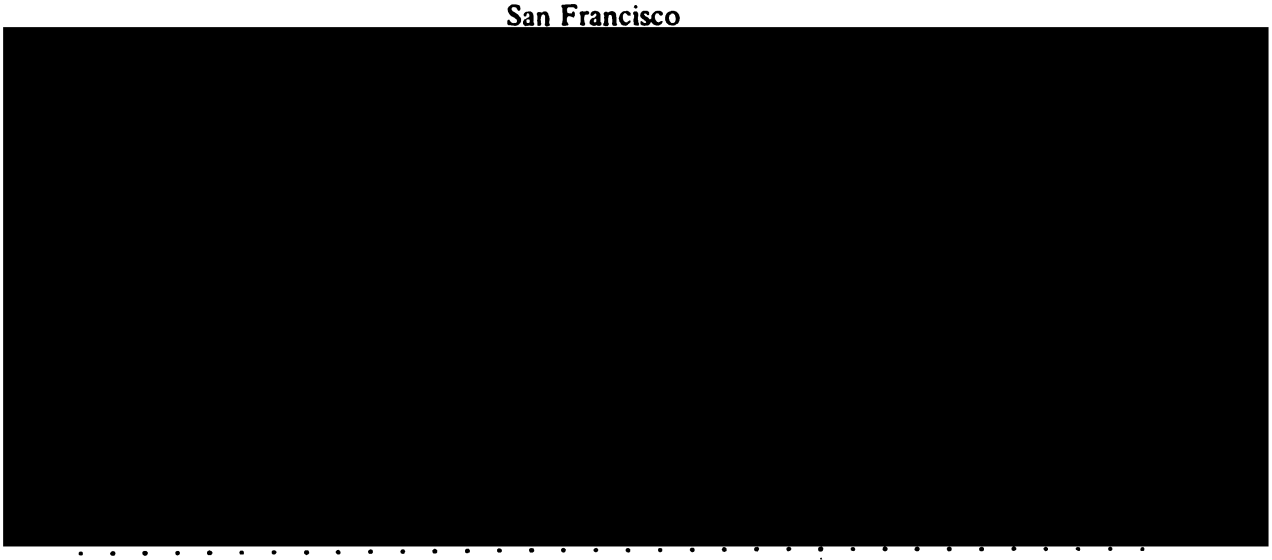
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



. . . . . .

Date University Librarian

DEC 29 1985

Degree Conferred: . . . . . .

To my parents,

for their love and support.

Acknowlegdements

I would like to thank Dr. Neal Castagnoli, Jr. for his support and guidance.

Also, I thank the members of the Castagnoli research group, other graduate students and postdoctoral fellows for their patience and support.

I acknowledge Dr. A.L. Burlingame for the use of the mass spectral facilities and Esther Roitman for her technical assistance.

Finally, I would like to thank my friends and family for sharing both the good and the bad times.

STEREOCHEMICAL STUDIES OF THE CYTOCHROME P-450 CATALYZED OXIDATION OF (S)-NICOTINE TO NICOTINE Δ-1',5'-IMINIUM ION

Lisa A. Peterson

Abstract of the Dissertation

Mammals metabolize the tobacco alkaloid (S)-nicotine principally to the lactam (S)-cotinine by a pathway involving an initial cytochrome P-450 catalyzed two electron oxidation at the prochiral 5'-carbon atom. The stereochemical course of this reaction was examined with rodent and human microsomal preparations using the (E)- and (Z)-diastereomers of (S)-nicotine-5'-d_1. The unstable iminium ion intermediates were trapped as the corresponding α-cyanoamines which were analyzed for deuterium content by capillary column gas chromagraphy-mass spectral selected ion monitoring techniques. Analysis of ether extracts of these incubation mixtures indicated that the oxidation proceeds with the stereoselective abstraction (75-90%) of the least hindered (E)-5'-proton. Possible complications due to deuterium isotope effects and subsequent oxidation of the α-cyanoamines to cotinine proved to be insignificant. These findings suggest that diastereotopic interactions are an important feature of the complex formed between (S)-nicotine and the active site of cytochrome P-450.

The stereochemical course of the α-carbon oxidation of (S)-nicotine then was examined in a series of model oxidizing systems in order to understand more fully the events taking place at the enzyme's active site. Iodosobenzene and cumene hydroperoxide were substituted for NADPH/O_2 to probe the molecular mechanism of oxidation and were found to have no effect on the regioselectivity of the reaction. Only slight

oxidant-dependent variations in the diastereoselectivity of the 5'-α-carbon oxidation were observed with preferential abstraction of the (E)-5'-proton/deuteron in all cases.

The role of the heme iron environment in directing the enzymatic process was examined with the synthetic tetraphenylporphyrin catalyst. Although the iodosobenzene-tetraphenylporphyrin catalyzed reaction was considerably less regioselective than the enzymatic process, the (E)-5'-proton/deuteron was selectively lost. These observations demonstrate that interactions of (S)-nicotine with the porphyrin system, in part, directs the stereochemical course of the enzyme catalyzed oxidation.

The influence of the protein environment of the heme iron on the stereochemical course of the cytochrome P-450 catalyzed reaction was explored with several hemoprotein models including chloroperoxidase, horseradish peroxidase, and methemoglobin. In all cases, the reaction displayed little stereochemical control; the loss or retention of the 5'-proton/deuteron was determined primarily by deuterium isotope effects. These results demonstrated that cytochrome P-450 is unique among the hemoproteins studied in its ability to selectively remove the (E)-5'-proton during the 5'-α-carbon oxidation of (S)-nicotine.

The chemical mechanism of the cytochrome P-450 catalyzed oxidation was investigated by studying the stereo- and regiochemical course of the methylene blue-sensitized photochemical and electrochemical oxidation of (S)-nicotine. In both reactions, the choice of the 5'-proton was determined by stereoelectronic effects with the selective loss of the (Z)-5'-proton/deuteron of the monodeuterated nicotine diastereomers. The diastereoselectivity of the chemical oxidations contrasted dramatically with that of the cytochrome P-450 mediated reaction which is accompanied

by the selective loss of the (Z)-5'-proton/deuteron. The chemical processes also were considerably less regioselective than the enzymatic system.

The data obtained from all the model systems revealed that the nature of the interactions of (S)-nicotine with the active site of cytochrome P-450 is important in determining the regio- and stereochemical course of this oxidation.

TABLE OF CONTENTS

List of Tables

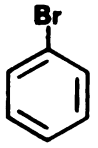
List of Figures

Chapter I

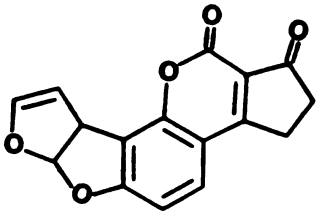
INTRODUCTION

The biotransformation of drugs and other foreign compounds plays an important role in determining the pharmacological and toxicological effects of these compounds. In general, xenobiotics are metabolized to more polar, water-soluble molecules which are excreted more rapidly than the parent substances. In most cases these enzymatic transformations lead to the formation of metabolites which are less biologically active. It is now well-known, however, that many relatively stable chemicals can be biotransformed to highly reactive metabolites by these same enzymes systems. Interactions of the toxic metabolites with cellular macromolecules and/or other cellular constituents may trigger changes in the cellular biochemistry leading to cell death or cell transformation.[1] Current knowledge suggests that these metabolically generated intermediates are involved in many kinds of chemically-induced toxic events, including carcinogenesis, mutagenesis, hepatic and renal necrosis, methemoglobinemia, and fetotoxicities.[2]

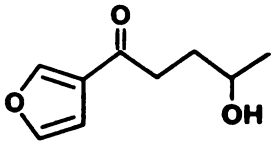
While the events leading to cellular damage are not well understood, experimental evidence suggests at least two ways in which metabolic events might lead to cellular damage. First, many toxins, such as bromobenzene (1),[2] aflatoxin B_1 (2),[3] 4-ipomeanol (3),[4] benzo(a)pyrene (4)[5] and acetominophen (5)[6] have been shown to be converted to reactive electrophiles which are capable of interacting with nucleophilic functionalities present on proteins, nucleic acids and other cellular constituents. These covalent modifications are believed to result in altered function of important cellular macromolecules.[1,7] Secondly,


Br

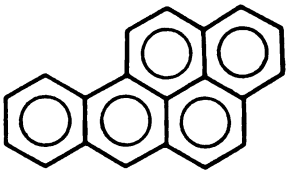
1


O
O
O
O
O

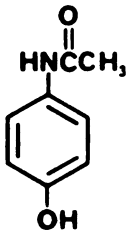
2


O
O
OH

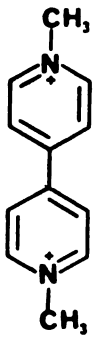
3



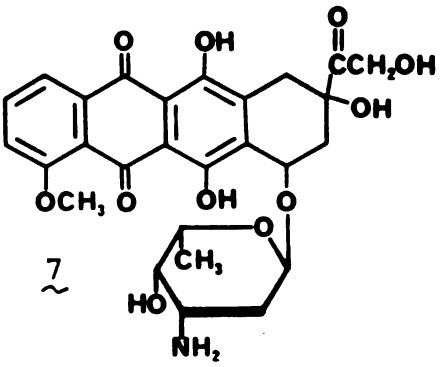
4


O
HNCCH3
OH

5


CH3
N
N
CH3

6

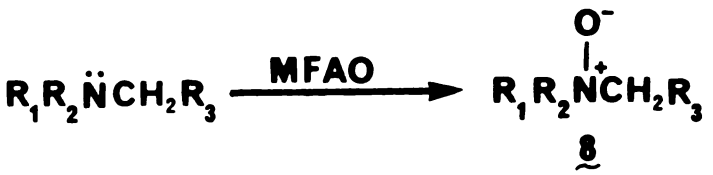

O
OH
O
CCH2OH
OH
OCH3
O
OH
O
O
CH3
HO
NH2

7

other toxins are activated to intermediates capable of reducing molecular oxygen to reactive species which then cause cellular damage. These compounds, in general, do not react directly with cellular functionalities but lead to a process referred to as oxidative stress and which involves formation of toxic oxy radicals.[1,7] Examples include quaternary bipyridyl compounds such as paraquat (6),[8] and semiquinone precursors such as acetominophen (5)[9] and adrimycin (7).[1]

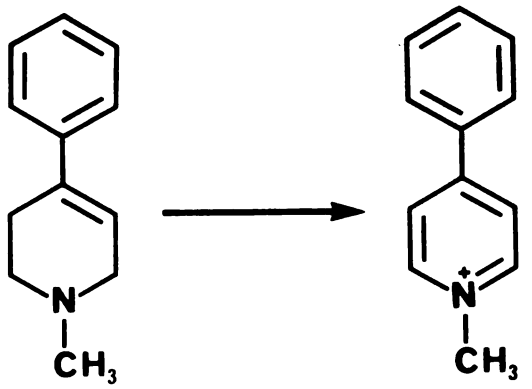
While all tissues are susceptible to toxic injury by xenobiotics, most chemicals exhibit their toxicity in specific organs and/or cell types. Portals of entry and exit are common targets as a result of exposure to high concentration of the drugs and their metabolites. Furthermore, tissues that are major sites of biotransformation are particularly susceptible to chemically-induced injury since many compounds are metabolized to toxic products which react at the site of formation.[10] A number of factors determine the tissue's response to toxicity including the distribution and pharmacokinetics of the toxin, the metabolic fate of the compound within the tissue, including the balance between bioactivation and detoxification processes within the cell, and the ability of the cell to respond to the deleterious effects of the toxin.[10]

A wide variety of enzyme systems are involved in the metabolism of xenobiotics to toxic and nontoxic products. The structural features of the xenobiotic and its metabolites, not differences in the nature of the catalytic reaction, determine whether the metabolic transformation produces an inert or potentially toxic metabolite.[1,11]



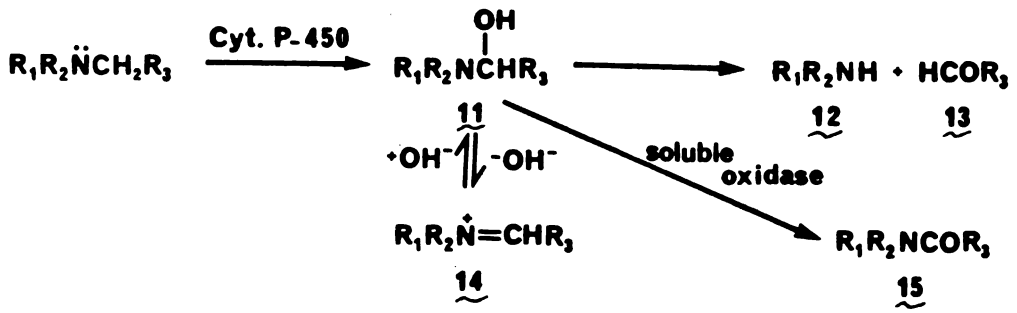
8

Many drugs, toxicants and environmental chemicals are tertiary amines. Two enzyme systems well-recognized to be important in the transformation of this class of compounds are the flavoprotein mixed function amine oxidase (MFAO) which catalyzes N-oxidation of these substrates to tertiary amine N-oxides (8) and the cytochrome P-450 system which catalyzes α-carbon oxidation reactions and, to a lesser extent, N-oxidation reactions.[12] Recently, mitochondrial monoamine oxidase also has been shown to catalyze the oxidation of the neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (9) in a two step process to the N-methyl-4-phenylpyridinium species 10.[13,14,15] This oxidation is believed to be responsible for the bioactivation of 9 since the toxicity[14,16] and the *in vitro*[13] and *in vivo*[14,16] conversion of 9 to 10 is inhibited by pargyline and deprenyl, known inhibitors of monoamine oxidase. This observation suggests an important role for monoamine oxidase in the oxidative transformation of exogenous tertiary amines.



9 10

Of central interest to this thesis is the α-carbon oxidation reactions catalyzed by the cytochrome P-450 system. This family of enzymes possesses the unusual ability to mediate the oxidative metabolism of a diverse group of substrates.[11] In the case of tertiary amines, a cytochrome P-450 system catalyzes the oxidation of the α-carbon atom leading eventually to the formation of a carbinolamine (11). This metabolite may spontaneously cleave to form a secondary amine (12) and a carbonyl product (13). In cyclic systems, the carbinolamine or the isoelectronic iminium species (14) may undergo a second two electron oxidation to yield an amide (15) in a reaction catalyzed by a soluble oxidase.[12]



While the mechanism by which the cytochrome P-450 system catalyzes the oxidation of compounds is not well-understood, there is general agreement that the stoichiometry of the overall reaction is as follows:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+.$$

Substrate oxidation is believed to involve an oxenoid mechanism whereby the enzyme generates a reactive oxene species bound to the cytochrome's heme iron (enzyme-Fe^{3+}:Ö:). This reactive oxygen species is then transferred to the bound substrate.[17]

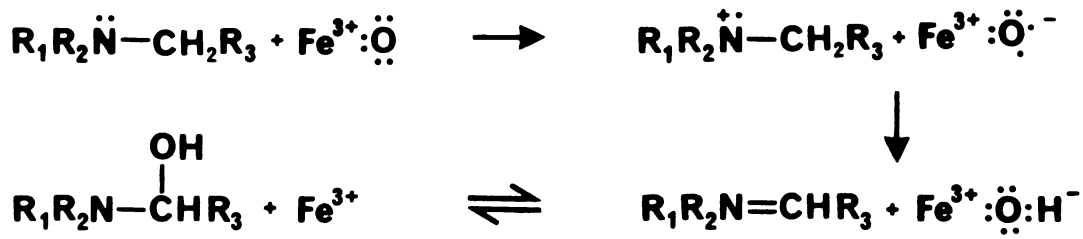
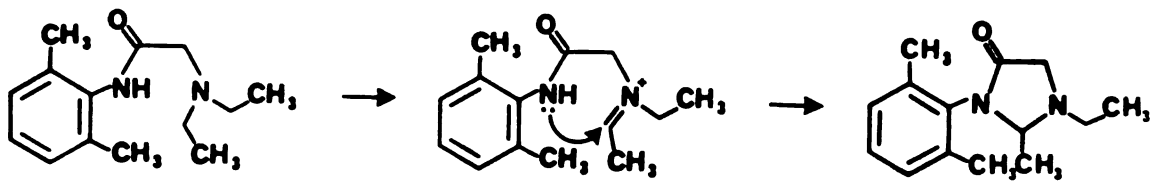


Figure 1. Proposed mechanism for the cytochrome P-450 catalyzed α-carbon oxidations of tertiary amines.

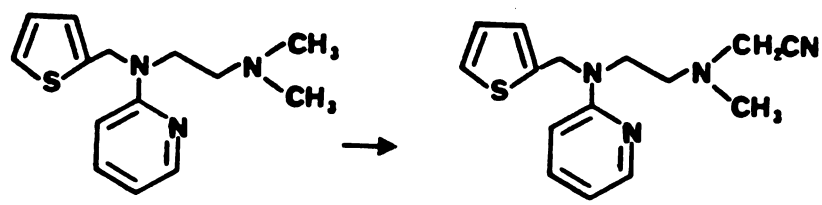
The precise chemical mechanism for α-carbon oxidation remains unclear. Mechanisms which have been suggested for this process include direct oxene insertion,[12] N-oxide formation,[18] and aminium radical cation formation.[19-22] The existing experimental evidence discussed in detail in Section III.A. primarily supports a two-one electron reaction sequence as shown in Figure 1. This mechanism predicts initial iminium ion formation; however, one cannot exclude direct formation of a carbinolamine. In any case, carbinolamines will be in equilibrium with the corresponding iminium ions.[23] The possible formation of the reactive, electrophilic iminium ions during the α-carbon oxidation of amines may explain the deleterious effects of a number of tertiary amines since these intermediates have the potential to interact with cellular nucleophiles.[24] A review of this subject recently has appeared.[24]



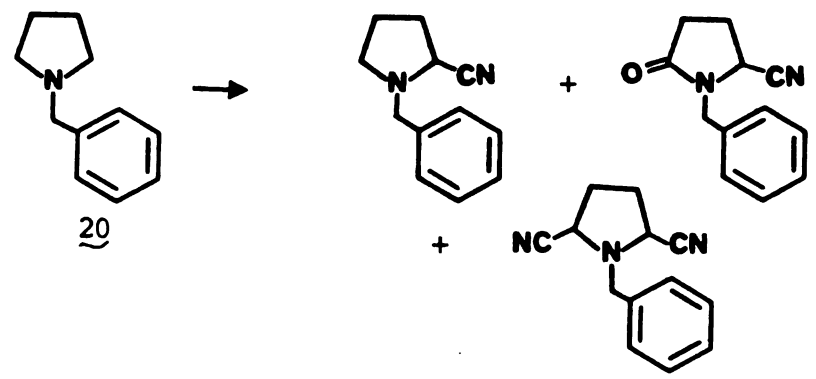
16 18 17

Several lines of evidence support the importance of iminium ions in the cytochrome P-450 catalyzed metabolism of tertiary amines. The formation of intermediate iminium ions in the microsomal oxidation of lidocaine (16) was suggested by the characterization of the intramolecular cyclization product 17.[25] The formation of the imidazolidinone 17 can be explained most easily by the intramolecular trapping of the intermediate 18.

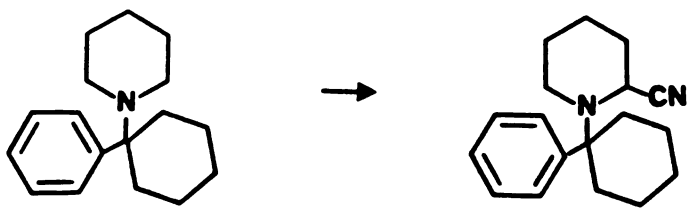
Further support for the formation of electrophilic iminium ions during the oxidative metabolism of methapyrilene (19),[26] 1-benzylpyrrolidine (20),[27] phencyclidine (21),[28] (S)-nicotine (22)[29-31] and MPTP (9)[32] has been provided through the characterization of the corresponding α-cyano adducts isolated from NADPH-fortified microsomal incubations of the amines performed in the presence of sodium cyanide. The cyanoamines are formed by the reaction of the metabolically generated iminium ions with the nucleophilic cyanide ion.



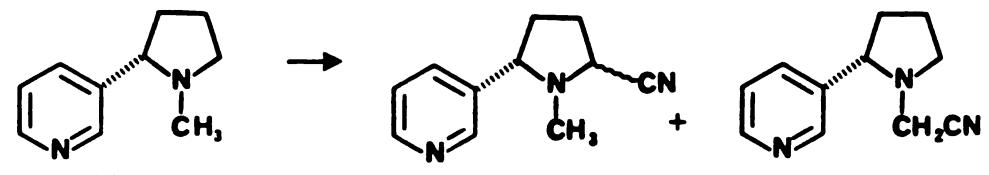
19



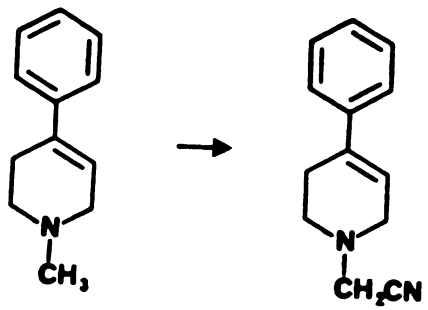
20



21

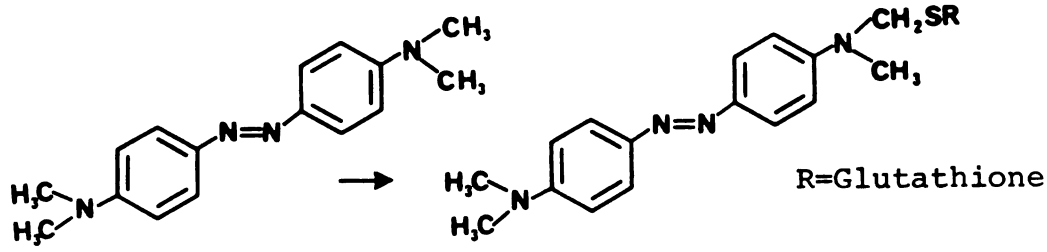


22

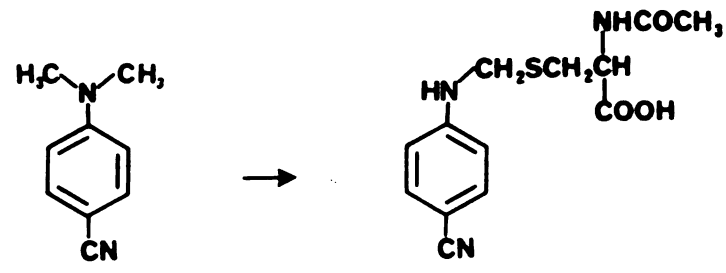


9

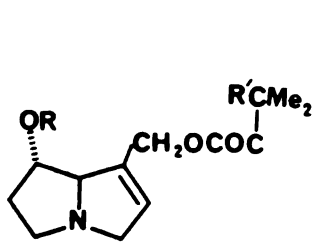
The physiological importance of iminium ions has been suggested by the identification of N-(glutathione-S-methylene)-4-aminoazobenzene (23) as a major biliary metabolite of the hepatocarcinogenic N,N-dimethylaminoazobenzene (24) in the rat.[33] N-Acetyl-S-(4-cyano-anilinomethyl)-cysteine (25) is a major metabolite of 4-cyano-N,N-dimethylaniline (26) isolated from rat and mouse urine.[34] These two metabolites are believed to be the products of the reaction between glutathione and the corresponding methylene iminium ions.



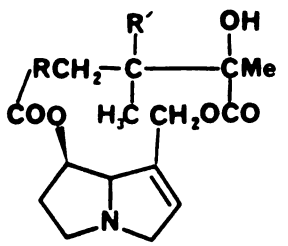
24 23



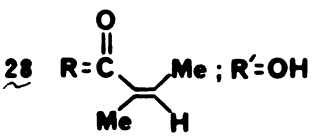
26 25



27 R=H;R′=H



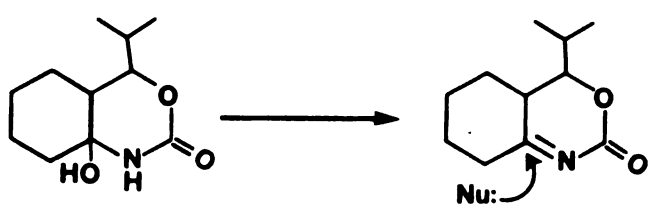
29 R=CHMe; R′=OH



28 R=C(=O)CH=CMe; R′=OH

30 R=C=CMe; R′=H

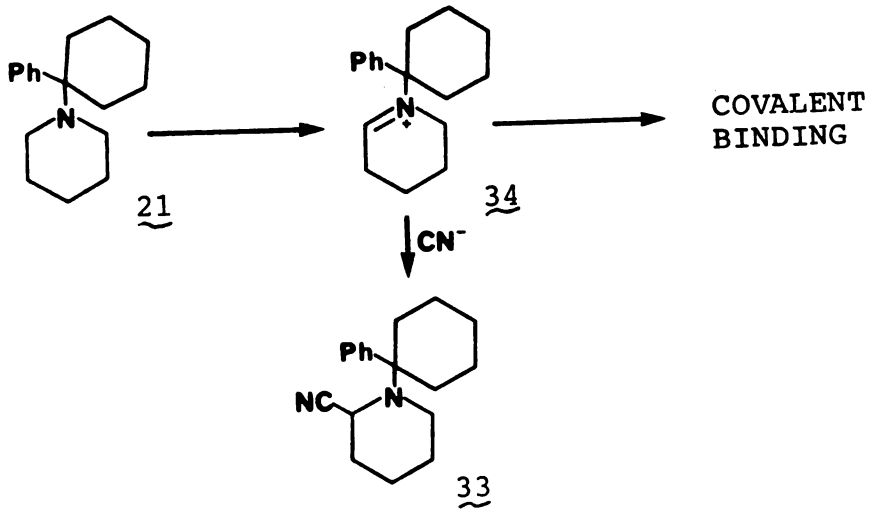
Evidence for a role of the electrophilic iminium ion in the pharmacological profile of a number of tertiary amines has been reported. Iminium ions are believed to be involved in the metabolism-dependent hepatotoxicity of the pyrrolizidine alkaloids which include heliotrine (27), lasiocarpine (28), monocrotaline (29) and retrorsine (30).[35] The antitumor activity of maytansine (31) is believed to be a result of the formation of a carbinolamide (32) which is capable of interacting with nucleic acids.[36]



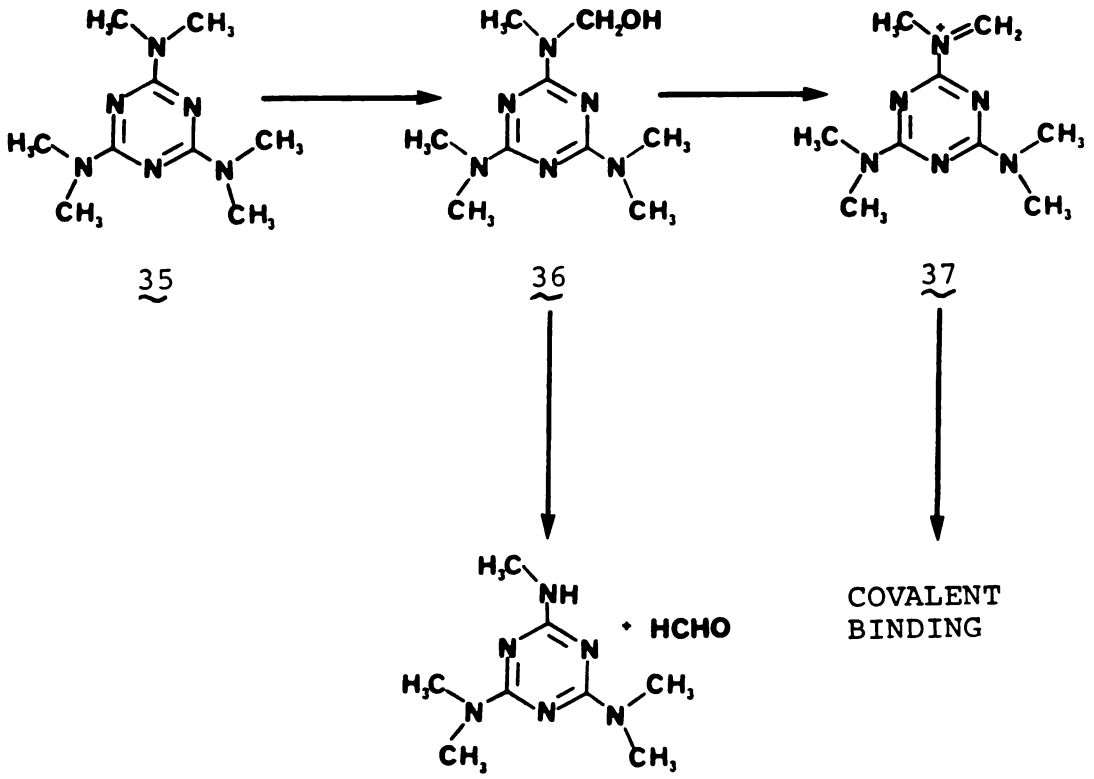
31

32

The iminium ion generated during the metabolism of the hallucinogen phencyclidene (21) has been suggested as a possible species involved in the neurotoxicity of this drug.[28] This hypothesis was based on the observation that the metabolism-dependent binding of this drug to liver microsomal protein was reduced by 75% when the incubations were carried out in the presence of cyanide ion at levels which did not inhibit metabolism.[28] These results, along with the isolation of the α-cyanoamine 33 from the incubations mixtures, implicated the iminium ion 34 as the bioalkylating species.[28] More recent studies with the synthetic iminium ion 34 (as the perchlorate salt) have demonstrated that this species requires further metabolic transformation to yield the actual agent that binds to protein.[37]



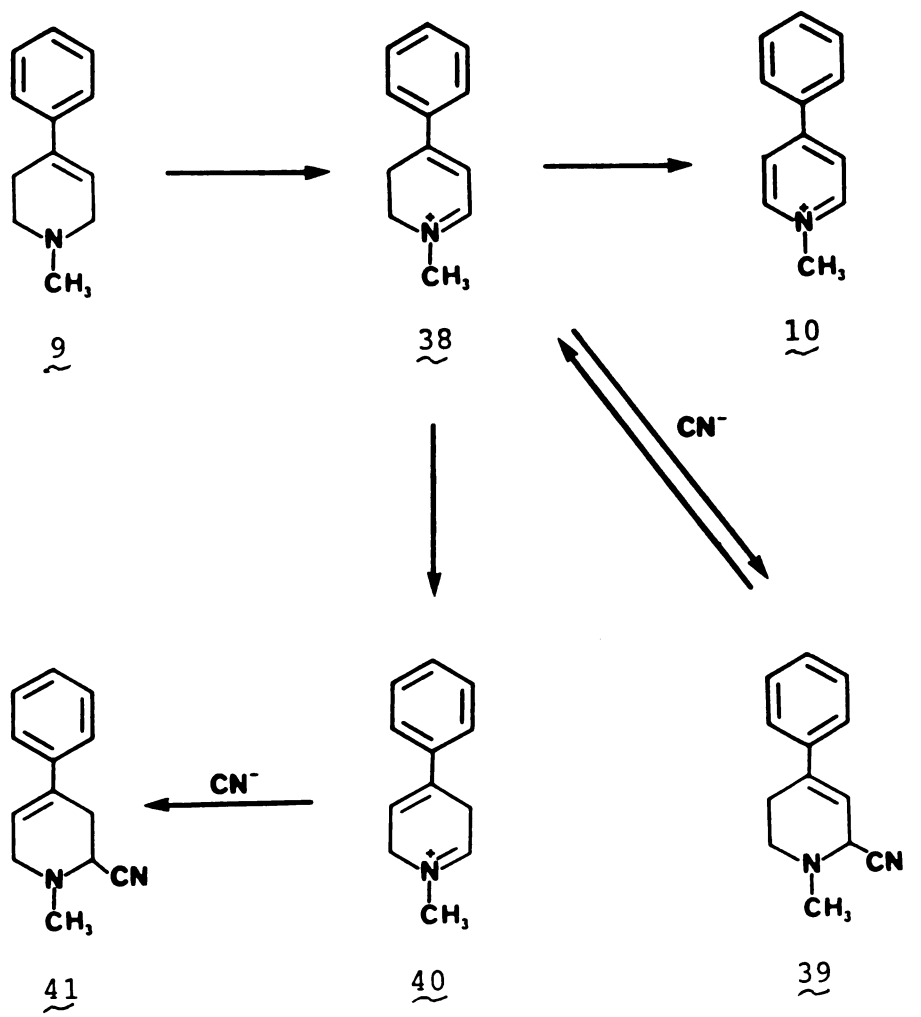
The antineoplasic agent hexamethylmelamine 35 requires metabolic activation to elicit its cytotoxic effects. In vitro studies have demonstrated that the metabolite N-(hydroxymethyl)pentamethylmelamine (36) is cytotoxic and it has been suggested that this compound is the cytotoxic species in vivo as well.[38] While the carbinolamine is a precursor to



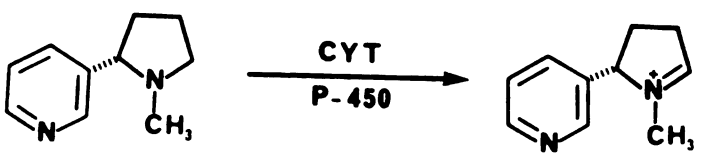
the toxin formaldehyde, ^{14}C-labeling studies have established that significant levels of the entire molecule become bound to cellular macromolecules following metabolic activation. Since the radiolabel of ^{14}C-ring labeled 36 also becomes bound to DNA and proteins in a metabolism-independent process, it was proposed that the carbinolamine decomposes to an iminium ion (37) which interacts covalently with cellular nucleophiles.[38]

Electrophilic iminium ions also have been encountered in the monoamine oxidase catalyzed oxidation of the neurotoxin 9 to the pyridinium species 10. Direct HPLC analysis of incubation mixtures of 9 with mitochondrial fractions led to the isolation and characterization of the unstable 1-methyl-4-phenyl-2,3-dihydropyridinium species 38 as an intermediate in the 4-electron oxidation of 9.[39] The inclusion of sodium cyanide in these incubation mixtures led to the formation of 6-cyano-1-

methyl-4-phenyl-1,2,3,6-tetrahydropyridine (39), confirming the intermediacy of 10 in the oxidation of 9.[40] The cyanide trapping experiments also revealed the formation of a second electrophilic iminium species, the 1-methyl-4-phenyl-2,5-dihydropyridinium ion 40 which was characterized as 2-cyano-1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (41). Deuterium labeling studies with synthetic 38 demonstrated that 40 results from the double bond rearrangement of 38.[40] These studies established that a number of reactive molecules were formed during the metabolic activation of MPTP by monoamine oxidase. The role of these intermediates in the toxicity of MPTP to nigrostriatal cells is unknown.



The data reviewed above demonstrate the importance of iminium ion intermediates in the metabolic and pharmacological profiles of tertiary amines. The goal of the studies described in this thesis was to examine the mechanism of formation of iminium ions in the cytochrome P-450 catalyzed oxidation of tertiary amines using (S)-nicotine (22) as a model compound. The role of iminium ions in the biotranformation of this pharmacologically important tertiary amine has been well documented and is reviewed in Chapter 2. We have studied the stereochemical course of the cytochrome P-450 catalyzed oxidation of 22 to the iminium ion 42. The results of these investigations are compared to those obtained in a variety of model reactions in order to gain some insight into the events taking place at the enyzme's active site.



22 42

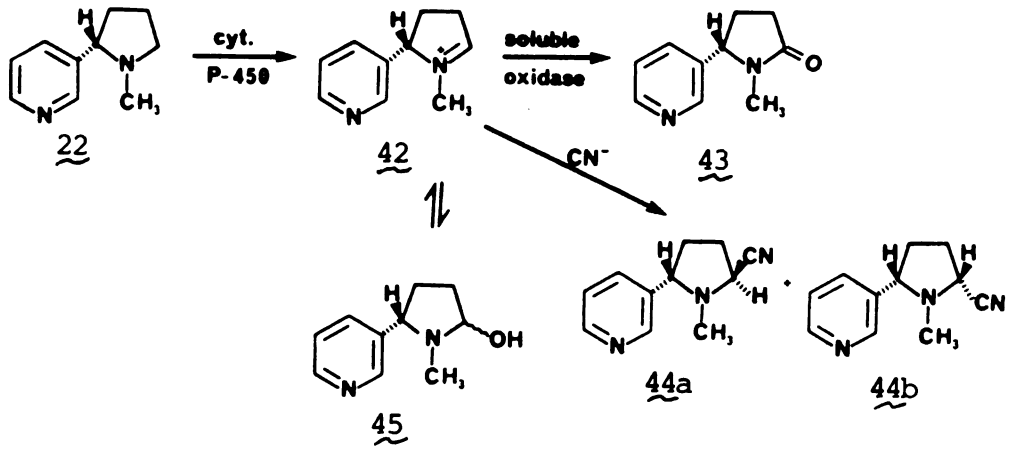
Chapter II.

STEREOCHEMICAL STUDIES OF THE CYTOCHROME P-450 CATALYZED OXIDATION OF (S)-NICOTINE

A. Introduction.

The use of tobacco products is believed to be a major risk factor in the development of cancer, cardiovascular disorders and pulmonary diseases.[41,42] While tumor initiators such as polycyclic aromatic hydrocarbons and co-carcinogens such as catechols are present in tobacco and appear to be largely responsible for the carcinogenic effects observed in laboratory animals,[43] some investigators have suggested that (S)-nicotine (22), the principal tobacco alkaloid, and its pyrolytic and metabolic breakdown products may contribute to the higher incidence of these diseases in smokers.[44-49] This possibility, in addition to nicotine's important pharmacological properties, has stimulated considerable interest in its metabolic fate in man and experimental animals.[50,51]

The metabolic profile of (S)-nicotine is quite complex--over 10 metabolites have been partially or fully characterized.[50,51] The major pathway in mammalian species involves a four electron oxidation at the prochiral 5'-position to form (S)-cotinine (43).[50] The initial step in this oxidative biotransformation is a two electron oxidation catalyzed by membrane bound cytochrome P-450 to yield an intermediate which, in turn, undergoes a second two electron oxidation catalyzed by a soluble cytosolic oxidase.[29-31,52-55]



The exact nature of the intermediate is unknown. Attempts to isolate the intermediate for precise structural characterization have been unsuccessful due to its instability.[52,56,57] However, the net process is likely to produce the iminium species 42. Evidence in support of this proposal includes the isolation of the intermediate as the stable diastereomeric 5'-cyanonicotines 44a and 44b from microsomal incubations of nicotine performed in the presence of the required cofactors and potassium cyanide.[29-31] These adducts most likely result from the reaction between cyanide ion and 42. Further evidence for the importance of 42 in the conversion of 22 to 43 was obtained from ^{18}O-studies. ^{18}O-labeled cotinine is not obtained when nicotine is incubated with both microsomal and soluble fractions of rabbit liver preparations under an $^{18}O_2$ atmosphere.[29] Since classical cytochrome P-450 catalyzed oxidations involve incorporation of molecular oxygen into the substate, the absence of label in the lactam is consistent with either initial 42 formation or loss of the label through equilibration of 5'-hydroxynicotine (45) with 42. NMR studies with synthetic 42 bisperchlorate provided evidence for the latter possibility by demonstrating that 42, not 45, is the major species (>90%) in pH 7.4 phosphate buffer.[58]

Both the carbinolamine 45 and the iminium ion 42 may participate in the overall mechanism involved in the cytochrome P-450 catalyzed α-carbon oxidations of tertiary amines. The available experimental evidence reviewed in Section III.A. best supports a stepwise reaction sequence involving initial electron transfer from the nitrogen atom to the electron deficient iron-oxo species to generate an aminium cation radical intermediate 46 as shown in Figure 2.[19-22,59-60] The aminium radical can then follow one of several pathways. Loss of a proton forms a neutral carbon-centered radical (47). This species can combine with the enzyme-bound oxygen moiety to form the carbinolamine 45 or give up another electron to generate the iminium ion species 42. Alternatively, the aminium radical can lose a hydrogen atom directly to generate the iminium species in a single step. The iminium ion can be released directly or combine with the hydroxide ion generated during the reaction and be released as the corresponding carbinolamine 45. The carbinolamine then could be in rapid equilibrium with the iminium ion in solution.

The two C-5'-protons of (S)-nicotine are diastereotopically related and, therefore, the two electron oxidation of 22 may proceed with the stereoselective loss of the pro-(R) or pro-(S) proton. The stereochemical course of this reaction could be influenced by several factors including the relative C-H bond energies of the reactive species undergoing C-H bond cleavage and the interactions between the substrate and the enzyme's active site. In order to gain some understanding of these factors, we have undertaken studies to determine if this biotransformation is under stereochemical control and to compare the stereochemical outcome of this enzyme-catalyzed reaction with various model systems.

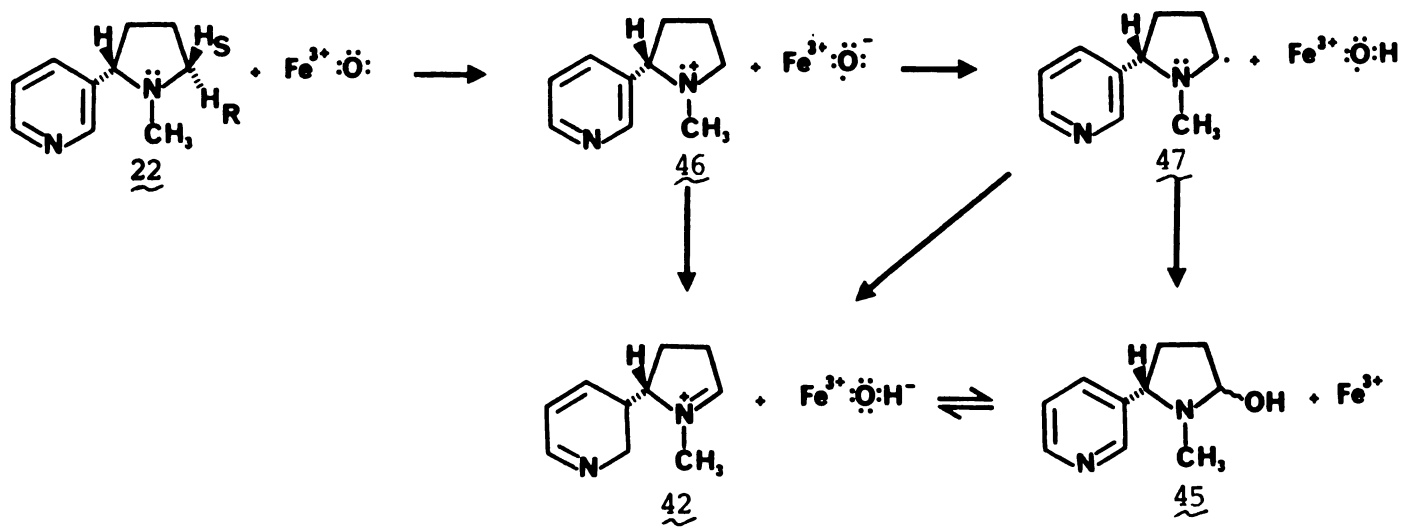
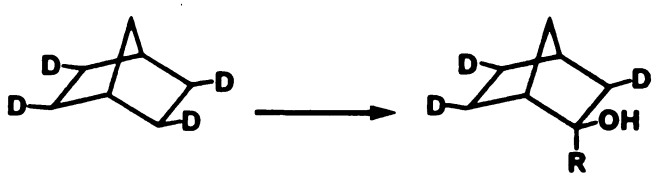


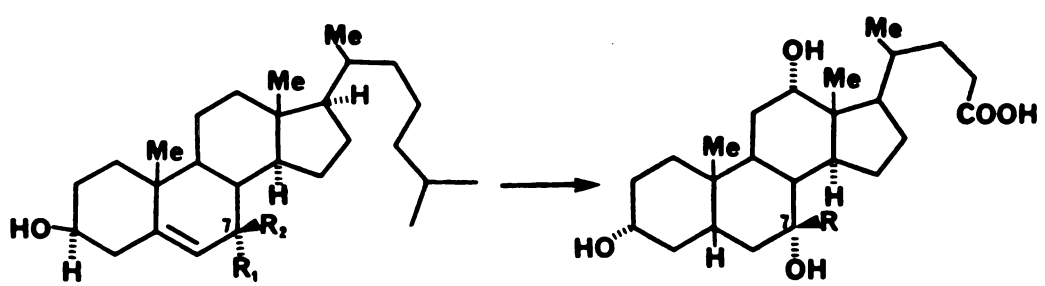
Figure 2. The proposed mechanism of the cytochrome P-450 catalyzed α-carbon oxidation of tertiary amines.

Stereoselective conversions related to the proposed study have been observed in a number of oxidative biotransformations through the use of stereoselectively labeled substrates and have been used as mechanistic probes for the enzymatic reaction. A number of hydroxylations at diastereotopic positions have demonstrated that the process proceeds with retention of configuration; that is, the oxygen atom is added to the same side of the molecule from which the hydrogen atom is removed. For example, the aliphatic hydroxylation of norbornane-exo,exo,exo,exo-2,3,5,6-d_4 (48) by reconstituted cytochrome P-450 to yield exo-norborneol (49) proceeded with 75% abstraction of the exo-hydrogen.[62] The 7-



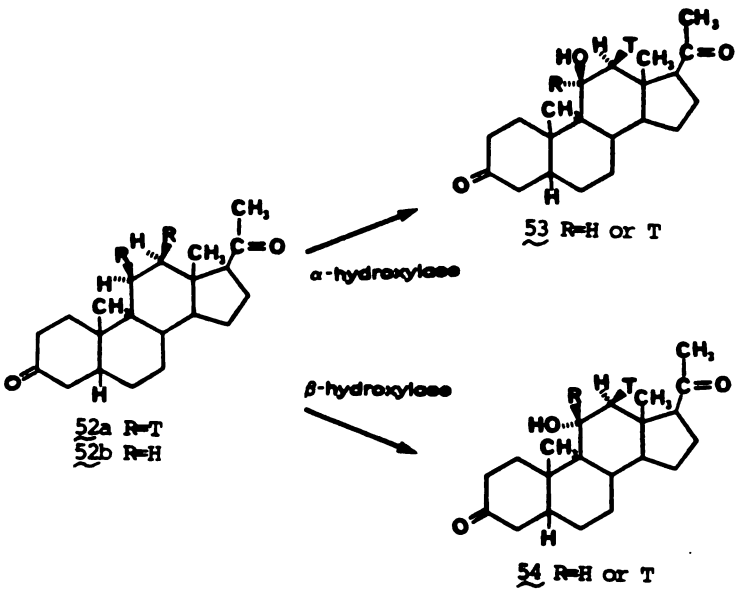
48 49 R=H or D

α-hydroxylation of cholesterol (50) to cholic acid (51) in rats also proceeded stereoselectively, with greater than 90% loss of the 7-α-hydrogen atom as demonstrated with cholesterol-7-α-t_1 (50a) and cholesterol-7-β-t_1 (50b).[63] When the stereochemical course of the 11-α-



50: R_1, R_2=H
50a: R_1=H; R_2=T
50b: R_1=T; R_2=H

51: R=H or T

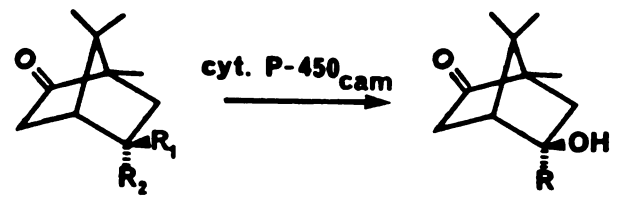


52a R=T
52b R=H

53 R=H or T

54 R=H or T

and 11-β-hydroxylation of pregnanedione-11-α-12-α-t_2 (52b) was studied in perfused bovine adrenal glands, the hydroxylation of the 11-α-position occurred with 31% retention of the radiolabel in the oxidation product 53 whereas complete retention of the label was observed for the 11-β-hydroxylation product 54.[64] The microbiological oxidation of pregnane-3,20-dione (52b) also has been shown to proceed with stereospecific displacement of the 11-α-hydrogen atom as demonstrated with pregnane-3,20-dione-11-β-d_1 and -11-α-d_1.[65] On the other hand, the 5-exo-hydroxycamphor (55) isolated from incubation mixtures of either camphor-5-exo-d_1 (56a) or camphor-5-endo-d_1 (56b) with bacterial cytochrome P-450_{cam} was enriched with the heavy isotope. The absence of

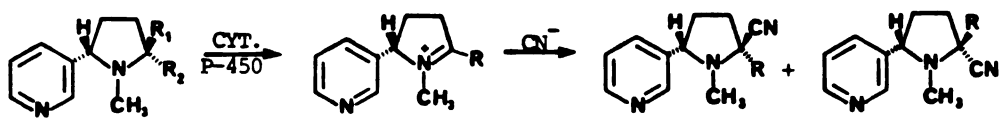


56a R_1=D; R_2=H
56b R_1=H; R_2=D

55 R=H or D

stereochemical control in this reaction suggests that the enzyme has access to either the 5-endo- or 5-exo-hydrogen atom.[66] According to our information, the stereochemistry of cytochrome P-450 catalyzed α-carbon oxidations to tertiary amines has not been investigated.

In order to examine the stereochemical course of the cytochrome P-450 catalyzed oxidation of the prochiral 5'-position of (S)-nicotine, the specifically labeled (E)- and (Z)-5'-monodeutereo diastereomers (57 and 58, respectively) of (S)-nicotine were required. Subsequent analysis of the deuterium content of the 2-electron oxidation product then would provide a measure of the stereoselectivity of the reaction. Our synthetic approach to 57 and 58 have provided the required diastereomers in about 90% isomeric purity. Structure assignments were made unambiguously on the basis of ^{1}H-NMR analysis. Our approach to the determination of the loss or retention of the deuterium label in the oxidation product required the development of a mass spectral assay. This was achieved through the in situ conversion of the metabolically generated oxidation product (presumably the iminium species 42) to the corresponding α-cyanoamines 44a and 44b and analysis of these adducts for deuterium content by capillary GC-EIMS selected ion monitoring techniques.



57: R_1=D; R=H
58: R_1=H; R=D

42 R=H or D

44a R=H or D 44b

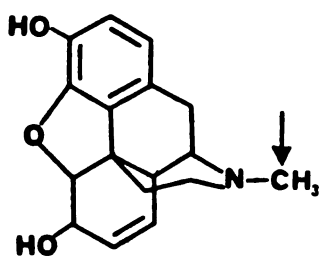
A potential complicating factor in these studies is the possible influence of a deuterium isotope effect on the stereochemical course of 5'-α-carbon oxidation of (S)-nicotine. A primary isotope effect could bias the reaction towards proton versus deuteron abstraction as has been observed in the P-450$_{cam}$ catalyzed 5-exo-hydroxylation of both camphor-5-endo-d_1 (56b) and camphor-5-exo-d_1 (56a).[66] The analysis of deuterium isotope effects has been a useful mechanistic probe for microsomal and reconstituted cytochrome P-450 catalyzed reactions.[67,68] The large isotope effects observed for aliphatic,[62] allylic,[69] and benzylic[70] hydroxylation reactions as well as O-dealkylations[67,71,72] mediated by cytochrome P-450 have led to the hypothesis that these types of reactions involve homolytic cleavage of the C-H bond to generate a carbon-centered radical prior to recombination with a hydroxy radical to yield the final product.[62,67,69-72] On the other hand, N-dealkylation reactions catalyzed by these enzymes proceed with only modest kinetic isotope effects.[19,20,61,73,74] These differences suggest that the α-carbon oxidation reactions of amines proceed through an alternate pathway.[19,68,75]

Mechanistic interpretations of isotope effects observed in our studies may be difficult. While the intrinsic or actual magnitude of a deuterium isotope effects is directly observed when the reaction in question is a single step process, it can be masked in multistep processes such as enzymatic reactions when they are determined by intermolecular competition, i.e., competition between labeled and unlabeled substrates. This suppression occurs because any one of the steps can be partiallly rate determining. Thus, the observed isotope effect reflects the relative rate of the chemical conversion in the overall process. A more accurate estimate of the intrinsic isotope effect can be achieved

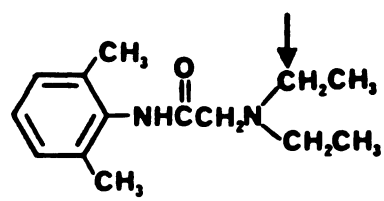
through intramolecular competitions between isotopically different but otherwise equivalent positions within the same substrate molecule. Even in these studies the true isotope effect can be suppressed if the two groups are not equally accessible to the enzyme's active site following binding of the substrate to the enzyme.[76]

In the proposed studies, the reaction under investigation involves a choice of two positions, one of which is labeled. However, the 5'-protons are diastereotopic due to the chirality at the 2'-position. As a result, the two atoms will react through diastereomeric transition states that will have differences other than those generated by isotopic substitution.[77] Thus, the possible influence of isotope effect on the stereochemical course of the α-carbon oxidation may be masked.

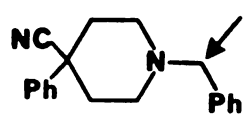
In general, modest inter- and intramolecular isotope effects have been observed for metabolic α-carbon oxidations of aliphatic tertiary amines. Intermolecular isotope effects of 1.23 for the N-demethylation of morphine (59),[78] 1.49 for the N-deethylation of lidocaine (16)[73] and 1.46 for the N-debenzylation of 1-benzyl-4-cyano-4-phenylpiperidine (60)[74] have been reported. A value of 1.0 ± 0.1 was found for the



59

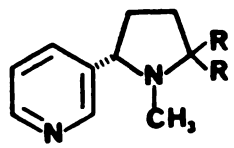


16

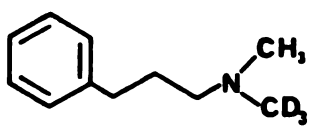


60

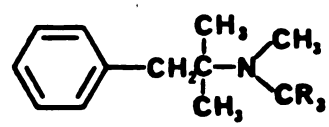
metabolism of (S)-nicotine (22) versus nicotine-5',5'-d_2 (22-5',5'-d_2).[79] These small intermolecular isotope effects indicate that cleavage of the α-C-H bond is partially rate-determining in the overall enzymatic process.



22 R=H
22-5',5'-d_2 R=D



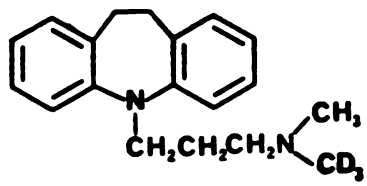
61



62 R=H
62a R=D

Intramolecular isotope effect studies also have revealed only modest values for this type of reaction. In 1975, Abdel-Monem reported that the N-demethylation of 1-(N-methyl-N-trideuteriomethylamino)-3-phenylpropane (61) proceeded 1.3 times faster at the N-methyl group than at the N-trideuteriomethyl group in microsomes from phenobarbital-pretreated and untreated rats.[60] similar studies with mouse microsomes demonstrated an isotope effect of 1.45 for this demethylation reaction.[60] While no intermolecular isotope effect was observed for the N-demethylation of N,N-dimethylphentermine (62), an intramolecular isotope effect between 1.6-2.0 was observed for this reaction when studied with N-trideuteriomethyl-N-methylphentermine (62a).[61] These moderate isotope effects have been compared to those seen in chemical oxidations of amines which involve aminium radical intermediates.[19] Shono and co-workers compared the intramolecular

isotope effects of microsomal and anodic N-demethylation of N-trideuteriomethyl-N-methylimipramine (63) (1.64 and 1.88, respectively).[19] The similarity of the values suggests a common mechanism for the two reactions. The mechanistic importance of such similarities will be discussed later.

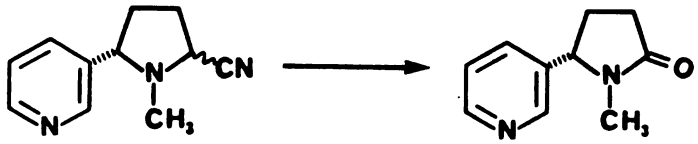


63

While the kinetic isotope effects observed for tertiary amines are modest, our concern that deuterium isotope effects could mask the stereoselectivity of 5'-α-carbon oxidation of 22 led to a investigation of the importance of these effects in the reaction. Furthermore, deuterium isotope effects could be helpful when comparing the results obtained for the cytochrome P-450-mediated reaction and the model systems. In order to distinguish between stereochemical effects and isotope effects, we have carried out experiments with both the 5'-(R) and 5'-(S)-deutereo diastereomers. If only stereochemical factors are important, we would expect to observe the same percent formation of unlabeled product from one diastereomer as labeled product from the other diastereomer. If the oxidation products from both diastereomers show an enrichment of the labeled material, then we may conclude that the stereochemical course of the oxidation has been influenced by kinetic isotope effects.

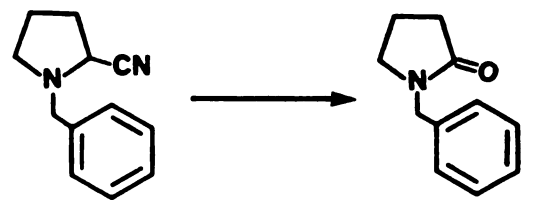
A second but perhaps less serious confounding factor concerns the possible influence of an isotope effect in the subsequent oxidation of the initial two electron oxidation products. The oxidation of the

iminium ion 42 or the corresponding carbinolamine 45 has been demonstrated to be catalyzed by soluble oxidases.[53] Our microsomal preparations, however, should contain only low levels of such oxidase activity.[53] Furthermore, the remaining activity should be inhibited by the cyanide ion present in the incubation mixtures since Hibberd and Gorrod demonstrated that 99% of the soluble oxidase activity in the 140,000 g supernatant fraction could be inhibited by 1 mM cyanide ion.[53] The inhibition of the soluble oxidase by cyanide ion also has been reported by others.[29,52,80,81] Therefore, under the conditions of this study, the soluble oxidase catalyzed further oxidation of the iminium ion should be neglible.

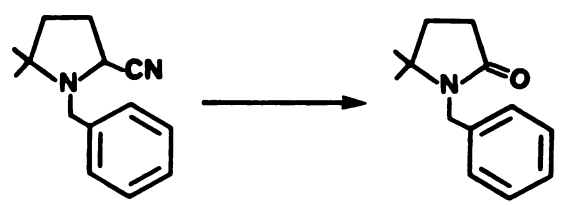


44a and 44b 43

It also is possible that the 5'-cyano adducts may be oxidized to cotinine in a reaction catalyzed by cytochrome P-450. Several α-aminonitriles including 1-benzyl-2-cyanopyrrolidine (64)[82] and 1-benzyl-2-cyano-5,5-dimethylpyrrolidine (65)[83] have been shown to be substrates for NADPH-dependent microsomal enzymes which catalyzed the formation of the corresponding lactams 66 and 67, respectively. These observations suggest that the 5'-cyanonicotines may be further oxidized to cotinine in our experiments. The importance of this reaction and its influence on the deuterium content of the α-cyanoamines observed in the stereochemical studies also will be considered in detail.



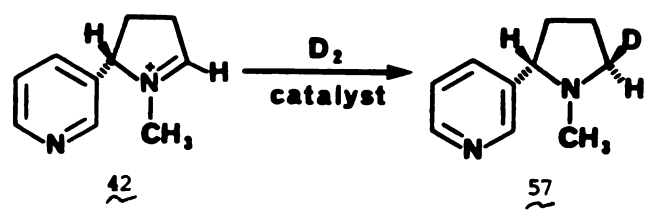
64 66



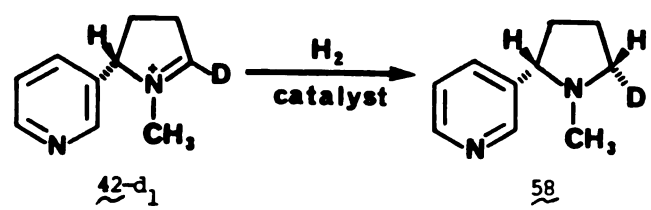
65 67

B. Synthetic Work.

Our approach to investigate this stereochemical problem required the two 5'-deuterionicotine diastereomers. Both diastereomers were required in order to study the possible influence of isotope effects on the stereochemistry of the reaction. While a number of methods for the synthesis of nicotine have been reported,[84-87] our approach to these products relied on the stereoselective catalytic reduction of the corresponding (S)-nicotine Δ-1'(5')-iminium species 42 and 42-5'-d_1. Catalytic hydrogenation is generally believed to result in cis addition to the less hindered side of the molecule since the molecule must be absorbed onto the surface of the catalyst prior to the addition of hydrogen.[88] A number of examples of stereoselective reductions have been reported.[88-90] Thus, reduction of 42 with D_2 would be expected to yield (S)-nicotine-(E)-5'-d_1 (57), that is the product resulting from the introduction of a deuterium atom to the least hindered side of 42. Similarly, reduction of 42-5'-d_1 with H_2 should provide (S)-nicotine-(Z)-5'-d_1 (58).



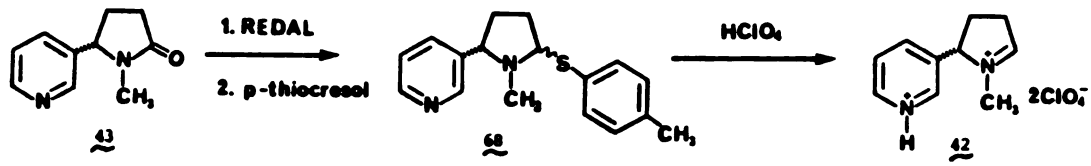
42 57



42-d_1 58

1. Nicotine-(E)-5'-d_1.

The synthesis of the required iminium ion as a crystalline bisperchlorate salt had been reported by Lindblom and Brandange.[58] This procedure involves the two electron reduction of (S)-cotinine with sodium dihydrobis(2-methoxyethoxy)aluminate (REDAL). The reduction product was purified by these workers as the thiocresol adduct 68. The bisperchlorate salt of 42 then was obtained in a 67% yield following treatment of the intermediate 68 with anhydrous perchloric acid in methylene chloride.



43 68 42

Our attempts to synthesize 68 by this pathway were unsuccessful. Addition of p-thiolcresol to the REDAL reduction mixture lead to the formation of a product which could be detected on thin chromatography (TLC). The nuclear magnetic resonance (NMR) spectrum also indicated the formation of a new product by the appearance of a broad doublet at 5 parts per million (ppm) and a series of multiplets at 4.05, 3.8 and 3.45 ppm (Figure 3). The reported literature values for the 2'- and 5'-proton signals are 3.83 and 5.5 ppm for the trans-isomer of 68 and 3.51 and 4.06 ppm for the cis-isomer.[58] The observed spectrum of our crude reaction mixture was similar to the literature values with the exception of the lower field doublet which occurred at 5.0 ppm instead of 5.5 ppm. Attempts to prepare the perchlorate salt of the suspected product gave an intractable gum. Lindblom reported that the rapid dimerization of 42 to yield 69 in aqueous solutions made the isolation of 42 bisperchlorate

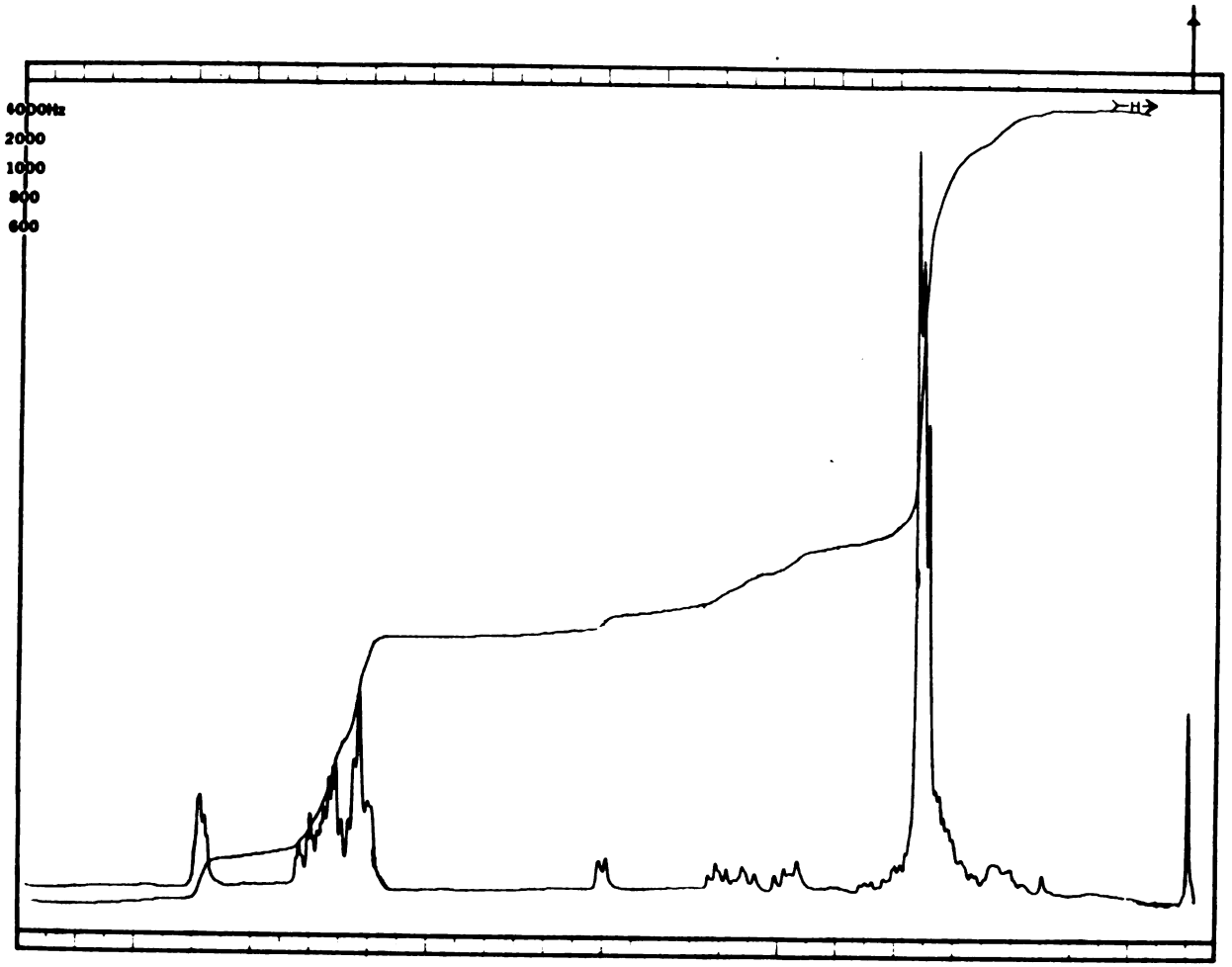
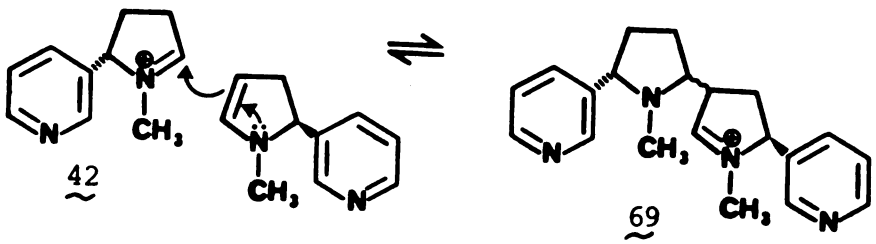


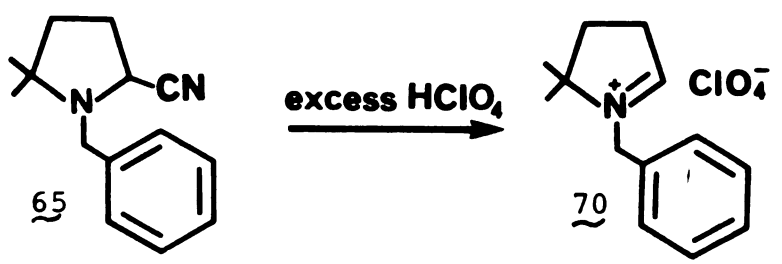
Figure 3. The 80 MHz ^{1}H-NMR spectrum of the product obtained from the reduction of cotinine by REDAL followed by the addition of p-thiocresol.



42 69

difficult unless performed under anhydrous conditions,[58] a precaution not followed in these initial efforts. Dissolution of the gum in water followed by the addition of potassium cyanide (KCN), however, led to the formation of the 5'-cyanonicotine isomers 44a and 44b. Confirmation of the assigned structures was obtained through ^{1}H-NMR analysis of the methylene chloride extract of the reaction mixture. This spectrum was similar to that reported for the diastereomeric mixture of (E)- and (Z)-5'-cyanonicotines[57,91] which will be discussed later in this section.

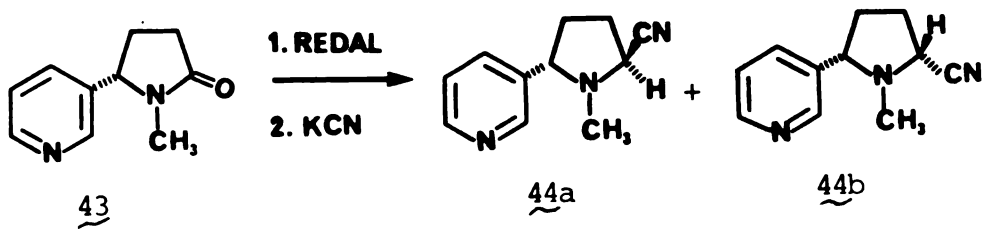
The formation of the α-aminonitriles in this system is analogous to the synthesis of 44a and 44b reported by Sanders et al.[57] Their method involved the controlled reduction of (S)-nicotine with sodium aluminum hydride and reaction of the product with aqueous potassium cyanide. Previous work in our laboratory by Sandro Probst had shown that 1-benzyl-5,5-dimethyl-1-pyrrolinium perchlorate (70) could be obtained from 1-benzyl-2-cyano-5,5-dimethylpyrrolidine (65) in quantitative yield by treatment with an ethanolic perchloric acid solution.[83] It was



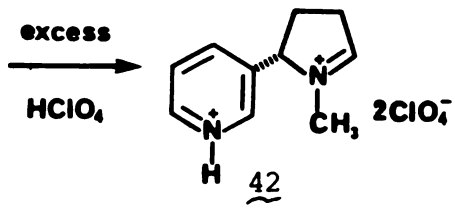
65 70

proposed that the bisperchlorate salt of 42 could be achieved in a similar manner from the 5'-cyanonicotines which, in our hands, were more easily handled than the thiocresol adducts.

The reduction of 43 with REDAL was repeated and the reaction was quenched with aqueous potassium cyanide. ^{1}H-NMR analysis of the reaction product following column chromatographic purification corresponded to that previously reported.[57,91] The presence of both diastereomers was indicated by two singlets at 2.30 and 2.32 ppm which are assigned to the N-methyl protons of (E)- and (Z)-5'-cyanonicotine, respectively (Figure 4).



43 44a 44b



42

Separation of the diastereomers by capillary gas chromatography demonstrated a 2:1 mixture of the stereoisomers (Figure 5). The capillary GC-EI mass spectral characteristics of both diastereomers were identical (Figure 6). The mass spectra shown in Figure 6 corresponded

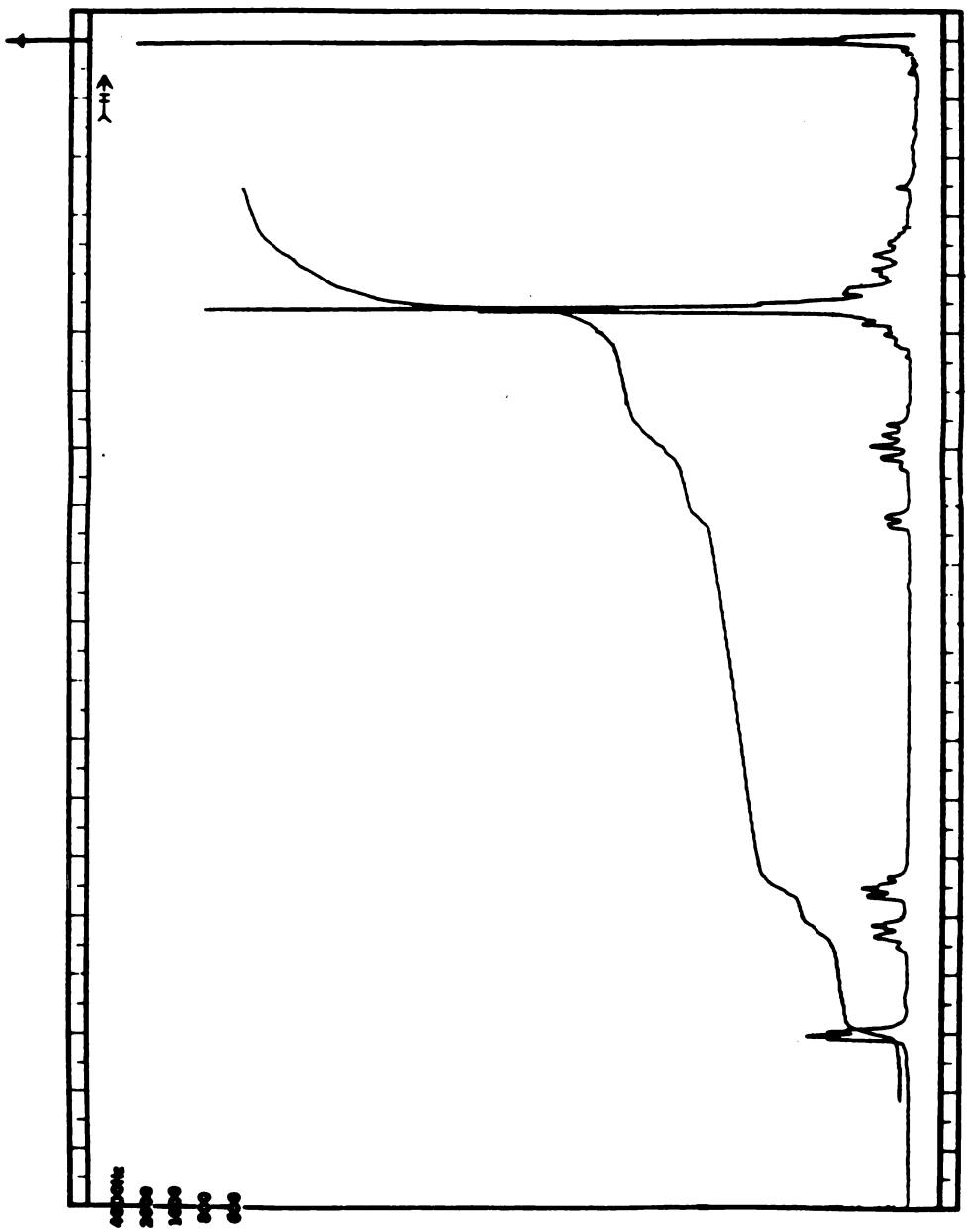


Figure 4. The 80 MHz ^{1}H-NMR spectrum of a mixture of the (E)- and (Z)-5'-cyanonicotines.

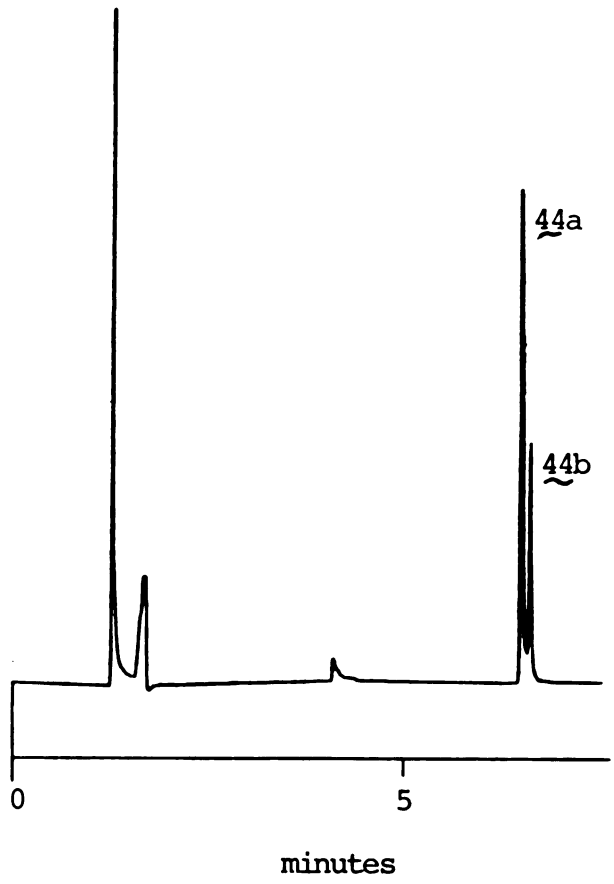


Figure 5. The capillary GC tracing of an n-butyl acetate solution of the synthetic (E)- and (Z)-5'-cyanonicotines (44a and 44b, respectively).

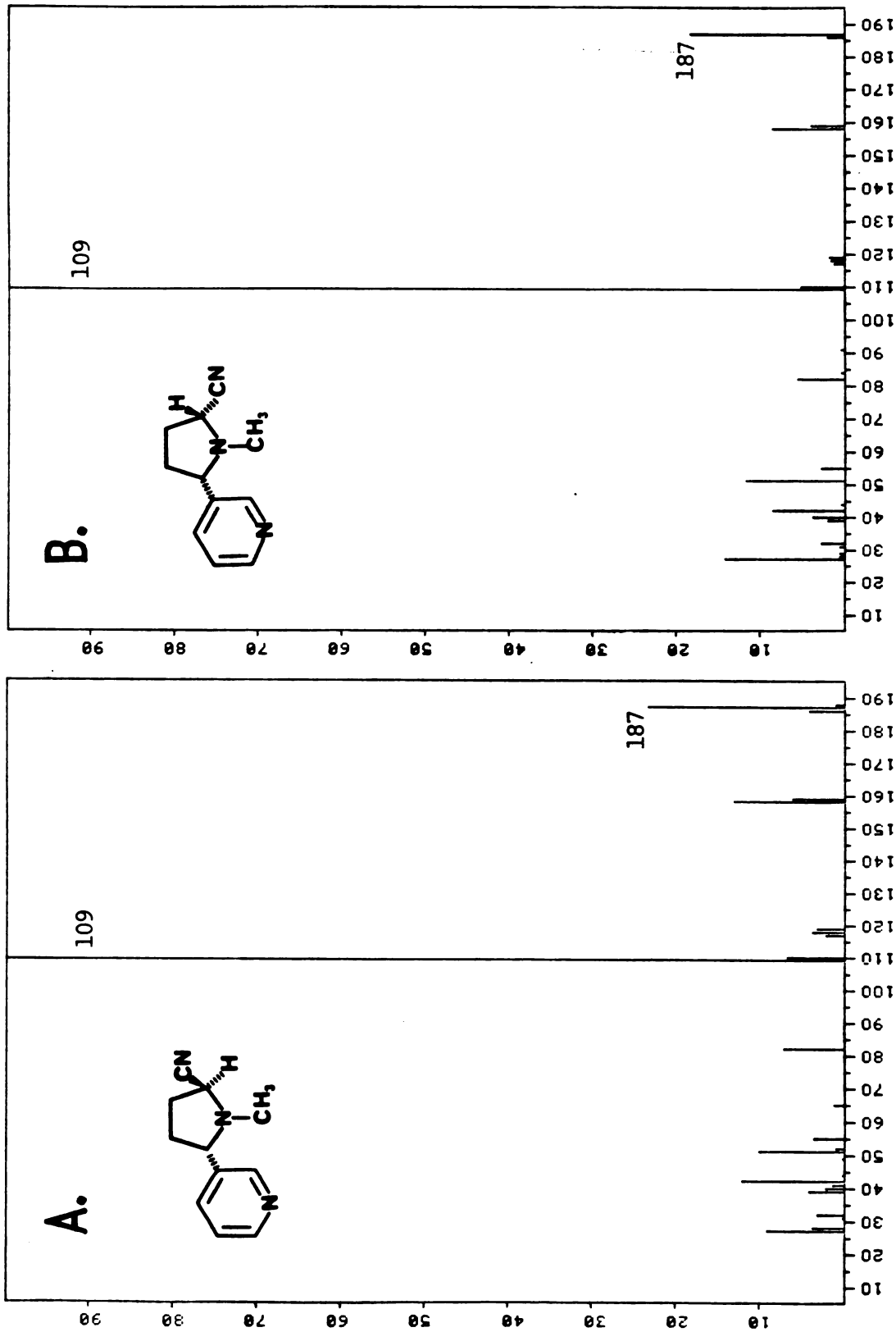
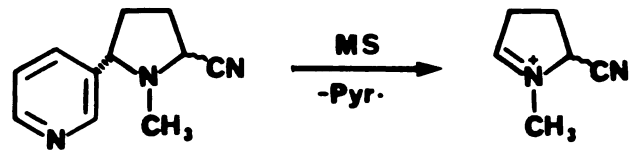


Figure 6. The EI-mass spectra of A.) (E)-5'-cyanonicotine and B.) (Z)-5'-cyanonicotine.

to that previously reported for 5'-cyanonicotine,[30] with the molecular ion (M^+) occurring at m/z 187. The base peak at m/z 109 corresponds to the fragment ion i resulting from the loss of the pyridine ring (M^+-C_5H_4N).



M^+: m/z 187 i: m/z 109

The diastereomers also were separable by normal phase high pressure liquid chromatography (HPLC) as shown in Figure 7. The assay was developed in collaboration with Dr. Y. Oka who used TLC to search for a mobile phase that would allow separation of the stereoisomers. The simplest system that gave baseline separation of the isomers consisted of ether saturated with ammonia (3:1). Reinjection of the purified components demonstrated that both compounds had epimerized under the conditions of analysis. The presence of the second isomer was believed to be due to epimerization of the purified isomer either on the column or in the collection flask. The epimerization was apparently an on-column phenomenon since the extent of epimerization decreased as the concentration of ammonia in the mobile phase decreased. A solution of 44a in ether-ammonia (3:1) was stable to epimerization for 1.75 days as demonstrated by ^{1}H-NMR analysis. The best separation with the least amount of epimerization was observed when the mobile phase consisted of a 2000:1 mixture of ether and ammonia. This system led to the isolation of the major isomer in 98% purity and the minor isomer in 91% purity as shown by HPLC analysis of the isolated peaks.

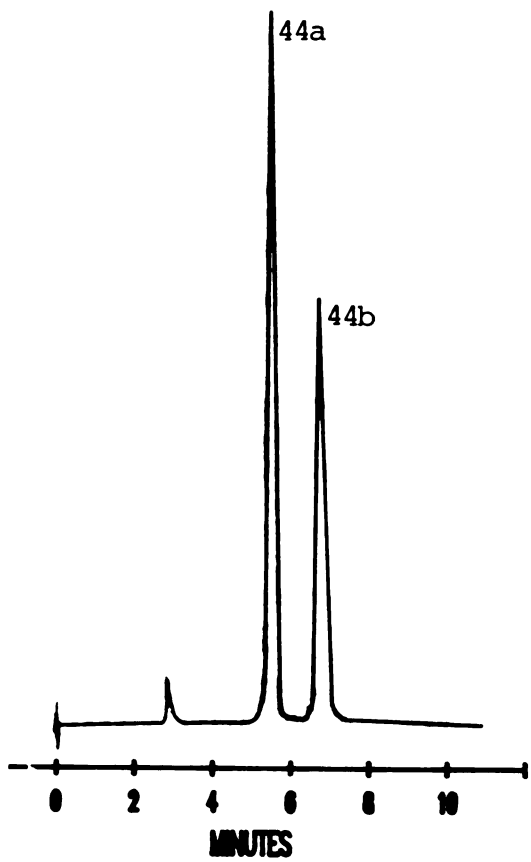


Figure 7. The HPLC tracing of the separation of the (E)- and (Z)-5'-cyanonicotine diastereomers (44a and 44b, respectively).

Isolation of each isomer from preparative-scale HPLC followed by ^{1}H-NMR analysis confirmed that the peaks corresponded to the two diastereomers. The major isomer was the first compound to elute and was assigned the structure of (E)-5'-cyanonicotine. The ^{1}H-NMR spectrum (Figure 8) displays the low field signals consistent with a 3-substituted pyridine ring. The 5'-proton resonates as a doublet of doublet at 4.1 ppm and is deshielded by the cis-lone pair of electrons of the nitrogen atom and the geminal cyano group.[92-100] The triplet at 3.1 ppm corresponds to the 2'-proton and the singlet at 2.30 ppm was assigned to the N-methyl protons.

The ^{1}H-NMR spectrum of the second isomer is consistent with the structure of (Z)-5'-cyanonicotine (Figure 9). The signal for the N-methyl protons occurs at 2.32 ppm. The upfield shift of this signal relative to that of the (E)-isomer is most likely due to the expected trans-relationship between the cyano and N-methyl groups in 44a. This orientation makes the N-methyl protons less susceptible to the anisotropic shielding effects of the cyano moiety than in 44b where the N-methyl and cyano substituents are situated cis to one another. The 5'-hydrogen atom resonates at 3.1 ppm and overlaps with the signal for the 2'-proton. The 5'-proton signal appears upfield relative to the corresponding signal in the ^{1}H-NMR spectrum of 44a as a result of the shielding effects of the cis methyl group and trans-lone pair of the nitrogen atom. These shielding effects have been documented for (S)-nicotine[92-94] and other cyclic tertiary amines.[95-100]

These data demonstrate that both 5'-cyano diastereomers are formed in the reaction of the iminium ion with cyanide ion. The observation that 44a is the preferred stereoisomer suggests that the attack of the cyanide ion on the Re face of the iminium ion is hindered by the bulky

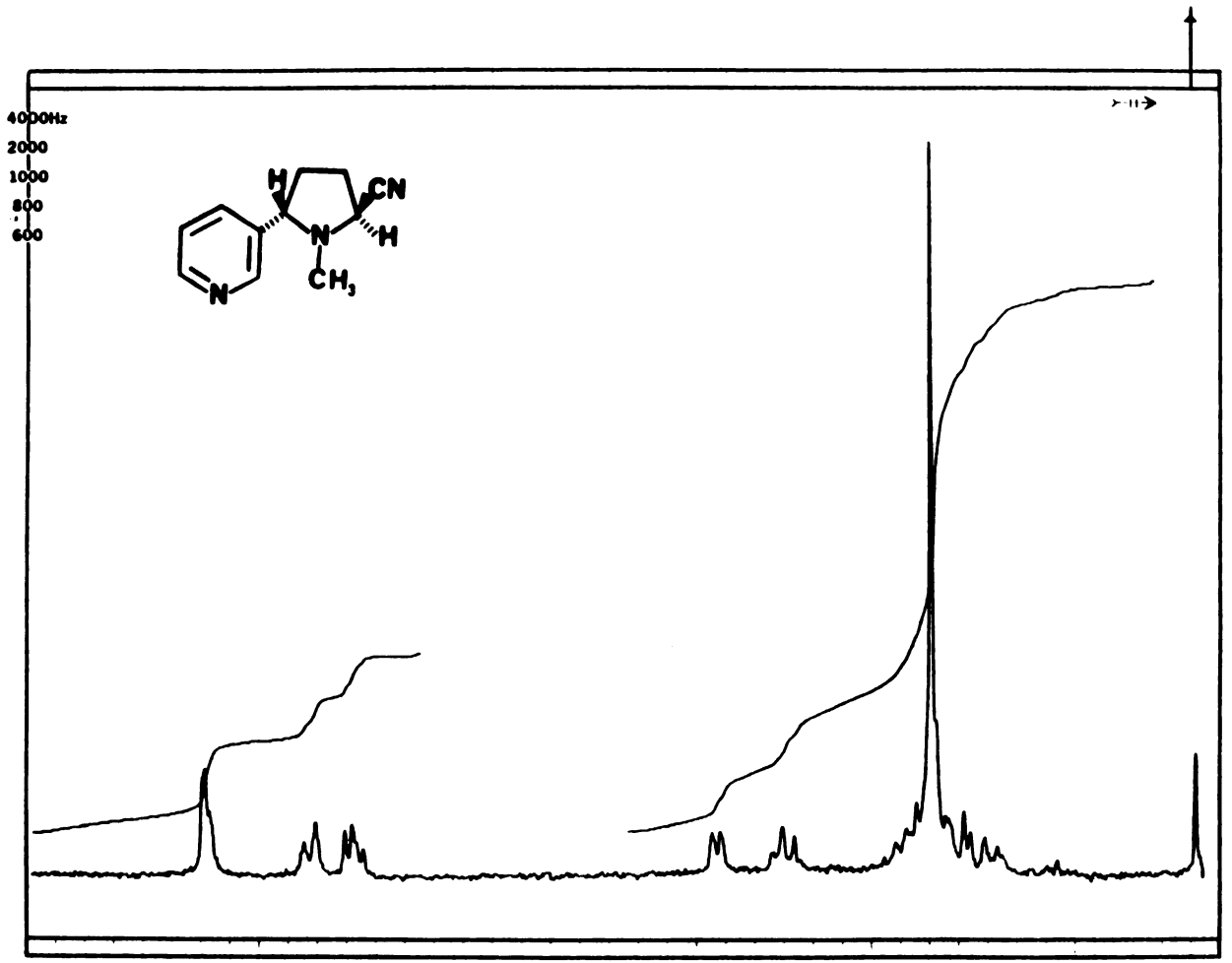


Figure 8. The ^{1}H-NMR spectrum of (E)-5'-cyanonicotine (44a) in $CDCl_3$.

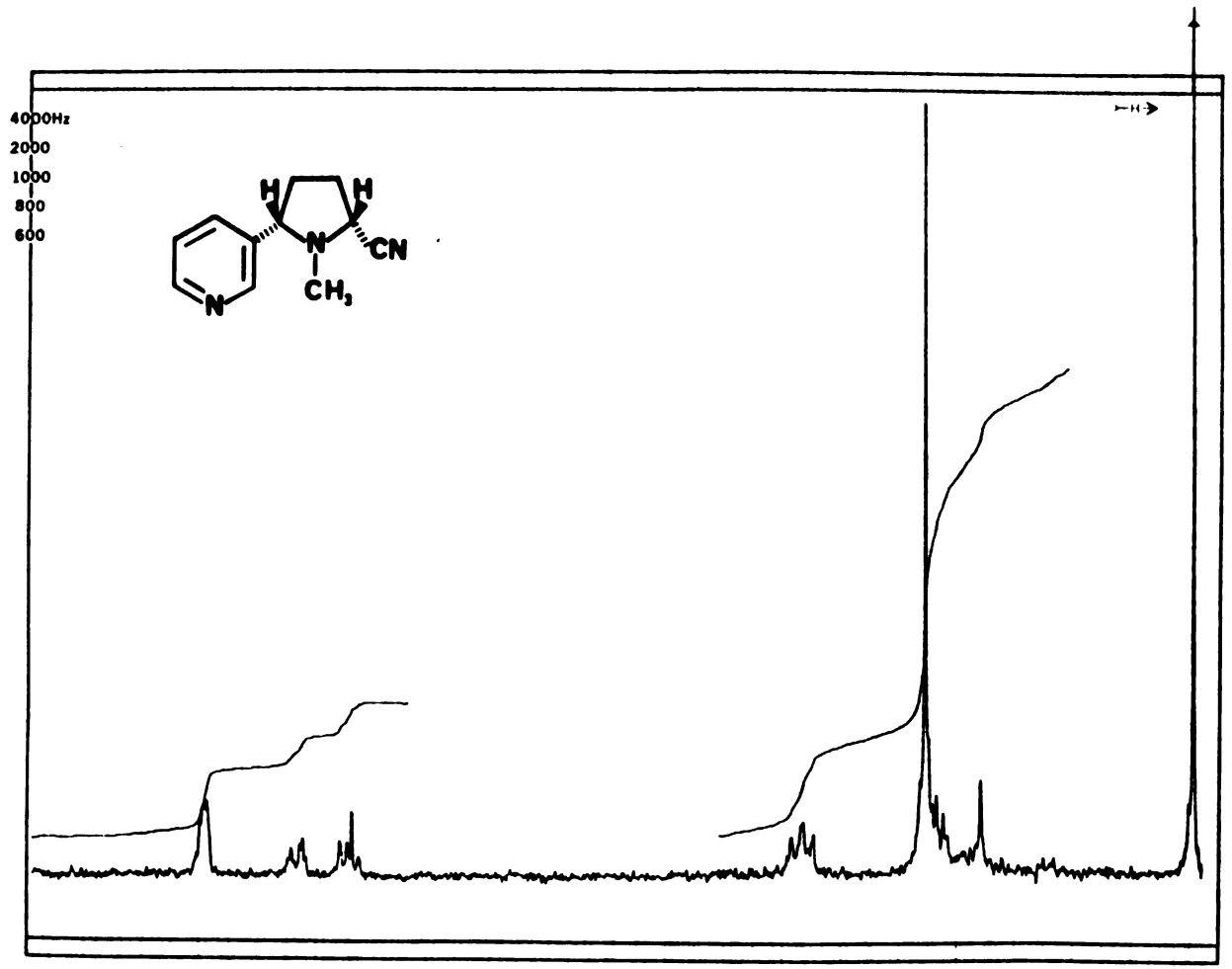


Figure 9. The 80 MHz ^{1}H-NMR spectrum of (Z)-5'-cyanonicotine (44b) in $CDCl_3$.

pyridine group. The same conclusion was reached by Sanders et al., who hydrolyzed the cyano group to the corresponding carboxylic acids, and, using NMR analysis, determined that the major species was the trans-isomer.[57]

Treatment of the purified mixture of 5'-cyanonicotine diastereomers with perchloric acid in ethanol yielded the crystalline bisperchlorate salt of 42 in 42% yield. The crystals melted between 240-242° C with some decomposition (literature[58] mp 230-232° C). The ^{1}H-NMR spectrum, taken in 10% deuterium chloride (DCl) in deuterium oxide (D_2O), was similar to that reported by Lindblom and Brandange (Figure 10).[58] The low field signals integrated for five protons. The pyridine protons account for four of these signals and the fifth signal can be assigned to $H_{5'}$. Protons analogous to $H_{5'}$ usually resonate in the 7.5-10.0 ppm range.[101] The signals resonating between 2.0 and 3.5 ppm integrate for seven protons. The singlet at 3.28 ppm is assigned to the N-methyl hydrogen atoms and is broadened by long range coupling with the 5'-proton. The complex multiplets appearing between 2.2 and 3.3 ppm are assigned to the C-3' and C4' methylene protons. The signal for C-2' hydrogen atom was not observed. This proton has been reported to resonate at 5.8 ppm.[58] The signal corresponding to HDO is centered at 5.9 ppm which obscures the C-2' proton signal. Elemental analysis agreed with the molecular formula of the bisperchlorate salt of 42. Further confirmation of the assigned structure was obtained by reduction of 42 with sodium borohydride to yield nicotine.

The catalytic deuteration of 42 required a solvent system in which the bisperchlorate of 42 was soluble. An investigation of the solubility characterics of this salt indicated that it was soluble in dimethyl sulfoxide (DMSO), dimethylformamide (DMF), acetonitrile (AcCN)

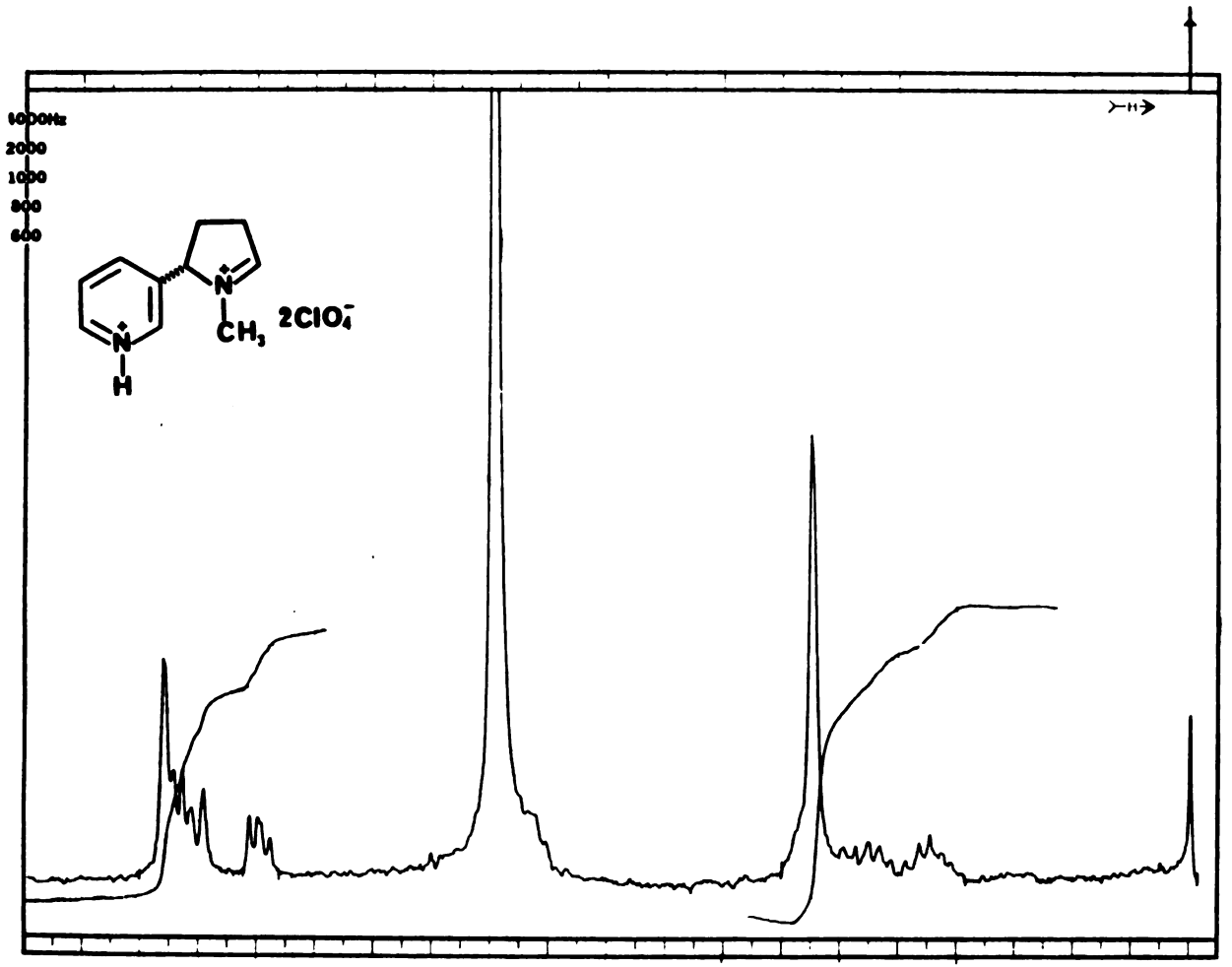


Figure 10. The 80 MHz ^{1}H-NMR spectrum of (S)-nicotine $\Delta^{-1'(5')}$-iminium bisperchlorate in 10% DCl in D_2O.

and water (H_2O). It was insoluble in ethyl acetate, acetone, dichloromethane, dioxane, tetrahydrofuran, methanol and diethyl ether. With this knowledge in hand, the catalytic deuteration of 42 bisperchlorate was attempted under a variety of reaction conditions. Platinum oxide (PtO_2), 10% palladium on carbon (Pd/C) or 10% palladium on calcium carbonate ($Pd/CaCO_3$) were used as catalysts. The reaction was tested in several solvent systems including dimethylformamide, water, deuterium oxide, acetonitrile, 2.5% deuterium chloride in deuterium oxide, and 2.5% hydrochloric acid in water. The reactions were monitored for the formation of nicotine either by GC or TLC. The results of these studies are summarized in Table 1.

Table 1. Deuterium composition of nicotine formed during the reduction of (S)-nicotine Δ-1'(5')-iminium ion under various conditions.

Catalyst	Solvent	Deuterium Incorporation into the Pyrrolidine Ring $d_0:d_1:d_2:d_3:d_4$*
PtO_2	D_2O	no reaction after 25 hrs
PtO_2	2.5% DCl in D_2O	11:72:17:0:0
PtO_2	2.5% HCl in H_2O	83:17:0:0:0
PtO_2	AcCN	46:46:8:0:0
PtO_2	DMF	no reaction after 45 hrs
Pd/C	D_2O	22:34:30:14:0
Pd/C	H_2O	37:38:23:2:0
Pd/C	AcCN	33:38:23:4:2
$Pd/CaCO_3$	D_2O	no reaction after 24 hrs

*The deuterium content of the reduction product was determined by capillary GC-EI mass spectral analysis of the reaction mixture. The extent of labeling was determined by the shift in the base peak ($M^+-C_4H_4N$) in the EI-mass spectrum of nicotine.

In all cases, the catalyst was prereduced by stirring the solvent-suspended catalyst in the presence of D_2 prior to the addition of substrate. The prereduction avoided a long induction period typical for the reduction of cyclic imino bonds.[88] No reduction was observed after 24 hours when palladium on carbon was used as the catalyst or after 45 hours when the reaction was attempted in the presence of platinium oxide in dimethylformamide. The reduction of 42 over platinum oxide in deuterium oxide was unsuccessful even upon heating (45° C) for more than 24 hours. In the presence of deuterium chloride, nicotine was formed after one hour. The acid presumably insures protonation of the starting material and also prevents the product amine from inhibiting the catalyst through protonation of the basic amine.[88] The reduction also proceeded in the presence of palladium on carbon in deuterium oxide in the absence of additional acid, suggesting that palladium on carbon is a more effective catalyst for this reaction. Both palladium on carbon and platinum oxide catalyzed the reduction in acetonitrile.

The extent of deuterium incorporation in the reduction product was determined by GC-EIMS analysis. The molecular ion could not be used to estimate deuterium content because M^+-H is a major fragment ion of nicotine.[102] Instead the base peak corresponding to ii, which occurs at m/z 84 $(M-C_5H_4N)^+$ for the unlabeled material, was used (see Figure 11). The only conditions which yielded monolabeled nicotine without significant amounts of unlabeled or multilabeled products were achieved with platinum oxide in deuterium oxide containing 2.5% deuterium chloride. The reduction product was contaminated with a small percentage of a

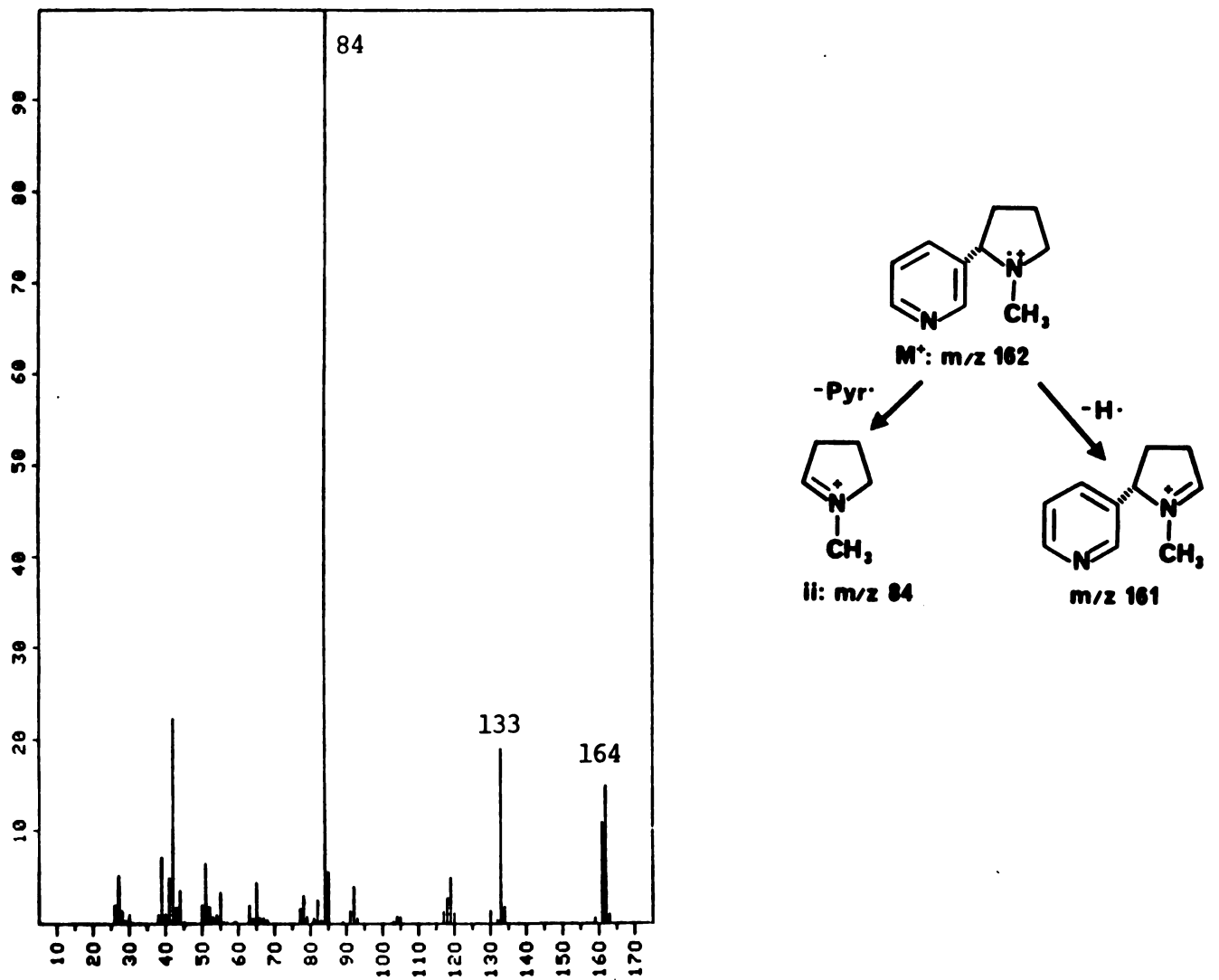
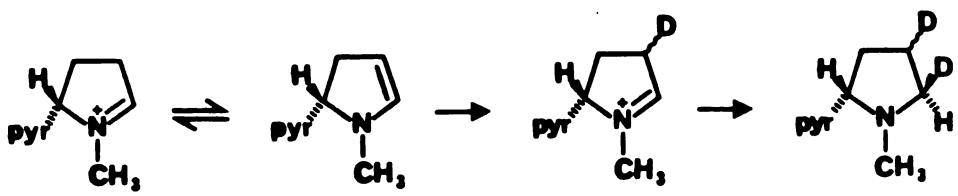


Figure 11. The EI-mass spectrum and fragmentation pattern of (S)-nicotine.



42 57a

dideuterated species (57a) which is presumably formed via proton-deuteron exchange at C-4' of the iminium perchlorate. Such an enamine-iminium ion equilibrium in basic solutions has been reported previously for this compound.[103].

When the deuteration was carried out in 2.5% hydrochloric acid in water, unlabeled nicotine was formed. Similar results were observed in the platinum oxide catalyzed reduction in acetonitrile; water in the solvent most likely was the source of protons. The protons of the solvent probably exchange with D_2 in the presence of the catalyst to form DH and H_2 which is then incorporated into the reduction product. This phenomenon, which has been observed by others,[104] stresses the importance of using a deuterated solvent or one containing no exchangeable protons for this type of reaction.

The stereoselectivity of deuterium incorporation was determined by ^{1}H-NMR analysis. Previous ^{1}H-NMR studies have established that the signal for the $H_{5'E}$ proton appears at 3.3 ppm, 1.0 ppm downfield from the corresponding signal for $H_{5'E}$ which appears at 2.3 ppm.[92-94] Initial analysis by 80 MHz ^{1}H-NMR proved inconclusive because the signals corresponding to $H_{5'Z}$ and $H_{2'}$ were not resolved. Attempts to improve resolution of these signals using the contact shift reagent tris(dipivalomethanato)europium were unsuccessful. These signals, however, were completely resolved in the 240 MHz ^{1}H-NMR spectrum of (S)-nicotine (Figure 12). The spectrum of unlabeled (S)-nicotine contains a

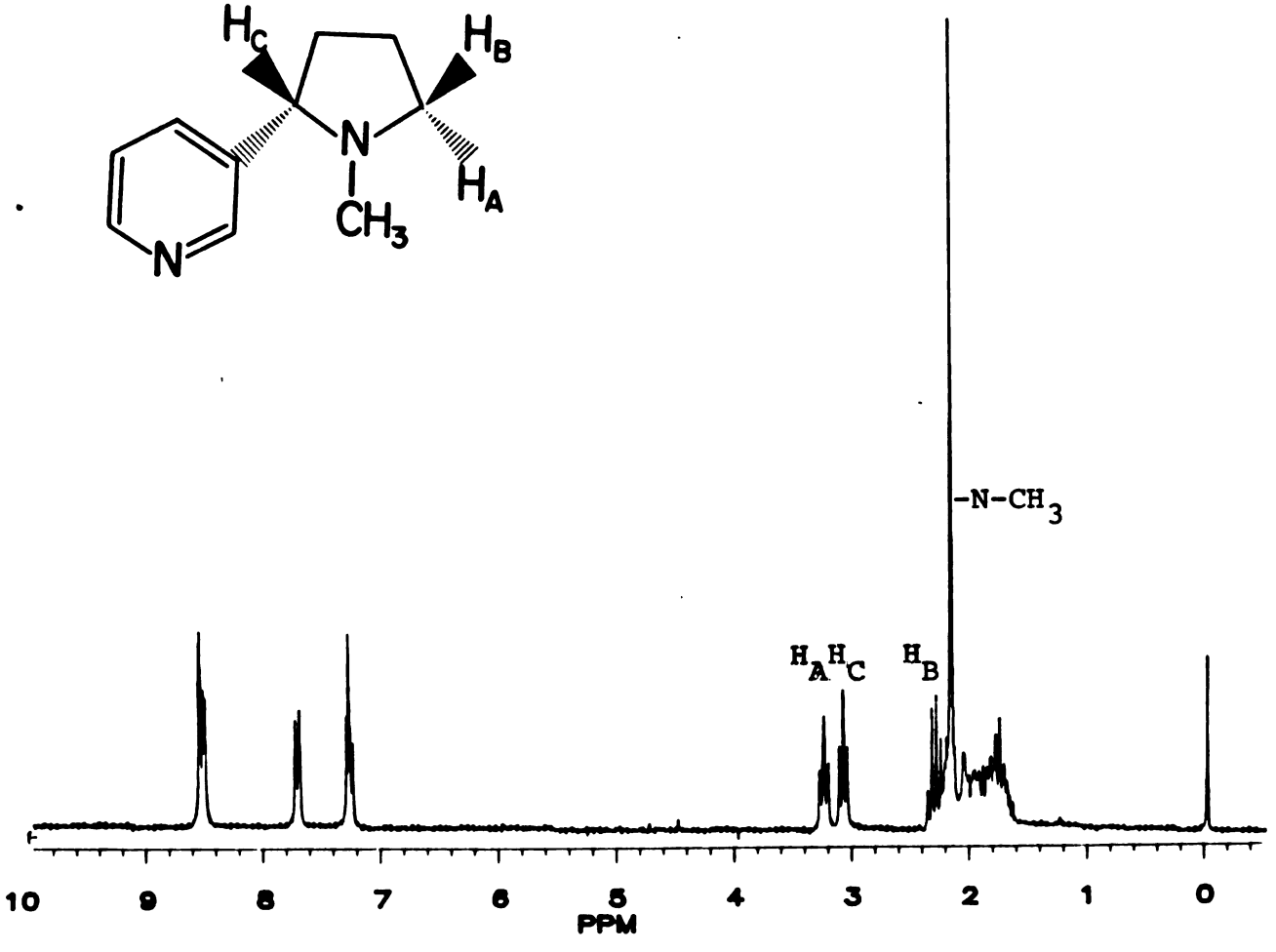


Figure 12. The 240 ^{1}H-NMR spectrum of (S)-nicotine in $CDCl_3$.

triplet at 3.3 ppm which corresponds to the pro-(R) 5'-proton. This hydrogen atom is deshielded relative to the pro-(S) proton by the cis lone pair on the nitrogen atom. The pro-(S) 5'-proton which resonates as a quartet at 2.3 ppm is shielded by the cis-methyl group.[92-100] The 240 MHz ^{1}H-NMR spectrum of the product obtained from the catalytic deuteration of 42 in the presence of platinum oxide in deuterium oxide containing 2.5% deuterium chloride suggested that the reduction had occurred in a stereoselective manner (Figure 13). The disappearance of the quartet corresponding to the signal for $H_{5'S}$ at 2.3 ppm and the simplification of the multiplet at 3.3 ppm for $H_{5'R}$ to a broad doublet reflecting the loss of the geminal coupling with $H_{5'S}$ are consistent with the addition of the deuterium atom trans to the pyridine ring to yield 57.

Since the catalytic deuteration of 42 under these conditions yielded the required monodeuterated nicotine, this procedure was used for the large scale synthesis of 57. Initial attempts resulted in low yields, possibly because of overreduction, irreversible absorption of the compound onto the catalyst and/or losses during the workup procedure. The yield of product was dramatically improved when the following precautions were taken. The progress of the reduction was monitored by direct analysis of the reaction by ^{1}H-NMR spectroscopy. The catalyst was removed upon disappearance of the N-methyl signal of the iminium ion at 3.5 ppm (about 3.5 hours). During the workup the dichloromethane extract was concentrated at atmospheric pressure rather than under reduced pressure to minimize losses of nicotine due to volatilization. The crude product was purified as its bis-l-tartrate salt which is stable and nonhydroscopic.[105] Furthermore, the salt can be used

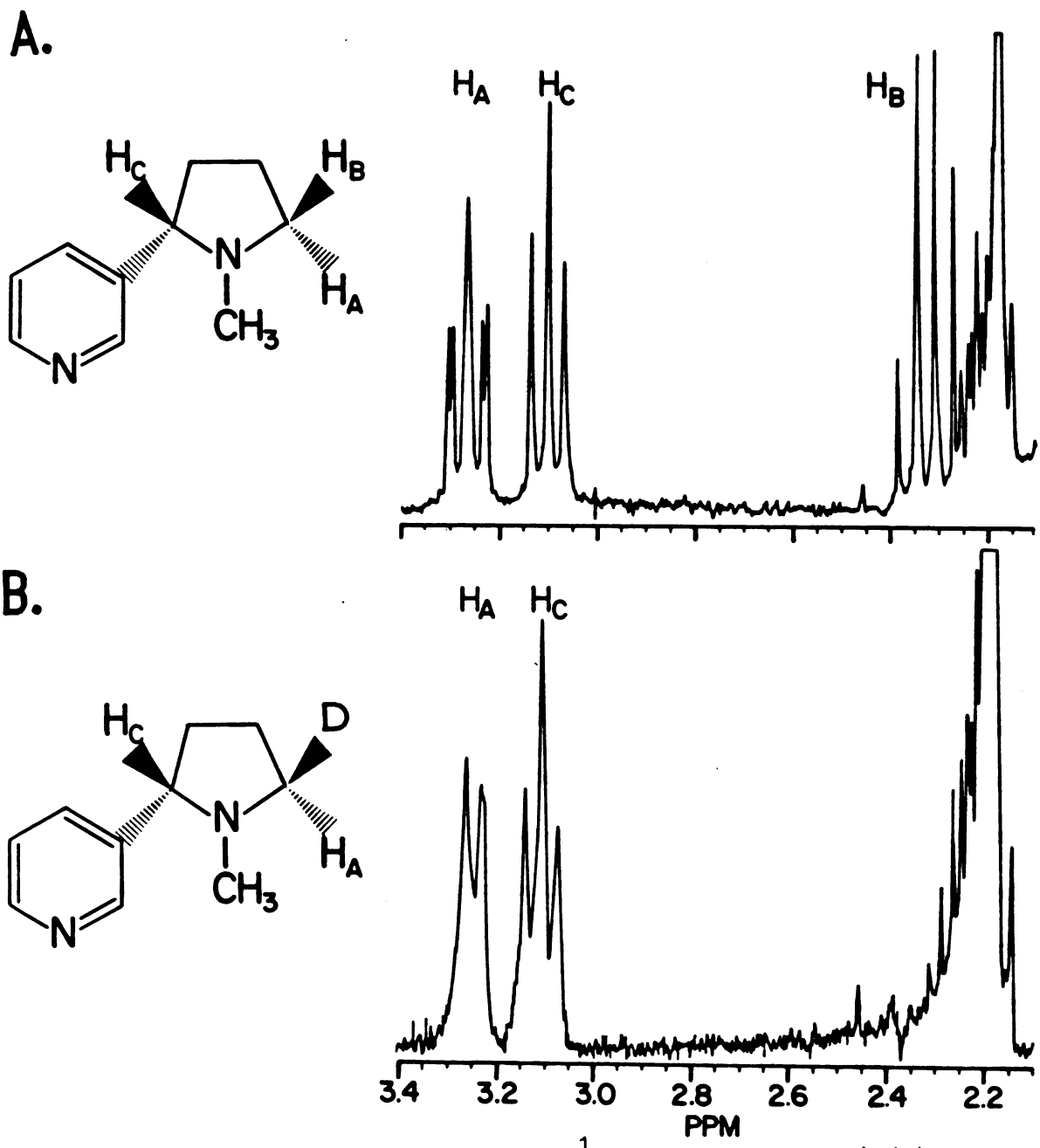


Figure 13. An expansion of the 240 ^{1}H-NMR spectra of A.) (S)-nicotine and B.) (S)-nicotine-(E)-d_1 in $CDCl_3$.

directly in metabolic studies since tartaric acid should not interfere with the enzyme systems.[105] Under these conditions, a yield of 56% was obtained.

Mass spectral analysis of the free base indicated that the reaction had proceeded primarily with the incorporation of one deuterium atom (Figure 14). The ratio of the ion intensities at masses 84:85:86 was found to be 2:88:10 (when corrected for the ^{13}C satellite contribution to the ion at mass 86). Analysis of the 240 MHz ^{1}H-NMR spectrum demonstrated that the incorporation had proceeded stereoselectively to yield the (E)-isomer 57. Repeated integration of the (Z)-5'- and 2'-proton signals and comparison of the areas indicated that the reduction had proceeded with 87 $\pm$ 2% (n=4) incorporation of the label in the pro-(S) position. Appropriate delay times were used to allow the protons to completely relax between radiofrequency pulses (delay time was greater than 5 times t_1) during the NMR experiments to insure accurate integration of the signals.[106] Since 2% of the observed (E)-5'-proton signal results from unlabeled material (determined by mass spectral analysis of the compound), the product contains 85% of the (E)-5'-labeled material.

The stereoselectivity of the reduction was confirmed by analysis of the ^{2}H-NMR spectrum of the bis-L-tartrate salt of 57 which is compared to that of nicotine-5',5'-d_2 in Figure 15. The ^{2}H-NMR spectra of the free base of a number of specifically labeled nicotine derivatives have been reported previously.[94,107] The ^{2}H-NMR spectrum of nicotine-5',5'-d_2 displays two signals, one at 3.8 ppm for $^{2}H_{5'Z}$ and the other at 3.4 ppm for $^{2}H_{5'E}$. The ^{2}H-NMR spectrum for 57 displays a strong signal at 3.4 ppm and a weaker signal at 3.8 ppm, confirming the location of the label in the (E)-5'-position. A weak signal is also observed in the aromatic region suggesting that a small amount of deuterium

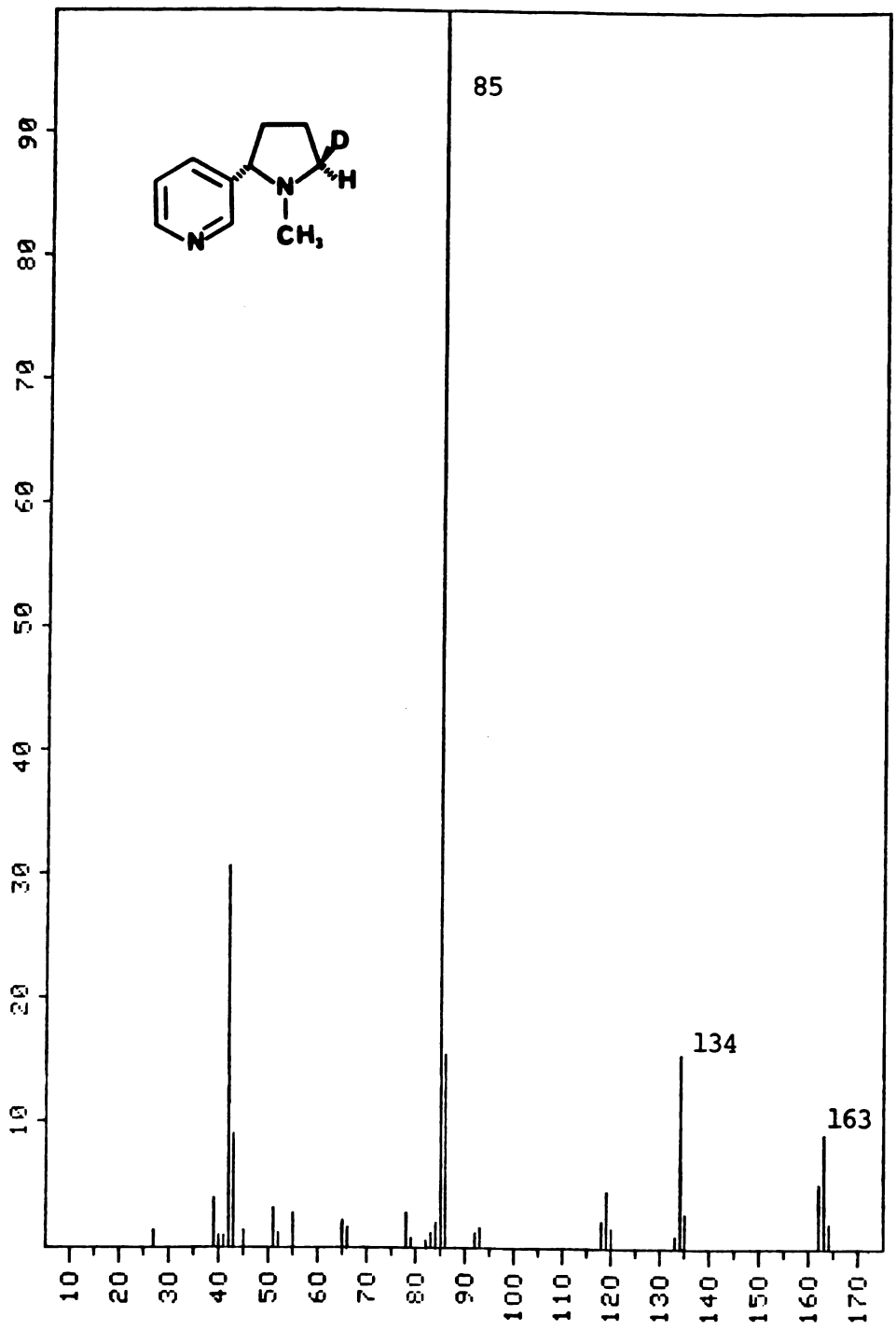
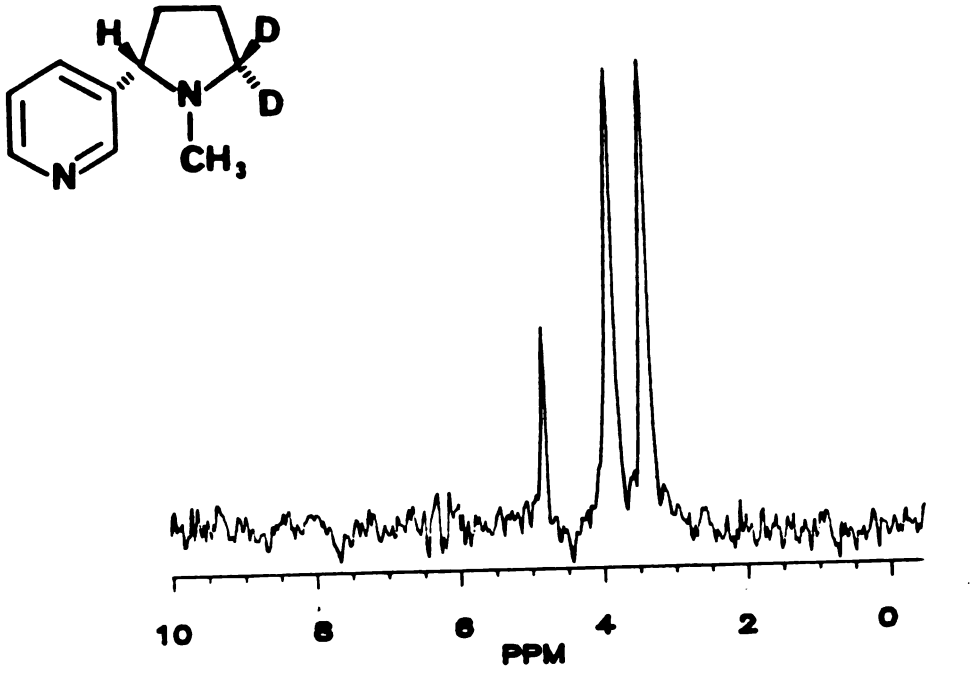


Figure 14. The EI-mass spectrum of (S)-nicotine-(E)-5'-d_1 (57).

A.



B.

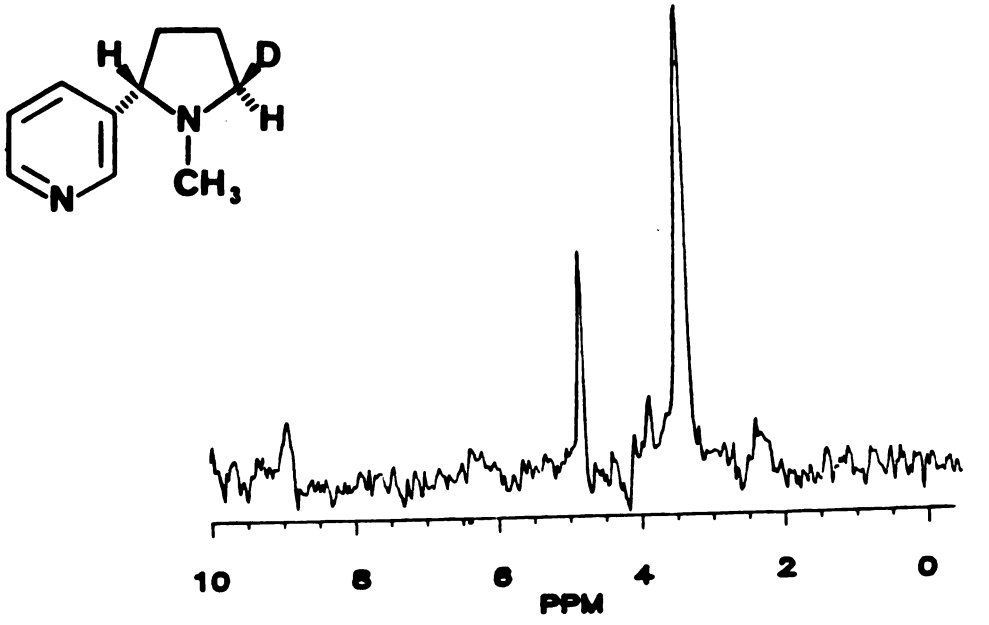


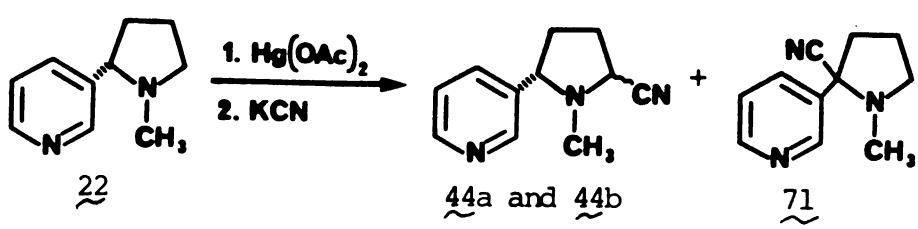
Figure 15. The ^{2}H-NMR spectra of A.) (S)-nicotine-5',5'-d_2 bis-l-tartrate and B.) (S)-nicotine-(E)-5'-d_1 bis-l-tartrate in D_2O.

incorporation has occurred in the pyridine ring. This contaminant can be ignored in the quantitative analysis of the metabolically generated cyano adducts since the deuterium content of these products are to be determined by analysis of the major mass spectral fragment which does not contain the pyridine ring ($M^+-C_4H_5N$: m/z 109. See Figure 6).

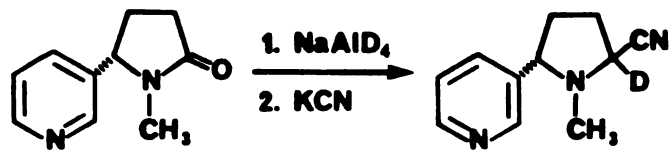
2. Nicotine-(Z)-5'-d_1

The second monodeutereo diastereomer, nicotine-(Z)-5'-d_1 (58), could be obtained in a manner similar to that used for the synthesis of 57 by the catalytic hydrogenation of 42-5'-d_1. The labeled iminium ion was to be prepared from the 5'-cyanonicotine-5'-d_1 diastereomers 44a-5'-d_1 and 44b-5'-d_1 by treatment with perchloric acid as described for 42. This synthetic pathway required the synthesis of the labeled 5'-cyanonicotine diastereomers. Several synthetic schemes were investigated. The most obvious method was to use the conditions described in the previous section for 44a and 44b by substituting REDAL with its deutereo analog, sodium dideuterobis(2-methoxyethoxy)aluminate. However, this reagent was not available commercially.

A number of procedures for the synthesis of the 5'-cyanonicotine diastereomers are reported in the literature[29,55,57,91] and presented us with several alternative routes to the corresponding deutero analog. One possibility involves the mercuric acetate oxidation of nicotine-5',5'-d_2 and reaction of the resulting intermediate mondeutereo iminium species with cyanide ion. Several authors have described the mercuric acetate oxidation of nicotine with varying results. Murphy reported the exclusive formation of the diastereomeric 5'-cyanonicotines,[29] while Hubert-Brierre et al. isolated both the 5'-cyano adducts and 2'-cyano adduct 71.[91] Sanders and co-workers obtained almost exclusively 71.[57]



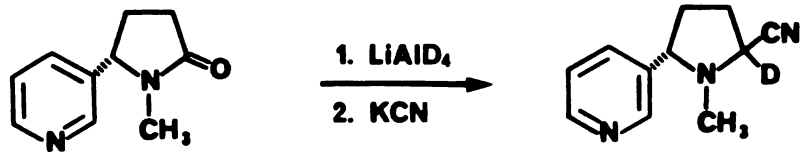
22 44a and 44b 71



43

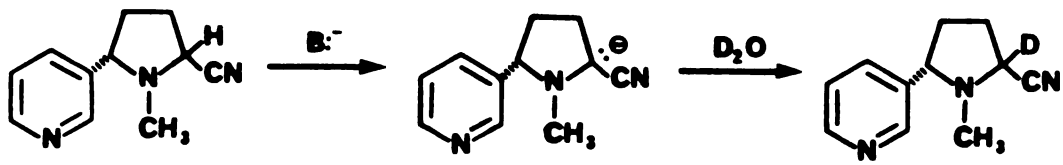
A second, more favorable route to the deuterated 5'-cyanonicotines involves the sodium aluminum deuteride ($NaAlD_4$) reduction of 43 followed by quenching the reaction with aqueous potassium cyanide according to the procedure described by Sanders et al.[57] This synthetic route should lead to only one positional isomer as was observed for the REDAL reduction. Although not available commercially, sodium aluminum deuteride can be readily prepared from sodium deuteride and lithium aluminum deuteride.[108]

A third possible route would proceed by the controlled reduction of cotinine with lithium aluminum deuteride to yield the iminium intermediate 42-5'-d_1. Although lithium aluminum hydride usually results in the full reduction of amides to amines,[109] the two electron reduction of tertiary amides to intermediates, which yield secondary amines and aldehydes, has been reported.[110]



43

A final possibility involves the base-catalyzed exchange of the 5'-α-proton of 44a and 44b. The α-aminonitrile carbanions of a number of tertiary amines are important intermediates in the preparation of a variety of mono- and disubstituted α-aminonitriles.[111,112] We proposed that treatment of 44a and 44b with a strong base such as n-butyl lithium should generate the carbanion 72 which would yield the desired labeled 5'-cyanonicotines following reaction with deuterium oxide.



44a and 44b 72

The mercuric acetate oxidation of nicotine led to a mixture of cyanoadducts in low yield (<45%). While the 5'-cyanonicotine diastereomers represented the major products (approximately 65%), significant amounts of 2'-cyanonicotine (15%) also were present in addition to unreacted nicotine (20%) as demonstrated by ^{1}H-NMR spectroscopy. The 80 MHz ^{1}H-NMR spectrum of purified 2'-cyanonicotine displayed signals in the aromatic region typical for a three-substituted pyridine ring (Figure 16). The N-methyl protons resonate as a singlet at 2.24 ppm. The multiplet centered at 3.3 ppm integrated as one proton and was assigned to the 5'-proton cis to the lone electron pair of nitrogen as observed for 22.[92-94] The multiplet at 2.55 ppm integrated for two hydrogen atoms and corresponds to the other 5'-hydrogen atom and a 3'-hydrogen atom. The remainder of the hydrogen atoms were assigned to the signals between 1.9 and 2.4 ppm which integrate for three protons. The values observed are different from those reported by Hubert-Brierre et

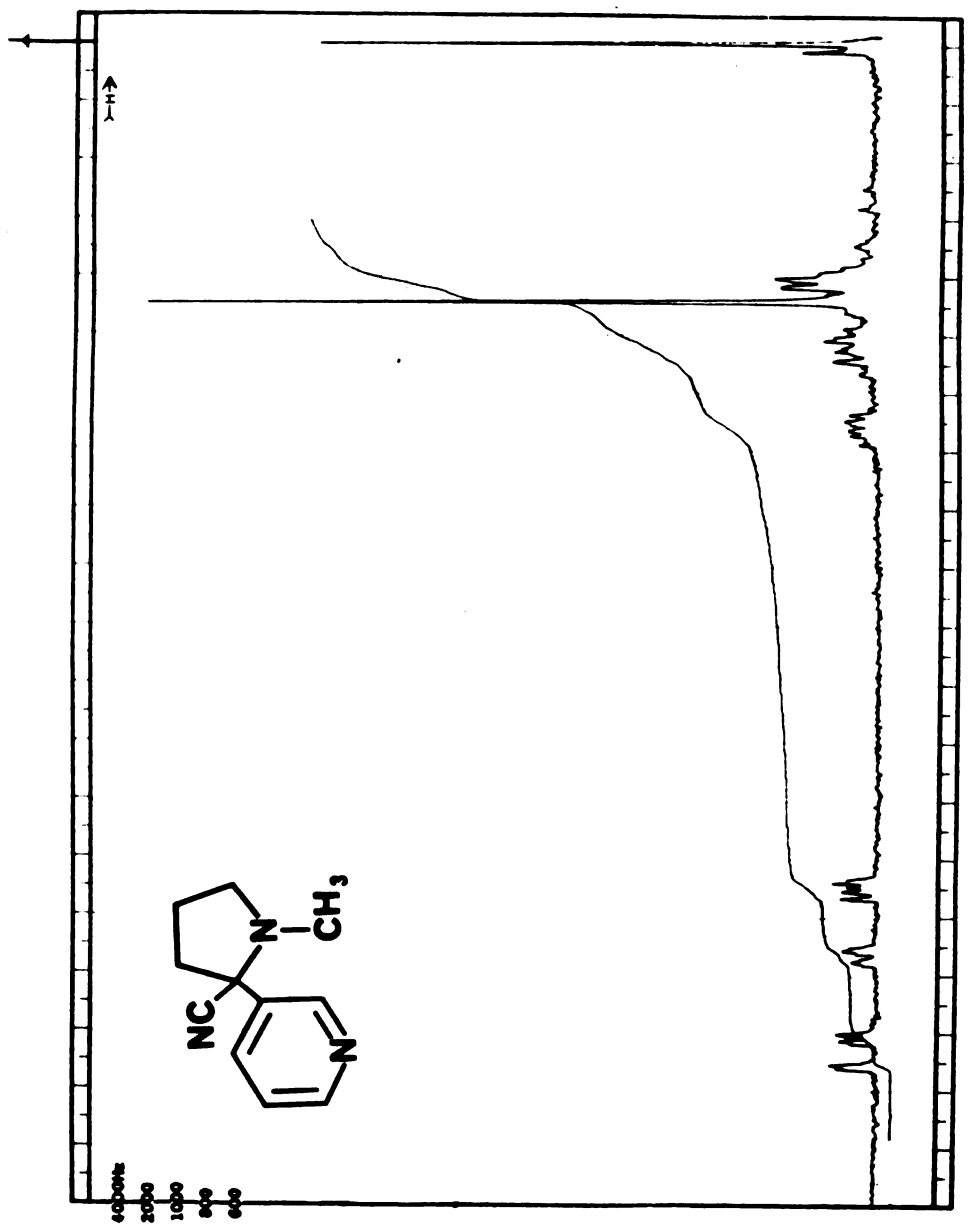
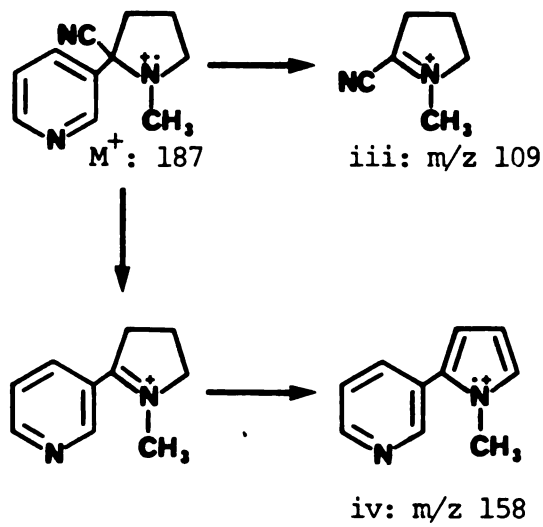


Figure 16. The 80 MHz ^{1}H-NMR spectrum of 2'-cyanonicotine in $CDCl_3$.

al.[91] who found a multiplet at 2.4 ppm for one 5' proton and a multiplet at 2.6 and 2.8 ppm for the other 5'-protons and a 3'-proton. The reason for these differences are unknown.

Capillary GC-EIMS analysis of 71 confirmed that this compound was a cyano adduct of nicotine since the mass spectrum contained a molecular ion at m/z 187 (Figure 17). The base peak is at m/z 109 and, like the 5'-cyanonicotines 44a and 44b, corresponds to the fragment iii which has lost the pyridine ring. Another major signal appears at m/z 158. This fragment (iv) most likely results from aromatization of the pyrrolidine ring. It is important to note that the mass spectrum of 71 does not contain a significant signal at m/z 82. This ion which is present in the mass spectra of 44a and 44b is derived from the loss of the pyridine ring and the cyano group. Such a fragment not possible for the 2'-cyanonicotine isomer.



The reduction of (S)-cotinine with freshly prepared sodium aluminum hydride or sodium aluminum deuteride in tetrahydrofuran followed by reaction with cyanide ion afforded a 50% yield of crude reaction mixture which was almost exclusively the 5'-cyanonicotine diastereomers

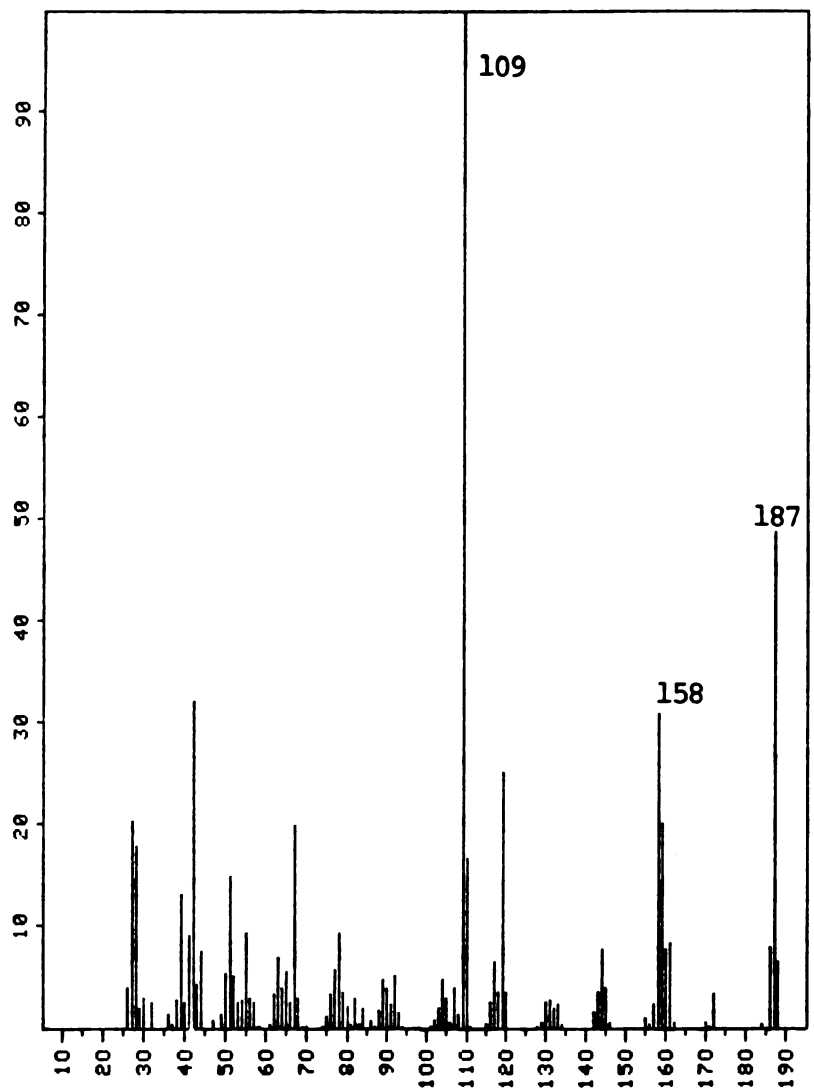


Figure 17. The EI-mass spectrum of 2'-cyanonicotine.

(>90%). This was established by ^{1}H-NMR analysis of the crude reaction mixture. Small amounts of cotinine (5%) and nicotine (3%) also were present.

The partial reduction of (S)-cotinine by lithium aluminum deuteride was performed at 0° C using only one hydride equivalent of reducing agent per mole lactam in order to reduce the extent of overreduction to nicotine. ^{1}H-NMR analysis of the crude product obtained following treatment with cyanide ion indicated the presence of cotinine, nicotine-5',5'-d_2 and the 5'-cyanonicotine-5'-d_1 diastereomeric mixture in a ratio of 1:7:3. The amount of nicotine relative to the other compounds was considerably greater than observed in the sodium aluminum deuteride reduction.

Attempts to exchange the 5'-proton of the 5'-cyanonicotine diastereomers using n-butyl or t-butyl lithium in dry tetrahydrofuran were unsuccessful.

From these studies it was determined that the best method for the preparation of the 5'-cyanonicotine-5'-d_1 diastereomers was the sodium aluminum deuteride reduction of (S)-cotinine followed by the reaction of the reduction product with aqueous sodium cyanide. ^{1}H-NMR analysis of the diastereomeric mixture confirmed the incorporation of deuterium at the 5'-position of both stereoisomers by the absence of both the 5'-proton signal of (E)-5'-cyanonicotine at 4.1 ppm and the 5'-proton signal of (Z)-5'-cyanonicotine at 3.5 ppm (Figure 18). Mass spectral analysis indicated the incorporation of one deuterium atom by the shift of the base peak at 109 to m/z 110 (Figure 19).

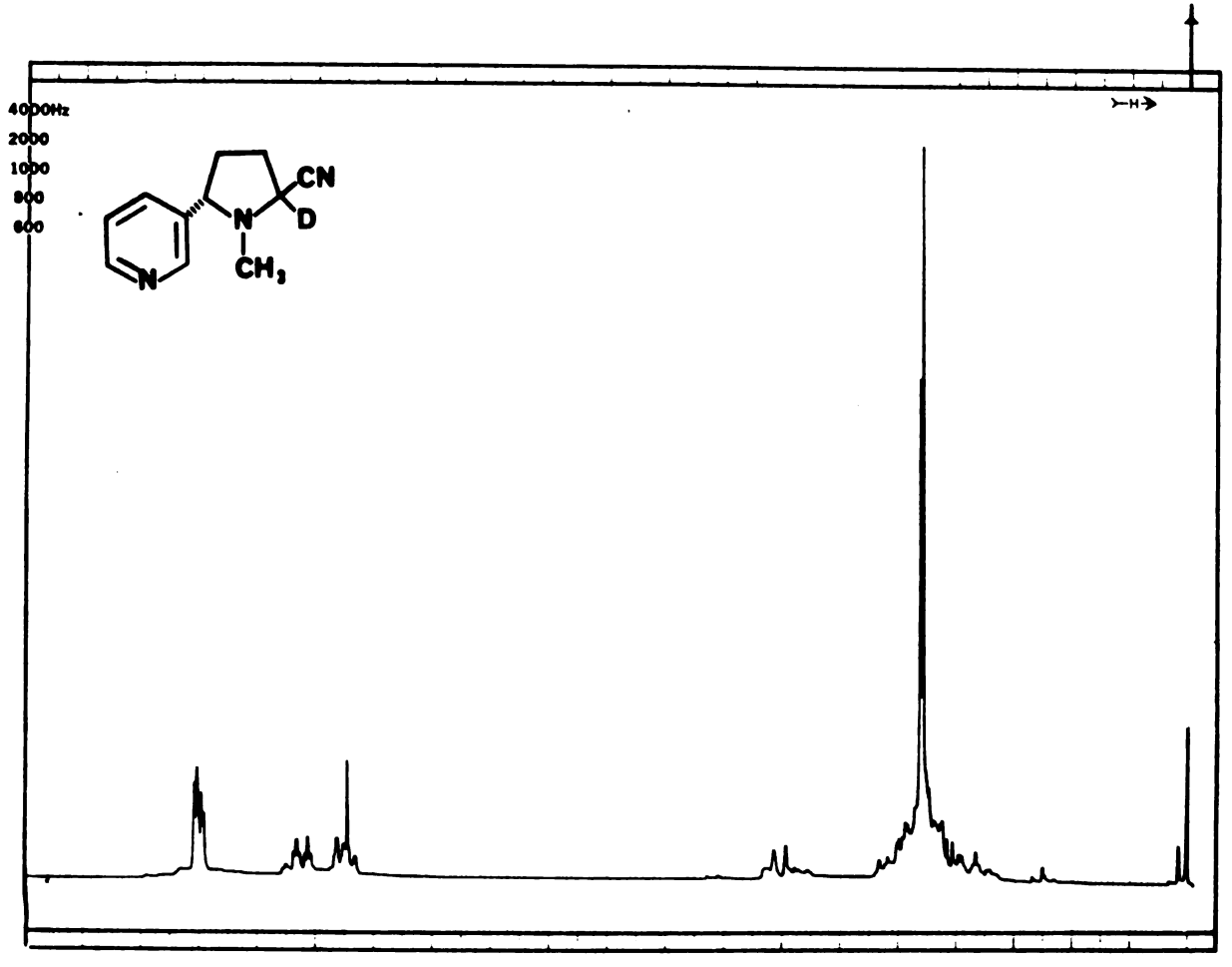


Figure 18. The 80 MHz ^{1}H-NMR spectrum of a mixture of (E)- and (Z)-5'-cyanonicotine-5'-d_1 in $CDCl_3$.

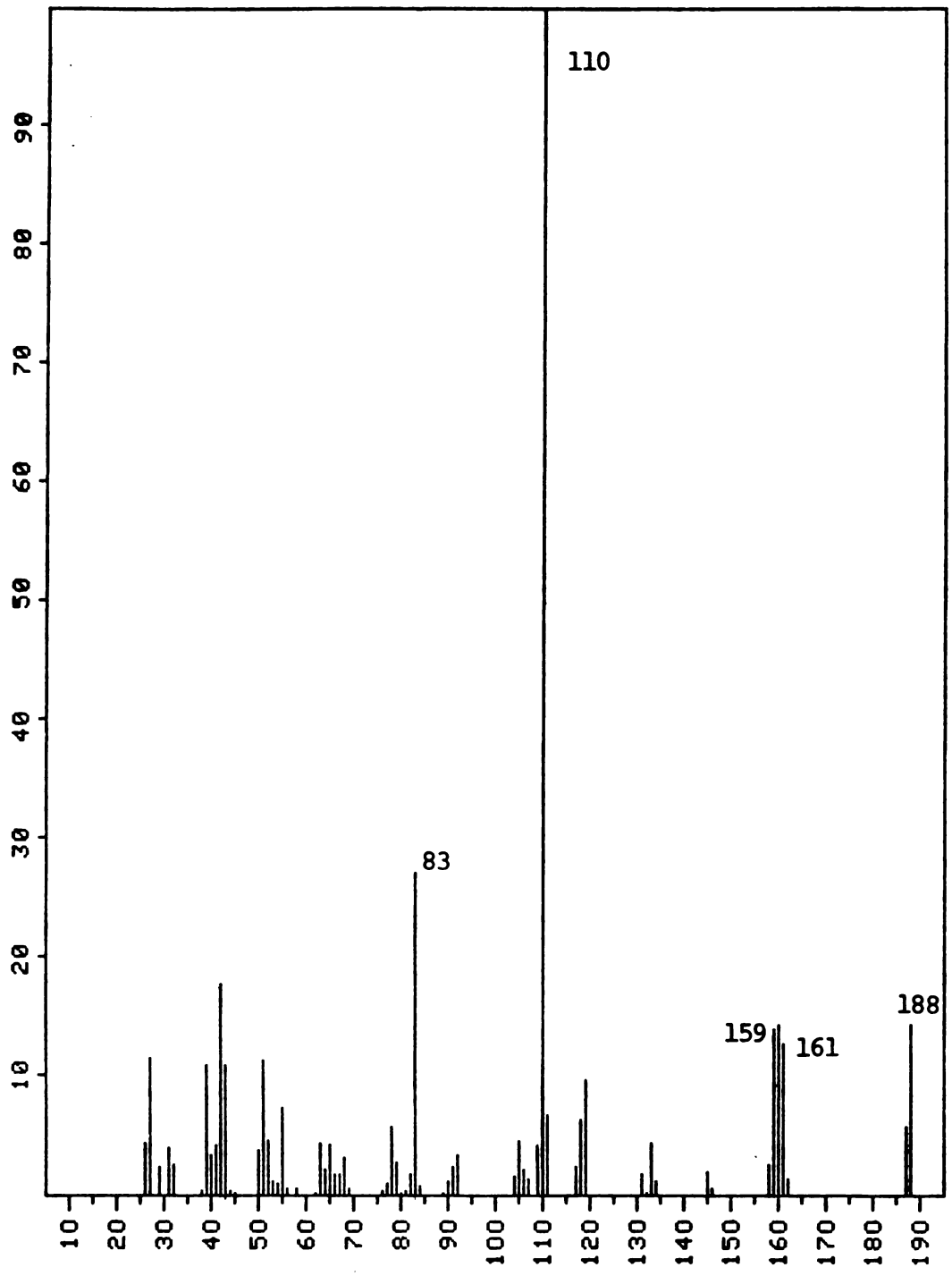


Figure 19. The EI-mass spectrum of (E)- and (Z)-5'-cyanonicotines-d_1.

The labeled α-aminonitriles were treated with ethanolic perchloric acid to generate Δ-$^{1'(5')}$-nicotine iminium bisperchlorate-5'-d_1 (42-5'-d_1). A yield of 45% was obtained after recrystallization from hot acidic methanol. The melting point was similar to that of the unlabeled material: 240-243° C with decomposition. The presence of deuterium in the 5'-position was demonstrated by the decrease in the broad signal at 8.9 ppm in the 80 MHz ^{1}H-NMR spectrum of 42-5'-d_1 (Figure 20). The remaining signals in this region integrated for the 4 protons assigned to the four pyridine protons.

Nicotine-(Z)-5'-d_1 was obtained following the reductive hydrogenation over platinum oxide of nicotine Δ-1'(5')-iminium ion bisperchlorate-5'-d_1 in 2.5% aqueous hydrochloric acid. The product displayed the spectral properties required for 58. Unlike the (E) isomer, 58 was not significantly contaminated with any multideuterated species as demonstrated by the GC-EI-MS spectrum which displayed the ion intensities of the base peak 84:85:86=5:94:1 (Figure 21). The 240 ^{1}H-NMR spectrum displayed only a weak signal for the 5'-(Z)-proton at 3.3 ppm (Figure 22). The replacement of this position with deuterium also led to the simplification of the 5'-(E)-hydrogen atom signal to a triplet as a result of the loss of the geminal coupling. Integration of the residual signal at 3.3 ppm suggested that 86.5 ± 0.1% (n=3) of the product contained a proton in the 5'-(Z)-position. After accounting for 5% unlabeled product indicated by mass spectral analysis, we have concluded that the hydrogenation proceeded with 92% stereoselectivity. The ^{2}H-NMR spectrum of the bistartrate confirmed the 5'-(Z)-position of the label by the presence of a single signal at 3.4 ppm (Figure 23).

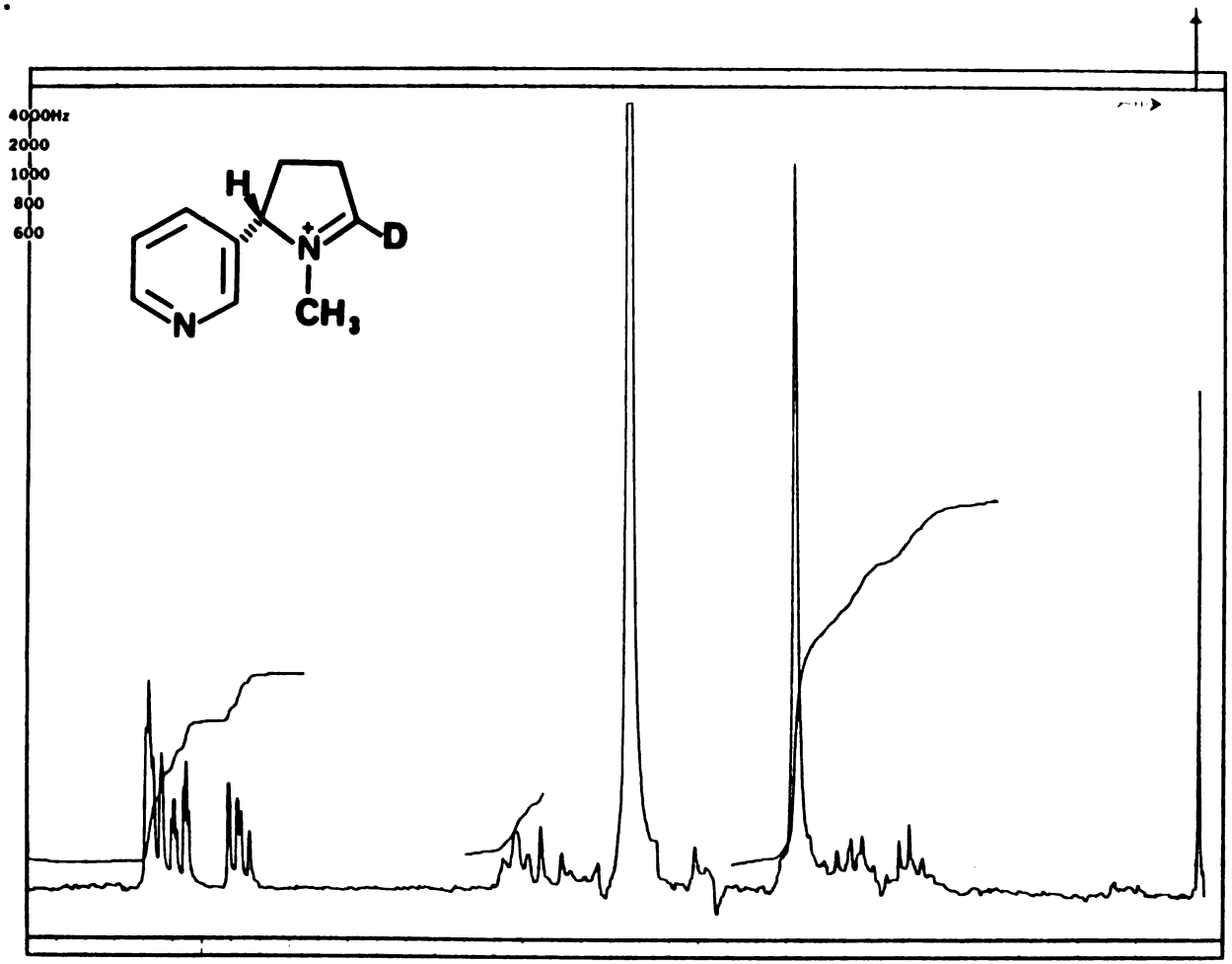


Figure 20. The 80 MHz ^{1}H-NMR spectrum of (S)-nicotine $\Delta^{-1'(5')}$-iminium bisperchlorate-5'-d_1 in D_2O.

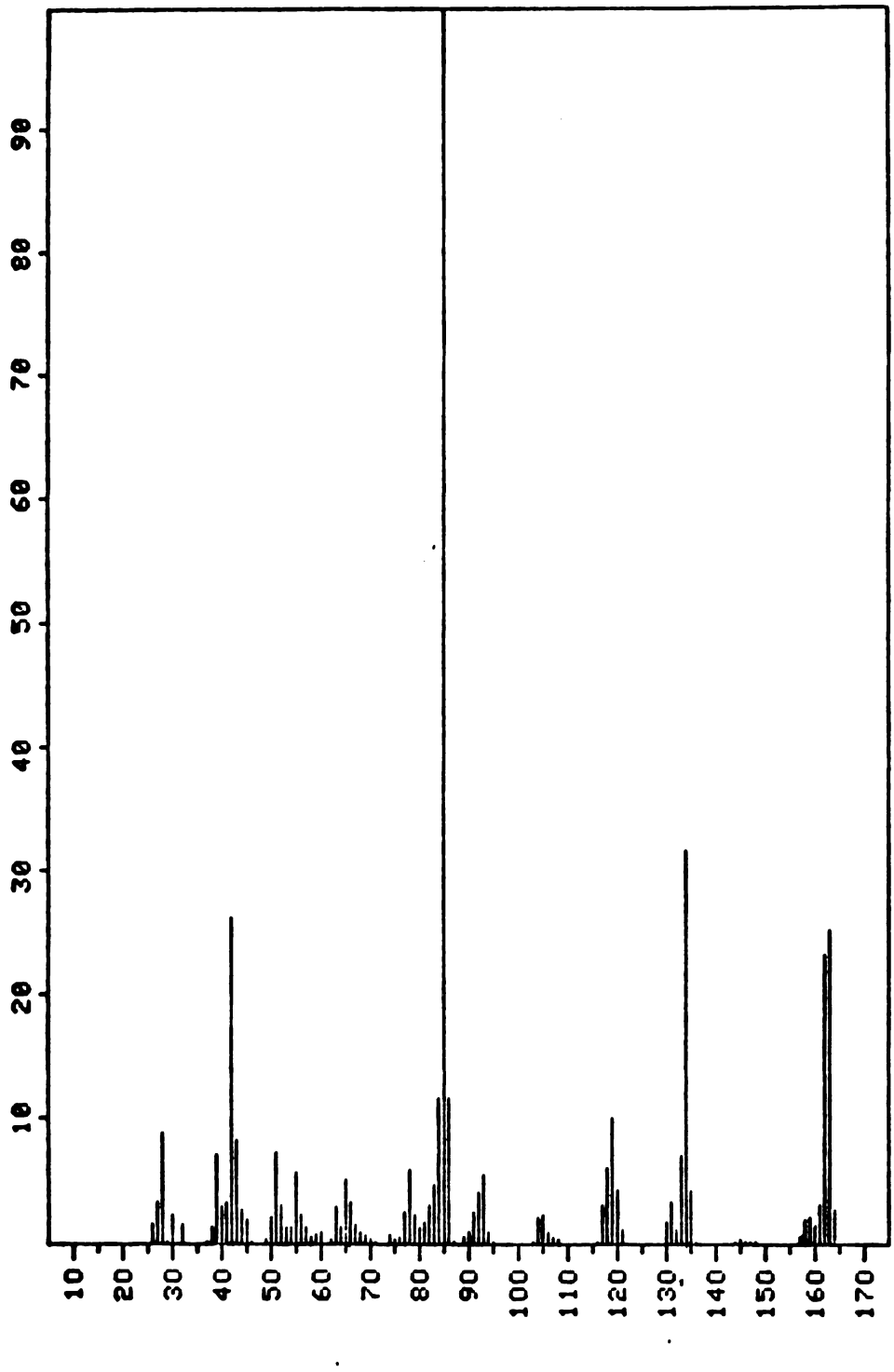


Figure 21. The EI-mass spectrum of (S)-nicotine-(Z)-5'-d_1.

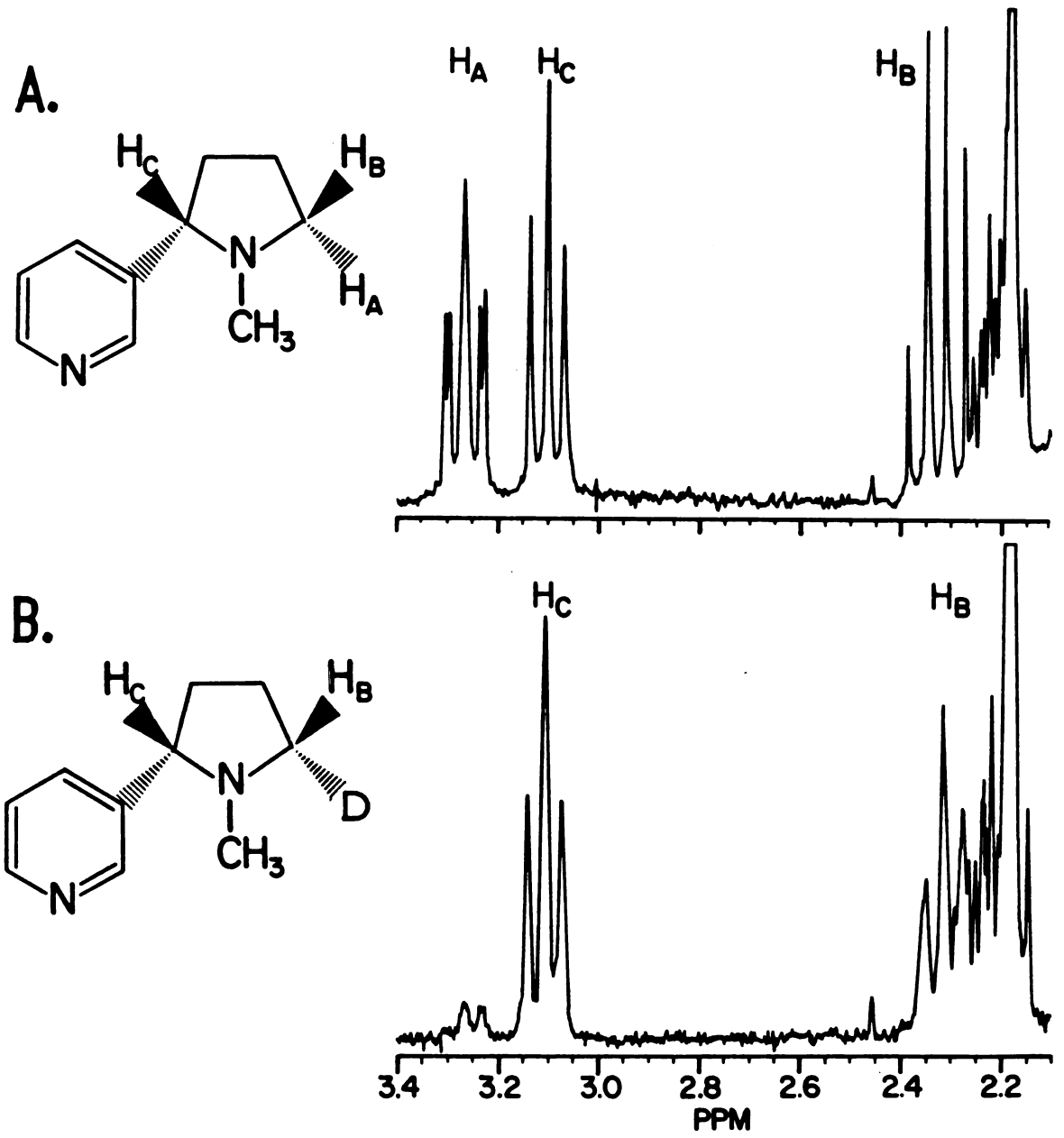
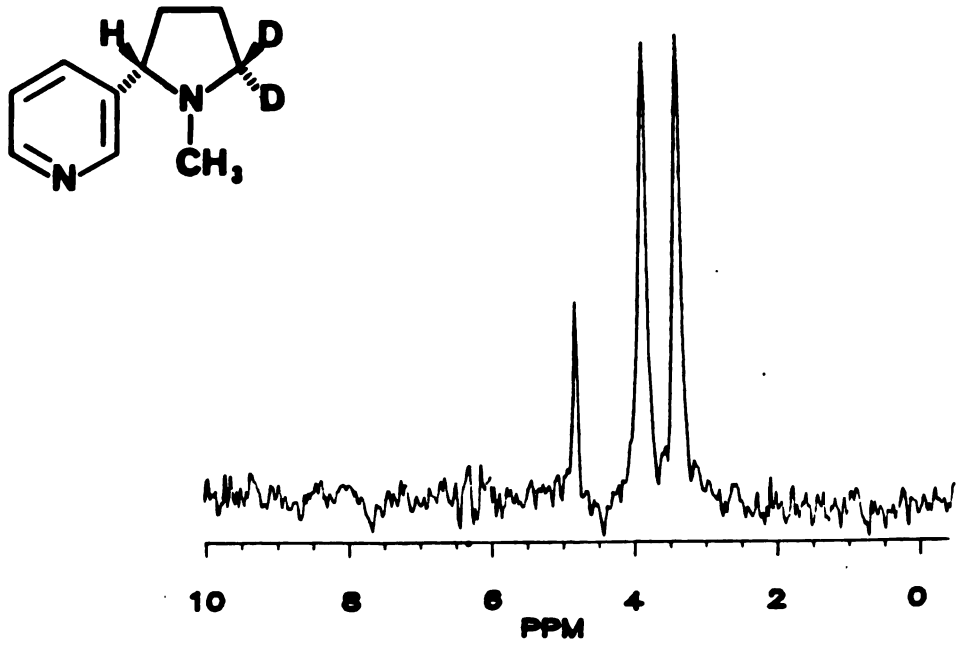


Figure 22. The 240 MHz ^{1}H-NMR spectra of A.) (S)-nicotine and B.) (S)-nicotine-(Z)-5'-d_1 in $CDCl_3$.

A.



B.

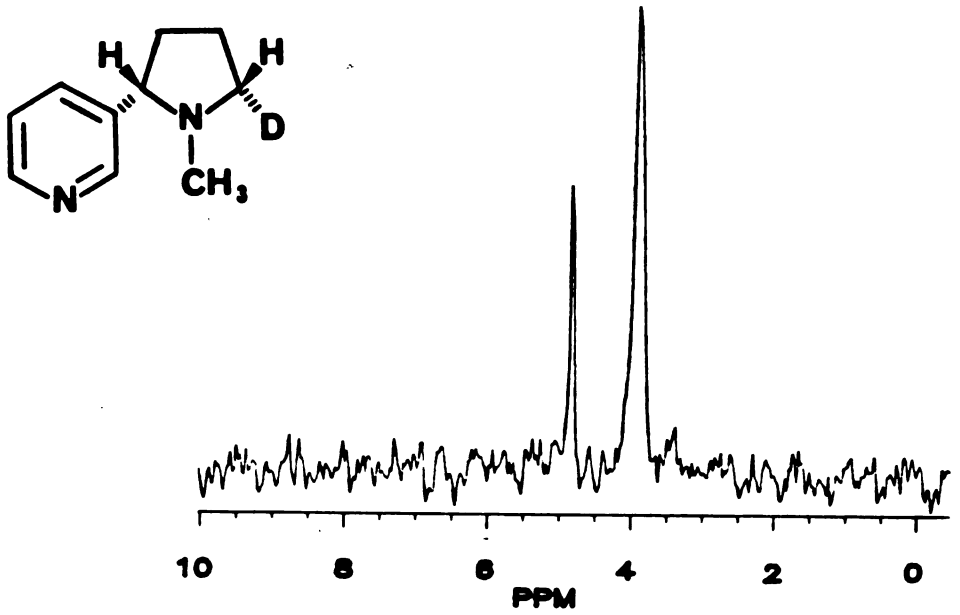


Figure 23. The ^{2}H-NMR spectra of A.) (S)-nicotine-5',5'-d_2 bis-l-tartrate and B.) (S)-nicotine-(Z)-5'-d_1 bis-l-tartrate in D_2O.

C. Analytical Gas Chromatographic Electron Impact Mass Spectral Assay.

With the availability of the (E)- and (Z)-5'-monodeuteronicotine diastereomers, we were prepared to initiate metabolic studies to determine the stereochemical course of the cytochrome P-450 catalyzed oxidation of (S)-nicotine. These studies required the development of an assay which would estimate the extent to which the deuterium is retained or lost during the course of the two electron oxidation of the substrate. To facilitate this analysis, the incubations were performed in the presence of cyanide ion to convert the metabolically generated iminium ion 42 to the corresponding diastereomeric α-cyano adducts 44a and 44b. The deuterium content of these adducts then was estimated by capillary column GC-EIMS selected ion monitoring. Since the deuterated substrates were not 100% stereospecifically labeled and contained small amounts of unlabeled and dideuterated material, the mass spectral measurements had to be corrected for isotopic and stereochemical impurities to provide accurate estimates of the deuterium content. In these initial calculations we have assumed that no significant isotope effects are involved in this biotransformation (see Section II.E.4 for further consideration of isotope effects).

Several methods for the detection of these cyano adducts were considered. Murphy, the first to characterize the metabolically generated 5'-cyanonicotines,[29] extracted the α-cyanoamines with dichloromethane and analyzed the extract by packed column GC techniques. Identification of the product was based on its coelution with a synthetic standard. Nguyen performed direct extractions of microsomal incubation mixtures with dichloromethane or diethyl ether.[31,79] Subsequent acid-base partitioning eliminated interfering biological materials.

Analyses of these extracts by GC-MS were performed on a 2% Dexyl columnn which gave only partial separation of the 5'-cyanonicotine diastereomers from N-cyanomethylnornicotine.[79]

We acheived baseline separation of the regio cyano isomers 2'-cyanonicotine, N-cyanomethylnornicotine, and the 5'-cyanonicotine diastereomers when n-butyl acetate solutions of the synthetic standards were analyzed by capillary GC linked to a nitrogen-phorphorus detector (Figure 24). This separation was obtained on SE 52, SE 54, DB1 or DB5 capillary columns. The diastereomers of 5'-cyanonicotine also were resolved on the capillary GC columms when the column was held isothermally at 180° C following the injection of the sample at 35' C.

The diastereomeric 5'-cyanonicotines could be extracted directly from aqueous buffer (pH 7.4) solutions with ether. The cyanoamines also could be extracted with toluene-isoamyl alcohol (9:1). In our hands, however, acid-base partitioning of an extract of a pH 7.4 buffer solution of 44a and 44b led to loss of more than 90% of the 5'-cyanoamines. This observation is consistent with the predictable instability of these adducts in acid. We decided from these studies to extract the microsomal incubations directly with ether.

Selected ion monitoring was used to determine the deuterium content of the generated cyanoamines. The EI-mass spectrum and fragmentation pattern of the 5'-cyanonicotines are shown in Figure 25. The principal peaks in both mass spectrum are at m/z 187 (M^+), m/z 158 (loss of hydrogen and hydrogen cyanide), m/z 109 (100%, loss of the pyridine ring), and m/z 82 (loss of hydrogen cyanide and the pyridine ring). The molecular ion at m/z 187 was not useful for deuterium composition studies because M^+-H is a major fragment ion for the 5'-cyanonicotines.

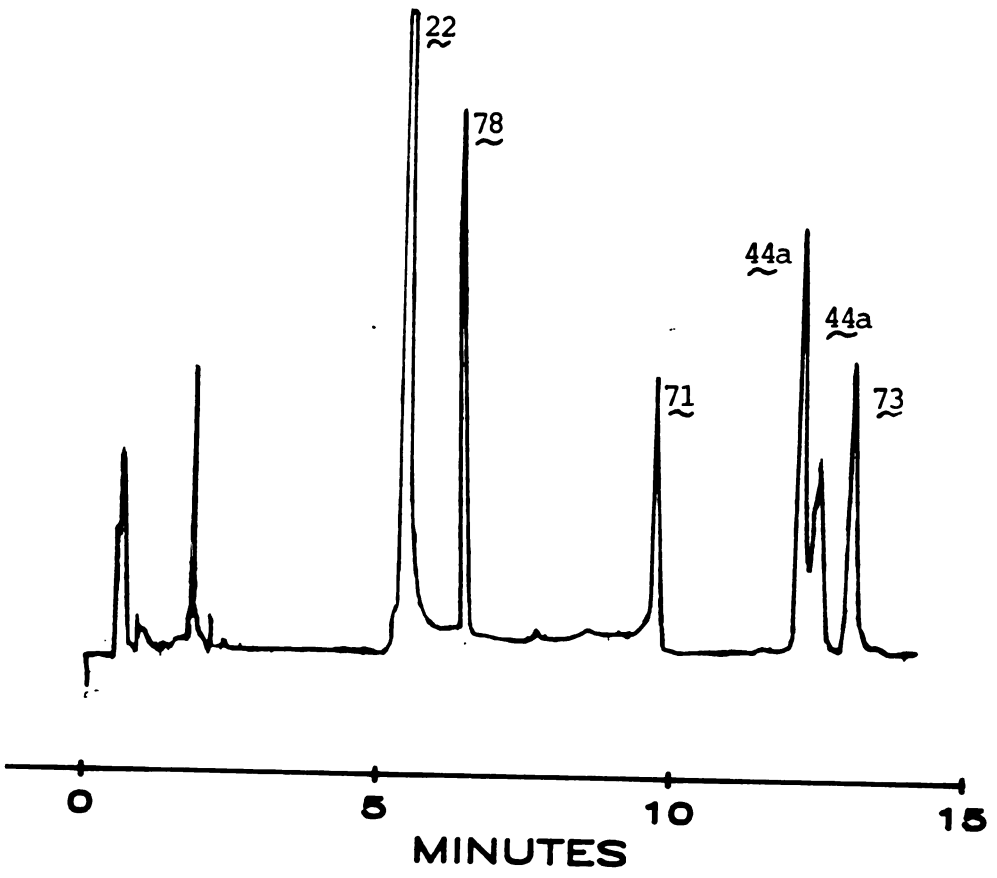


Figure 24. The GC tracing of a n-butyl acetate solution of (S)-nicotine (22), nornicotine (78), 2'-cyanonicotine (71), (E)- and (Z)-5'-cyanonicotines (44a and 44b, respectively) and N-cyanomethylnornicotine (73).

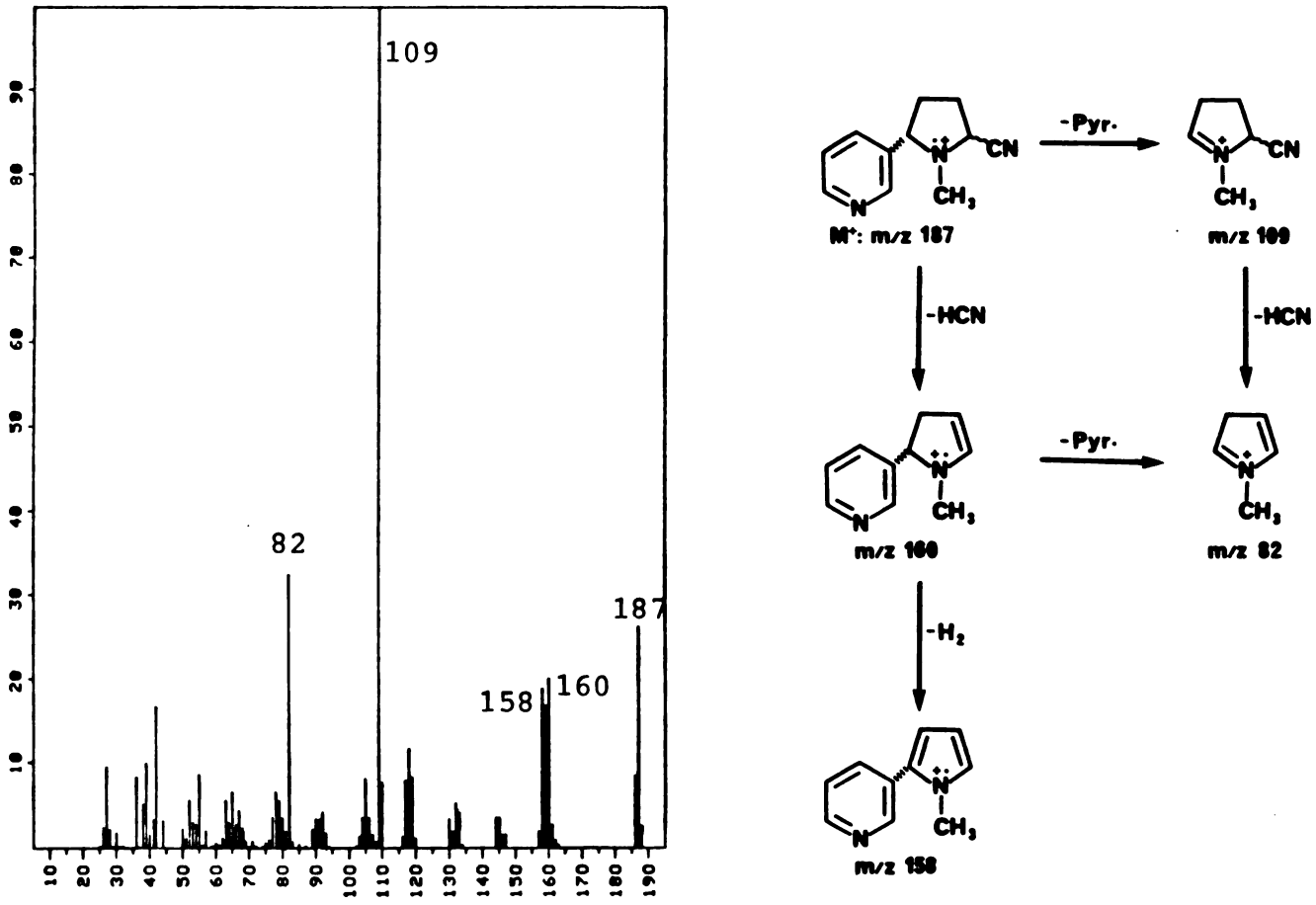
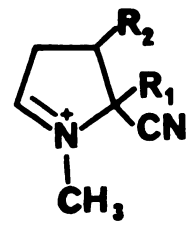


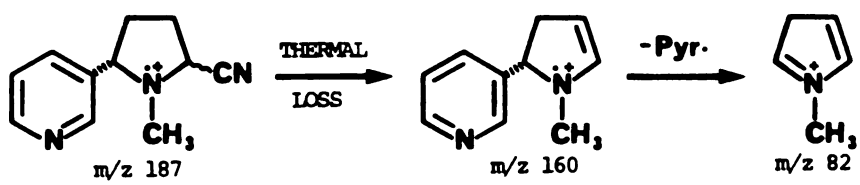
Figure 25. The EI-mass spectrum and fragmentation pattern of the (E)- and (Z)-5'-cyanonicotines.

Instead, the base peak at m/z 109 was used. This ion and the corresponding deuterium containing ion at m/z 110 were chosen for selected ion monitoring. In addition, the ion current at m/z 111 was monitored so corrections for the presence of the small d_2-contaminant in 57 could be made.



m/z 109: R_1, R_2=H
m/z 110: R_1=D; R_2=H
m/z 111: R_1=D; R_2=D

The metabolic extracts were analyzed with the mass spectrometer inlet line at roughly 200° C and the ion source at 150° C. The mass spectral characteristics of the 5'-cyanonicotines are extremely sensitive to ion source temperature (Figure 26).[31] At low source temperatures, m/z 109/110 is the base peak and the molecular ion is relatively intense. As the source temperature increases, these peaks become less intense and the ion at m/z 160/161 corresponding to the loss of HCN increases in intensity. Furthermore, the ion intensity at m/z 82/83 resulting from the loss of HCN and the pyridine ring also increases with temperature until it becomes the base peak. At a source temperature of 250° C, the signals at m/z 109/110 and 187/188 are no longer present and



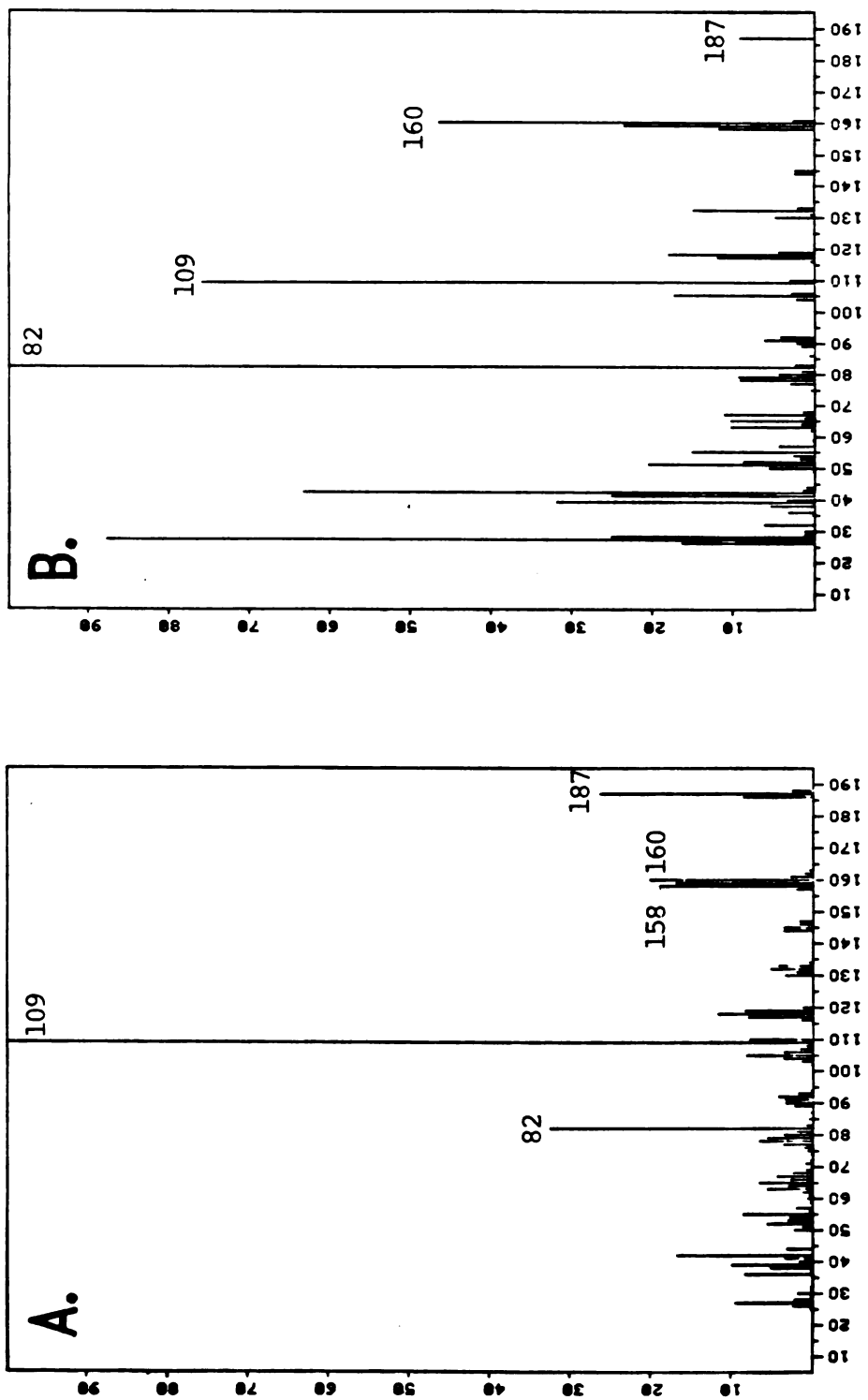


Figure 26. The EI-mass spectra of the 5'-cyanonicotine diastereomers at a ion source temperature of A.) 150° C and B.) 220° C.

the ions at m/z 82/83 and 160/161 are the dominant peaks. This temperature sensitivity of the mass spectral characteristics results from the thermal degradation of the 5'-cyanonicotines in the ion source prior to entering the ion chamber.[31] Thus, the analyses of the incubation mixtures were conducted at low source and inlet temperatures to optimize the production of the fragment ions corresponding to the ion currents at m/z 109, 110 and 111.

The thermal sensitivity of the 5'-cyanoamines was indicative of the general instability of these cyano adducts. Several other precautions were taken during the isolation and analysis of the metabolically generated compounds to insure their maximal detection by GC-EIMS analysis. First, the ether used to extract the metabolic incubations was prewashed with aqueous ferrous sulfate to remove any peroxides that could cause the destruction of the analytes. Second, the incubation mixtures were cooled on ice prior to the ether extraction. This step minimized the extraction of biological materials that seemed to enhance the extent of sample decomposition in the GC injector port. Third, the ether was separated from the aqueous phase by freezing the water layer using a dry ice bath and decanting the organic layer. This procedure helped to minimize the amount of water in the ether phase and allowed us to avoid drying agents. The samples decomposed upon analysis when the extracts were dried with anhydrous magnesium sulfate or potassium carbonate. This degradation apparently occurred in the GC injector port where the presence of residual drying agent were believed to catalyze the decomposition.

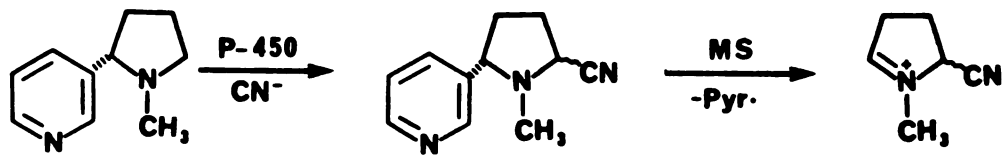
The samples in the ether extracts were stable. The best results were obtained if the sample was concentrated under a N_2 stream immediately before the GC-EIMS analysis. The dichloromethane used for reconstituting the sample was a better solvent than ether because of its higher boiling point. Ether tends to vaporize too quickly in the injection port, causing the sample to shoot past the front of the GC column. This phenomenon reduces the amount of material entering the GC column and the mass spectrometer.

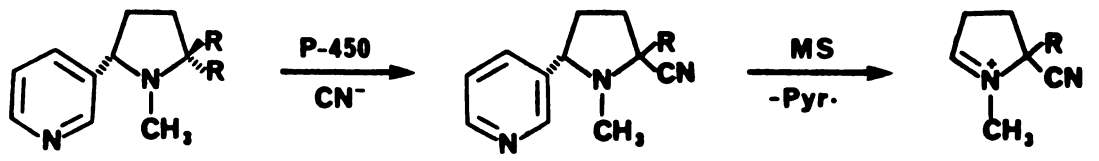
Detection of the 5'-cyanonicotines also was enhanced when the injector port and the GC-MS inlet line were properly silanized. In addition, any acidic or basic residues in the injector port catalyzed the decomposition of 44a and 44b making it necessary to clean the injector port on a regular basis. Standard solutions of the 5'-cyano adducts were used to test instrumental conditions prior to the analysis of metabolic samples.

In order for the ratio of the ion abundances at m/z 109 and 110 to represent accurately the amount of deuterium lost or retained during the oxidation, a correction had to be made for the natural ^{13}C abundance contribution of the m/z 109 ion to the m/z 110 ion and, in the case of 57, for the ^{13}C contribution of the m/z 110 ion to the m/z 111 ion. The ratio of ion intensities at m/z 110 to m/z 109 was found to be 0.0745 $\pm$ 0.001 in the EI-MS of synthetic diastereomeric 5'-cyanonicotine-d_0. The calculated ratio for C_6N_2 is 0.0748. The corresponding ion intensity ratio for m/z 111 to m/z 110 for synthetic 5'-cyanonicotine-5'-d_1 was 0.0790 $\pm$ 0.006. These values were determined at the outset of each experiment and were used to correct the data for that particular study since the values varied slightly from day to day. The ion current at m/z 110 was corrected for this natural ^{13}C abundance by multipling the

ion current at m/z 109 by 0.0745 ± 0.001 and substrating the product from the ion current at m/z 110 to yield the corrected value. The corresponding calculations were made to correct for the ^{13}C-satellite contribution of the m/z 110 ion to the m/z 111 ion.

In addition, corrections for the isotopic and stereochemical impurity of each compound were required. When 58 was employed as the substrate it was necessary to correct for the small amount of d_0-contaminant because the ion current at m/z 109 was attributed to the unlabeled 44a and 44b generated from both the unlabeled and monolabeled nicotines. First, the ion current only due to the 5'-cyanonicotines

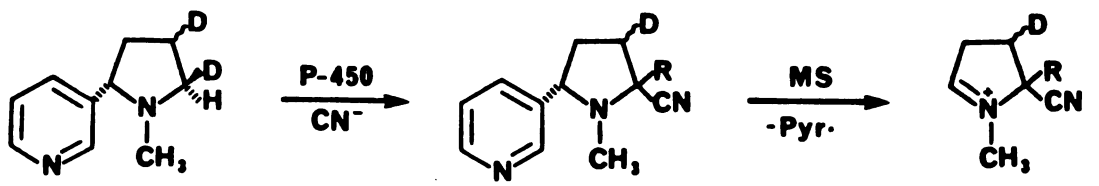




57: R_1=D; R_2=H
58: R_1=H; R_2=D

R=H or D

m/z 109: R=H
m/z 110: R=D



57a

m/z 110: R=H
m/z 111: R=D

resulting from the oxidation of the monolabeled material was obtained by multiplying the sum of the ion currents at m/z 109 and 110 by 0.95 (the fraction of 22-d_1 in 58). The ion current at m/z 110 pertains to the labeled oxidation products of 22-5'-d_1. The ion current at m/z 109 is caused by the unlabeled 5'-cyanonicotines derived from both 22-d_0 and 22-5'-d_1. In order to determine the amount of ion current at m/z 109 caused by the oxidation product of 22-5'-d_1, the ion current at m/z 110 was subtracted from the total ion current at m/z 109 and 110 calculated for the monolabeled material's oxidation products.

Corrections for the isotopic impurity of 57 were slightly more complicated since there was a small but significant contamination of 22-d_2 as well as unlabeled material. The dideuterated compound was assumed to be as selectively labeled in the 5'-position as the monolabeled material. Therefore, the ion current at m/z 109 in the spectrum of the metabolically generated cyano adducts is a measure of the product resulting from the loss of a hydrogen atom during the oxidation of 22-d_0 and the loss of a deuterium atom during the oxidation of 22-5'-d_1. The ion current at m/z 110 represents the products formed from the loss of the 5'-deuteron from the dideuterated substrate as well as the loss of a proton from 22-5'-d_1. The ion current at m/z 111 may be assigned to the product resulting from the removal of H from 22-d_2.

The following calculations were made to obtain the ion current values for the 5'-cyanoamines generated from the monodeuterated nicotine 57. The sum of the ion current at masses 109, 110 and 111 following ^{13}C-satellite corrections was multiplied by 0.90 to correct for the 10% d_2-contaminant. This value represents the total ion current for the oxidation products derived from 22-d_0 and 22-5'-d_1. The ion current at m/z 110 resulting from the monodeutero 5'-cyano adducts of 22-5'-d_1 is

calculated as the difference between this value and the ion current observed at m/z 109. Two percent of the sum of the ion currents at m/z 109, 110, and 111 was subtracted from the observed m/z 109 ion current value to obtain the ion current value for the mass 109 ion attributed to the product formed from the loss of the 5'-proton of 22-5'-d_1.

These corrected values then were used in our calculations to take into account the stereochemical composition of the monodeutereo diastereomers. The following equation was used to make these corrections:

$$x=(a-IC_{110})/(1-b/a); \quad \%x=(x/t)100$$

where for 58:

x=the ion current at m/z 109 of the 5'-cyanonicotines from 58
a=the total ion current of the 5'-cyanonicotines from 58
=0.92T
b=the total ion current of the 5'-cyanonicotines from the 57 contaminant
=0.08T
(1-b)/a=0.913

and for 57:

x=the ion current at m/z 109 of the 5'-cyanonicotines from 57
a=the total ion current of the 5'-cyanonicotines from 57
= 0.85T
b=the total ion current of the 5'-cyanonicotines from the 58 contaminant
= 0.15T
(1-b)/a=0.824

in all cases:

IC_{110}=the ion current at m/z 110 of the 5'-cyanonicotines generated from the unlabeled material.
T=the sum of ion currents at m/z 109 and 110 of the 5'-cyanonicotines generated from the monolabeled material (obtained from ^{13}C and isotope impurity calculations).

This equation was derived assuming no kinetic isotope effects in the oxidation of 57 versus 58 as a first approximation of the stereochemical course of this reaction. The derivation of the equation is outlined in Table 2.

Table 2. Derivation of the equation used for corrections of the stereochemical composition of the monodeutero nicotine diastereomers.

	IC_{109}	IC_{110}
IC contributions from the major isomer's 5'-cyanonicotines:	x	y
IC contributions from the minor isomer's 5'-cyanonicotines:	s	t

(IC=ion current)

eq. 1) $IC_{109}=x+s$

eq. 2) $IC_{110}=y+t$

eq. 3) $T=IC_{109}+IC_{110}$

eq. 4) a=x+y=IC from major d_1-diastereomer's 5'-cyanonicotines

eq. 5) b=s+t=IC from minor d_1-diastereomer's 5'-cyanonicotines

eq. 6) a+b=T

Assuming no isotope effects in the rate of H or D abstraction, the amount of H_R removal both (E)- and (Z)-d_1-diastereomers should be the same. In other words: x/a=t/b (eq. 7) and y/a=s/b (eq. 8).

From eq. 2: $t=IC_{110}-y$ and from eq. 7: t=bx/a.

So $bx/a=IC_{110}-y$.

However, y=a-x (from eq. 4) so $bx/a=IC_{110}-(a-x)$.

Rearranging: $bx/a=IC_{110}-a$ or $x(b/a-1)=IC_{110}-a$.

Or $x=(IC_{110}-a)/(b/a-1)$.

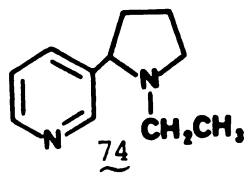
If multiply by -1/-1: $x=(a-IC_{110})/(1-b/a)$.

The corrected values for the ion current at m/z 109 and 110 then were used to determine the percent deuterium retained in the oxidation product formed from the specifically labeled 5'-monodeuteronicotine diastereomers. The ratio of the ion current at m/z 110 to the sum of the corrected values was used as an approximation of the deuterium content of 44a and 44b. The corrections described above made the data more accurate but they were not necessary. The general conclusions reached below in the metabolic and model studies were not modified if the data were not corrected for the isotopic and stereochemical impurities of 57 and 58.

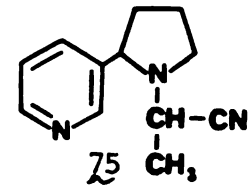
D. Quantitative Assays for Nicotine, the 5'-Cyanonicotines and Cotinine.

Since we were interested in determining the extent to which (S)-nicotine is oxidized to the iminium ion 42 in the overall *in vitro* metabolic degradation of nicotine, it became necessary to develop an assay to estimate metabolic yields of the cyanonicotines 44a and 44b. Furthermore, data obtained from such an assay could help us in assessing the importance of the further oxidation of the reaction products to cotinine. Finally, the assay could be used to determine the relative rates of cytochrome P-450 catalyzed oxidation of 57 and 58 at the 5'-position. Such information could reveal significant intermolecular isotope effects.

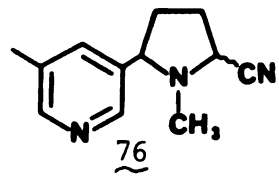
In order to quantitate the 5'-cyanonicotine diastereomers, we required a method to separate these cyanoamines from the other compounds present in the metabolic extracts. Several approaches were considered including analyses by HPLC, capillary GC outfitted with a nitrogen-phosphorus detector, and capillary GC-EI mass spectral techniques using a multideuterated internal standard. HPLC methods were not explored since attempts by Dr. Y. Oka to separate 44a and 44b from N-cyanomethylnornicotine (73) by liquid chromatographic techniques were unsuccessful. Gas chromatographic techniques, on the other hand, permitted separation of 44a and 44b from all other compounds present in the ether extracts[79] (see Section II.C). As detailed below, our attempts to develop a gas chromatographic assay utilizing a nitrogen-phosphorus detector were unsuccessful. These efforts failed primarily because we were unable to find a suitable internal standard. A number of candidate internal standards including N-ethylnornicotine (74), N-(1-cyanoethyl)nornicotine



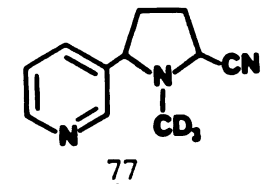
74



75



76



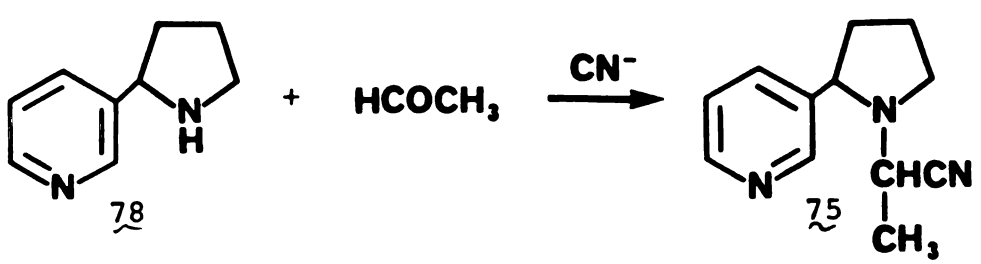
77

(75), and 5'-cyano-5-methylnicotine (76) were investigated and found unsatisfactory due to differences in their stability relative to 44a and 44b. These studies led us to turn our attention to a quantitative mass spectral assay where 5'-cyanonicotine-N-d_3 (77) served as the internal standard. Although this method requires further improvements, it provided reliable data for the relative amounts of the 5'-cyanonicotines formed from the deuteronicotine diastereomers 57 and 58. A quantitative HPLC assay developed by Mr. M. Shigenaga also was used to assess the importance of the α-carbon oxidation pathways in the overall microsomal metabolism of 22. Cotinine was estimated by the methods of Jacob et al.[113]

Initial experiments were performed using N-ethylnornicotine (74) as an internal standard. The analysis was done using a capillary GC column which separated the 5'-cyanonicotine diastereomers. The ratio of the sum of peak areas of 44a and 44b to that of the internal standard was used to establish a standard curve. Although the standard curve obtained for the 5'-cyanonicotines using this internal standard was satisfactory, there was a twenty percent variation in the ratio of peak areas between multiple injections of the same solution. This variability can be attributed to differences in chemical stability. N-Ethylnornicotine is not as sensitive to acid and heat as the 5'-cyanonicotines. In

addition, large fluctuations were observed between duplicate incubation mixtures when N-ethylnornicotine was used to estimate the concentration of nicotine. Such variations were not observed when the compounds were extracted from basic solutions.[114] Since we wanted to avoid strongly basic solutions to prevent decomposition of 44a and 44b, further efforts with this internal standard were not pursued.

These observations led to a search for an internal standard that was structurally more closely related to the 5'-cyanonicotines. The first compound tested was N-(1-cyanoethyl)nornicotine (75) which was chosen because it was an α-aminonitrile and could be easily prepared. It was synthesized from the reaction of nornicotine (78) with acetaldehyde and KCN following the method reported by Nguyen et al. for the preparation of N-cyanomethylnornicotine.[30] The 80 MHz ^{1}H-NMR spectrum of the reaction mixture suggested a 1:1 mixture the of N-(1-cyanoethyl)-nornicotine diastereomers by the pair of doublets resonating at 1.35 and 1.60 ppm (J=7 Hz) which were assigned to the protons of the methyl groups of the N-ethyl moiety (Figure 27).



GC analysis of the reaction product indicated the presence of two compounds in a 5:1 ratio (Figure 28) in marked contrast to the ^{1}H-NMR results. GC-EIMS analysis with the ion source temperature at 200° C demonstrated that these compounds were isomeric (Figure 29). The

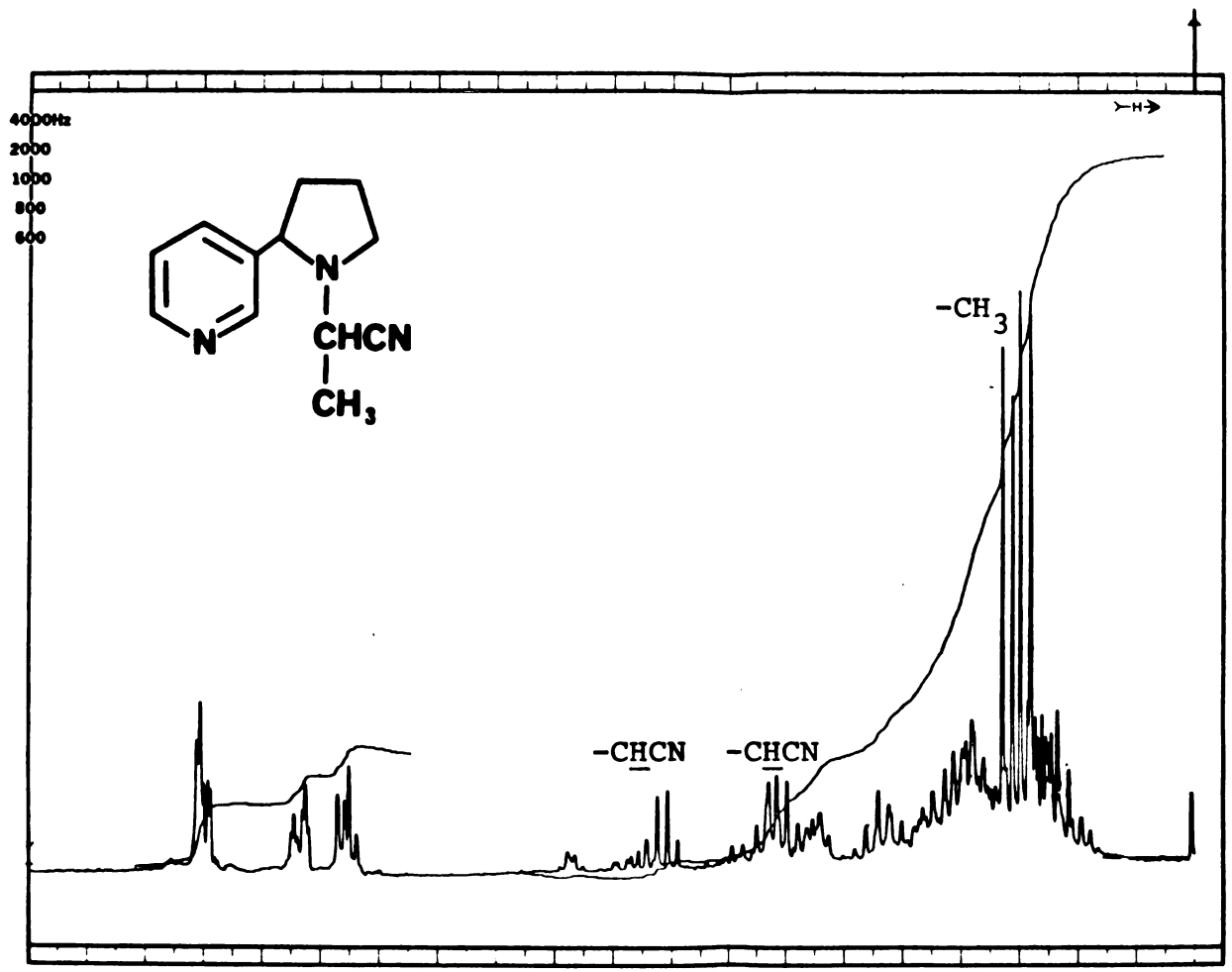


Figure 27. The 80 MHz ^{1}H-NMR spectrum of the N-(1-cyanoethyl)nornicotines.

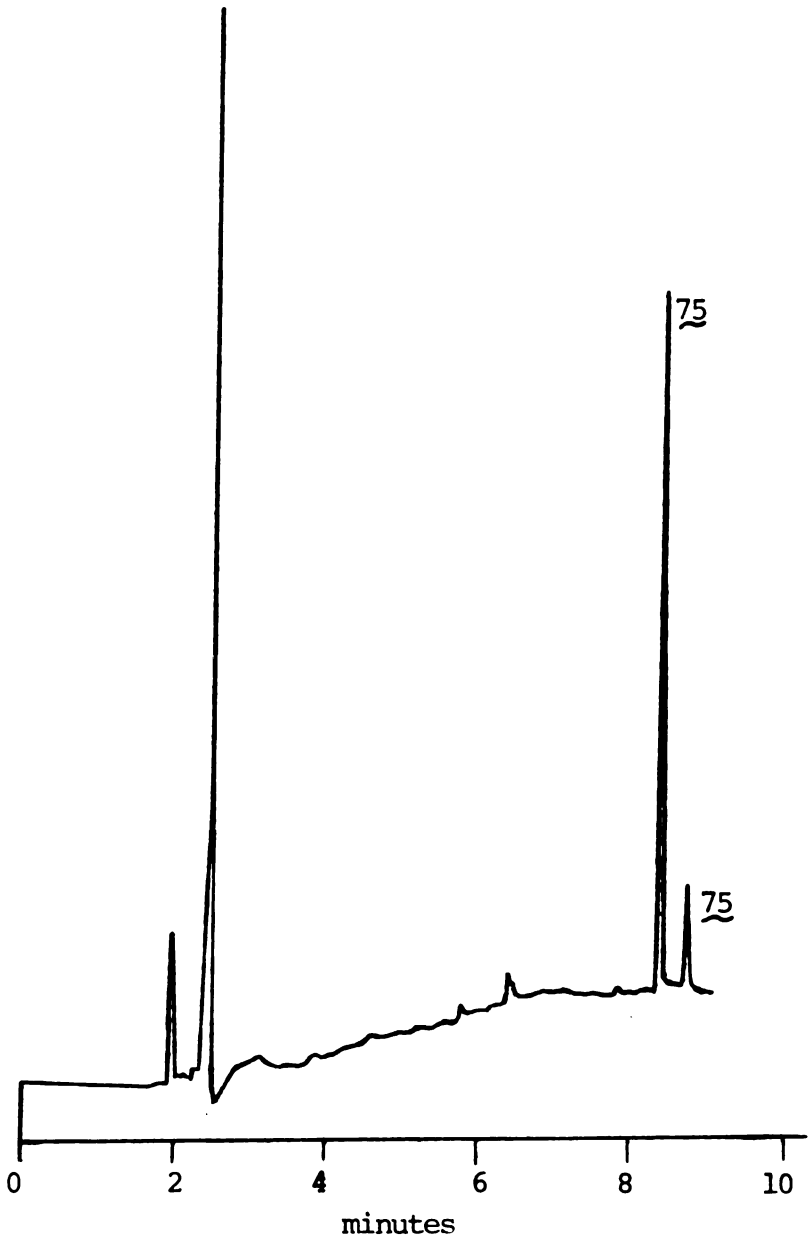


Figure 28. The GC tracing of a n-butyl acetate solution of crude diastereomeric N-(1-cyanoethyl)nornicotines.

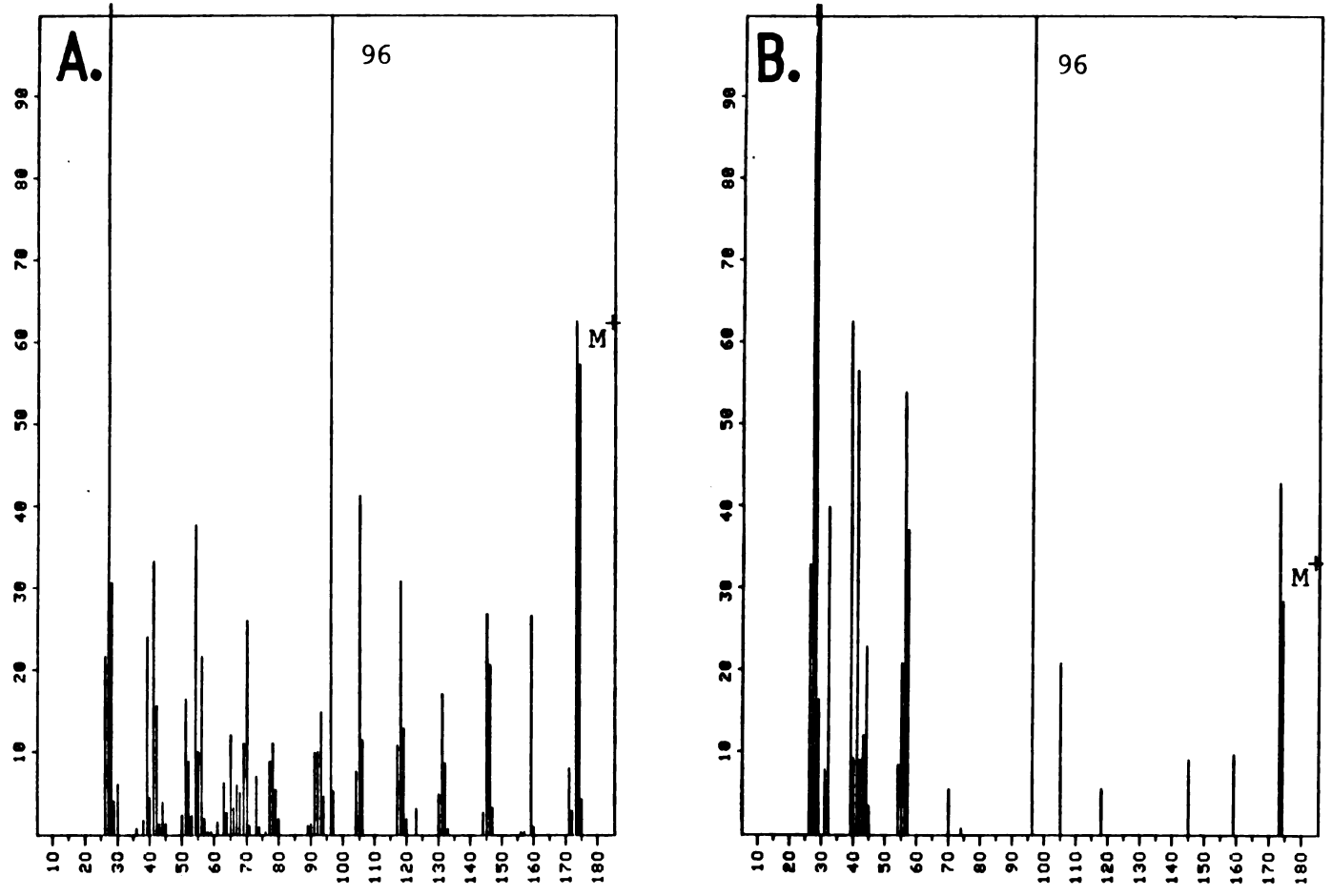
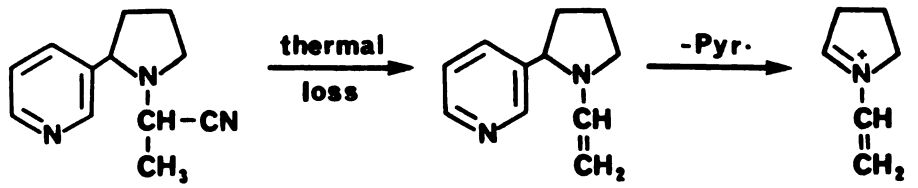


Figure 29. The EI-mass spectra of the N-(1-cyanoethyl)nornicotine diastereomers. A.) The major isomer. B.) The minor isomer.



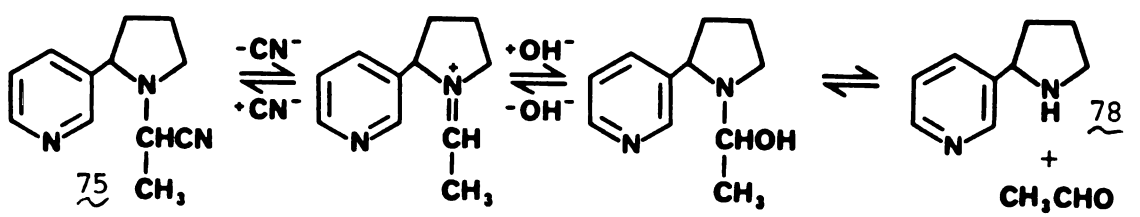
75 m/z 174 v: m/z 96

molecular ion for both compounds occurred at m/z 174 which corresponds to the molecular weight of the expected cyano adducts following the loss of HCN. The base peak of both spectra was at m/z 96. This peak is generated by the fragment v which has also lost the pyridine ring. Nguyen reported the mass spectral characteristics of this compound and found a prominent parent ion at m/z 201.[79] One possible explanation for the absence of this species in our spectra is that we used a higher ion source temperature and the mass spectral characteristics of this compound would be expected to be sensitive to the source temperature as is observed for the 5'-cyanonicotine diastereomers (see Section II.C). From these data the two compounds in the reaction products were thought to be isomeric cyanoethylnornicotines. If they are diastereomers as suggested by the NMR data, the 5:1 ratio observed in the GC analysis in contrast to the almost 1:1 ratio observed by NMR analysis may be explained by the selective decomposition of one diastereomer on the capillary GC column (injection port: 220° C; temperature program: hold 1 minute at 90° C, then heat to 180° C 30° C/min and hold).

The crude reaction mixture was used for initial GC studies since there were no peaks that interfered with 44a, 44b, or 73. A n-butyl acetate solution of the synthetic 5'-cyanonicotines and the reaction mixture was used for these studies. The GC peak area of the major N-cyanoethylnornicotine isomer in the reaction mixture was compared to the sum of the peak areas for the 5'-cyanonicotine diastereomers. The

variability in the peak area ratio between multiple injections of this solution was roughly 8%, a major improvement over that observed when N-ethylnornicotine was used as the internal standard.

The suitability of this internal standard was further investigated by extracting phosphate buffered solutions (pH 7.4) of nicotine and the cyanoadducts 44a, 44b, and N-cyanomethylnornicotine with n-butyl acetate. Both N-ethylnornicotine and the N-(1-cyanoethyl)nornicotines were present as the internal standards. GC analysis of the extracts demonstrated a poorer recovery of the N-(1-cyanoethyl)nornicotines relative to the 5'-cyanonicotines. When n-butyl acetate solutions of equal amounts of 5'-cyanonicotine and the N-(1-cyanoethyl)nornicotines were vortexed for 5 and 60 minutes, the size of the peak of the major N-cyanoethylnornicotine isomer decreased relative to that of the 5'-cyanonicotines in a time-dependent fashion (Figure 30). The disappearance of these compounds could be a result of their decomposition in aqueous solutions since the peak in the GC tracing corresponding to nornicotine did increase in size suggesting that the N-(1-cyanoethyl)-nornicotines break down to nornicotine and acetaldehyde in aqueous solution. These marginal results prompted us to examine additional possibilities.



75 78

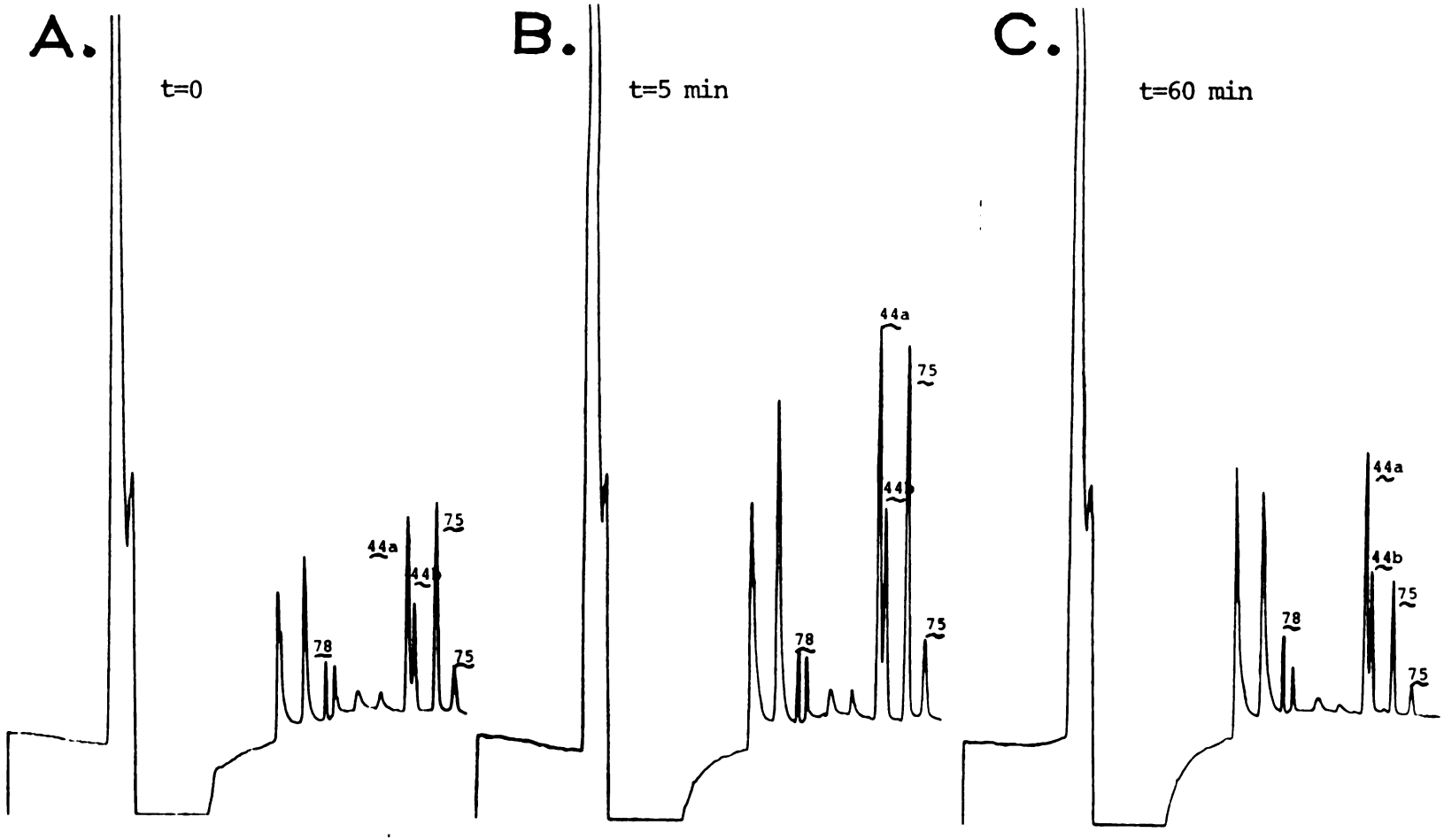


Figure 30. The GC tracing of a n-butyl acetate solution containing the 5'-cyanonicotine diastereomers (44a and 44b) and the N-(1-cyanoethyl)nornicotine reaction mixture (75). Nornicotine, 78.

The next internal standard candidate was 5-methyl-5'-cyanonicotine (76). Theoretically, this compound should be a suitable internal standard for 5'-cyanonicotine as it differs only by the methyl group in the 5 position of the pyridine ring. The synthetic scheme for the preparation of this compound is outlined in Figure 31. The 5-methylnicotine (79) was obtained via a pathway analogous to that developed for the synthesis of nicotine from ethyl nicotinate (80) and N-vinylpyrrolidinone (81).[115,116,117] The reaction scheme leading to 5-methylnicotine was worked out by Dr. P. Jacob. The mercuric acetate oxidation of 5-methylnicotine followed by reaction of the products with aqueous sodium cyanide yielded 5'-cyano-5-methylnicotine which was purified by column chromatography. The 80 MHz ^{1}H-NMR spectrum (Figure 32) established that the column-purified product was primarily a mixture of the diastereomeric (E)- and (Z)-5'-cyano-5-methylnicotines since the spectrum was similar to the ^{1}H-NMR spectrum of a mixture of 44a and 44b (Figure 4). The singlet resonating at 2.34 ppm was assigned to the 5-methyl group. Packed column GC analysis of the product indicated that the diastereomers elute as a single peak. A small amount of 5-methylnicotine was detected by GC analysis. ^{1}H-NMR analysis suggested that 5-methylnicotine represented less than 10% of the product whereas as packed GC analysis suggested that there was a 3:4 ratio of 5-methylnicotine to the 5'-cyano-5-methylnicotines. The discrepancy between the two methods of analysis suggests that the 5'-methyl-5-methylnicotines decompose under the conditions of the packed column GC analysis (injector port: 200° C; temperature program: 160° C, hold for 1 minute, then 4° C/minute).

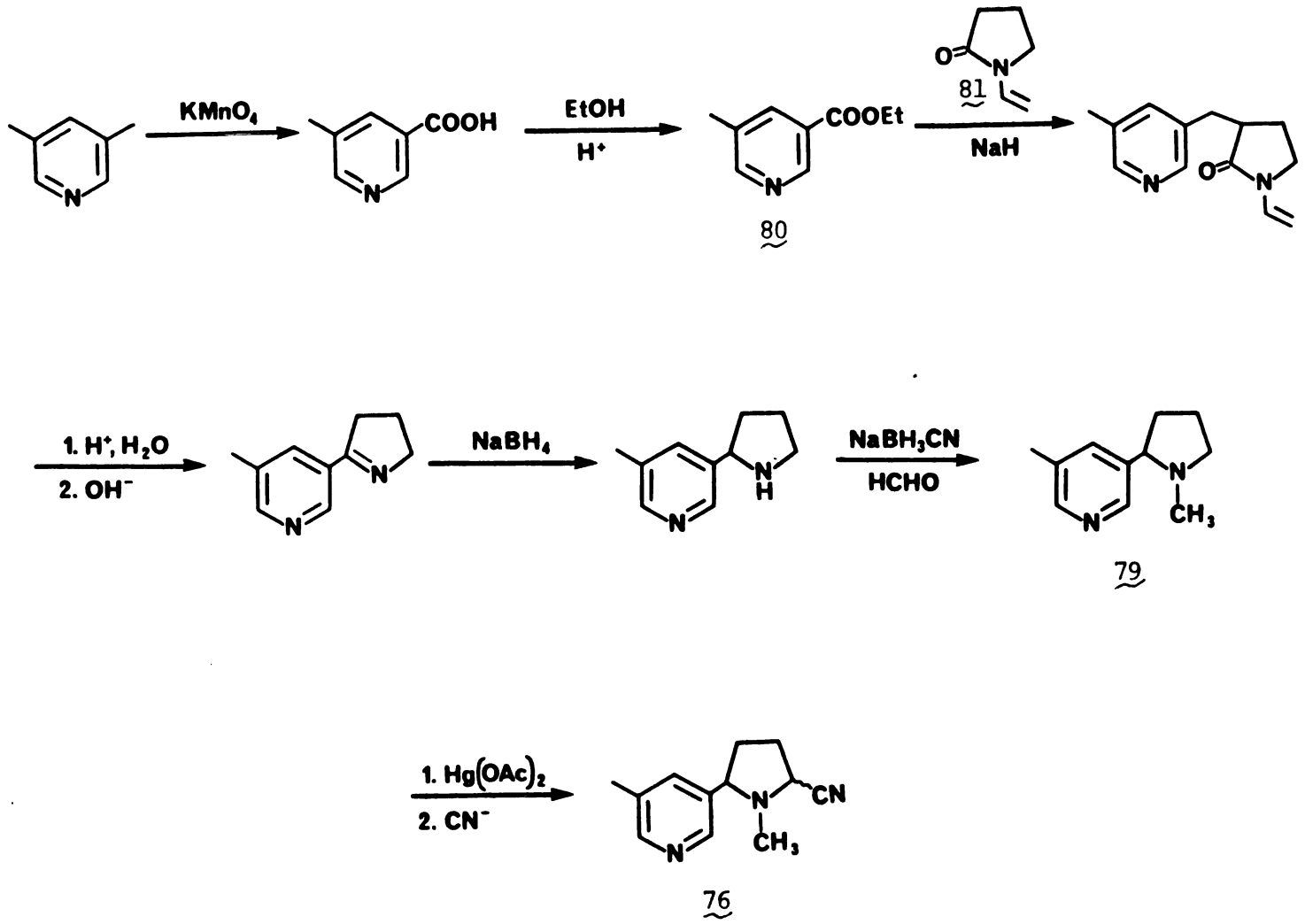


Figure 31. Synthetic pathway to 5'-cyano-5-methylnicotine.

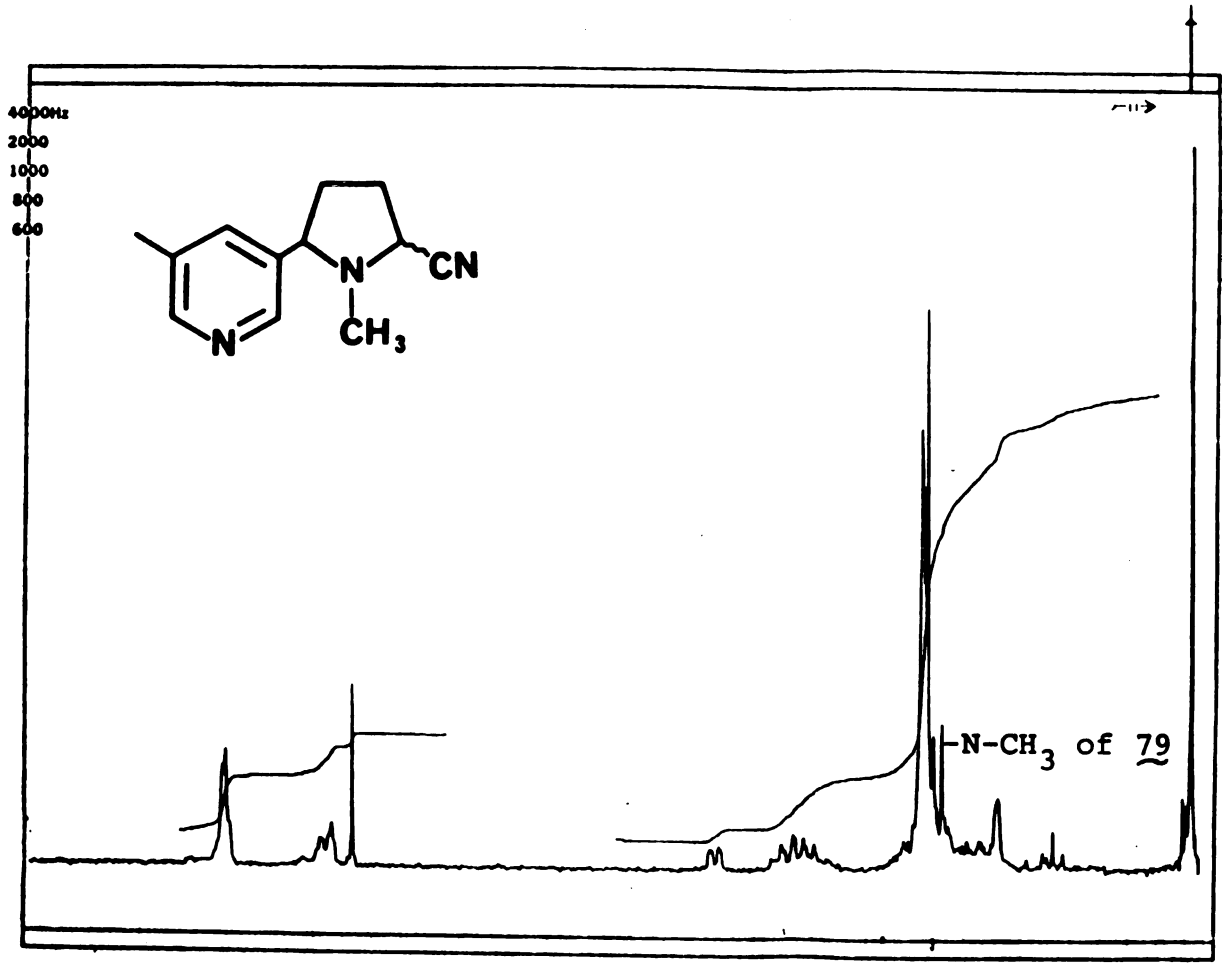
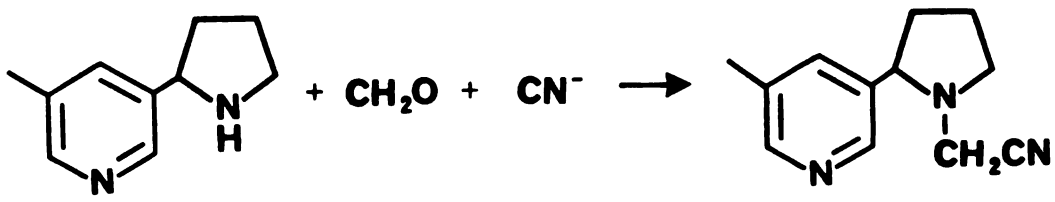


Figure 32. The 80 MHz ^{1}H-NMR spectrum of 5'-cyano-5-methylnicotine in $CDCl_3$.

The 5'-cyano-5-methylnicotines also were found to be very unstable in aqueous solutions. This instability was discovered by extracting a solution containing equal amounts of the 5'-cyano-5-methylnicotines and N-cyanomethyl-5-methylnornicotine (82) which was prepared by reacting 5-methylnornicotine with formaldehyde in the presence of cyanide ion as a



82

potential internal standard for N-cyanomethylnornicotine.[79] The compounds were solubilized in buffer with the aid of dimethyl sulfoxide. Analysis of the ether extract of this solution was done on a 3% OV 25 packed GC column (160° C then 4° per minute to 190° C). Baseline separation could not be obtained. The N-cyanomethyl isomer was present in much larger amounts than the 5'-cyano compounds (Figure 33). This observation suggests that either the 5'-cyano-5-methylnicotines are not extracted as well as the N-cyanomethyl isomer or that they are degrading in the buffer solution. The reasons for the low recovery of this compound were not further investigated since the initial problems suggested that it would not be a good internal standard for 44a and 44b.

Attempts also were made to develop a standard curve by extracting known quantities of the 5'-cyanonicotines from buffer solutions. The extracts were concentrated under a nitrogen stream and the residues were redissolved into a standard amount of solvent. A plot of the amount of the 5'-cyanonicotines versus the ion current observed for 44a and 44b

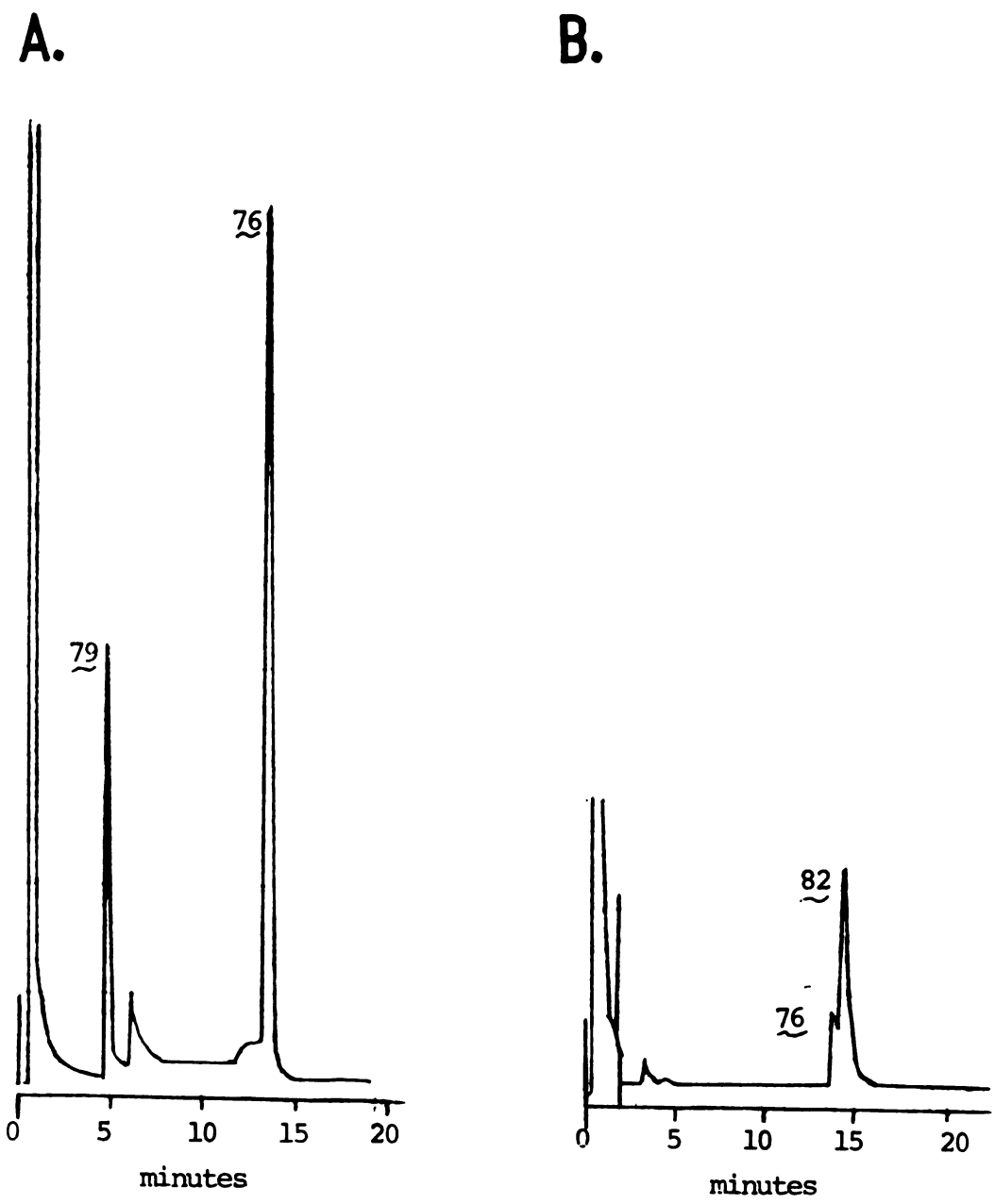
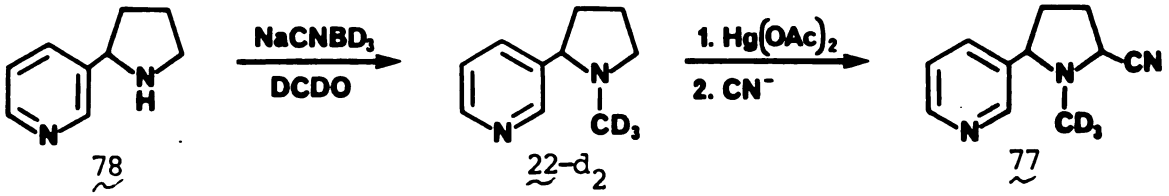


Figure 33. The GC tracing of A.) a standard ether solution of 5'-cyano-5-methylnicotine and B.) an ether extract of a pH 7.4 buffer solution of a 1:1 mixture of 5'-cyano-5-methylnicotine and N-cyanomethyl-5-methylnicotine. 5'-cyano-5-methylnicotine, 76; 5-methylnicotine, 79; N-cyanomethyl-5-methylnornicotine, 82 (3% OV 25 packed GC column; inject 160° C, hold 1 minute, then 4° C/min to 190° C).

when 1 μL of the solution was analyzed by GC-EIMS was generated. A straight line was not obtained. At low amounts of the α-cyanoamines (<5 μg) there was a significant amount of degradation of the compound within the GC-MS system and at higher amounts (>20 μg) saturation of the ion collector occurred. This nonlinearity prevented accurate determination of the 5'-cyanonicotines formed in the incubation mixtures. A similar analysis of peak height on packed GC did not allow good separation of the 5'-cyanonicotines and N-cyanomethylnornicotine.

One final effort to find a good internal standard for the 5'-cyanonicotines was made using 5'-cyanonicotine-N-d_3 (77). The synthetic route to this compound is outlined in Figure 34. Nornicotine (78) was allowed to react with formaldehyde-d_2 in the presence of sodium cyanoborodeuteride in deuterium oxide. Nicotine-N-d_3 (22-d_3) was purified by column chromatography on alumina. This material was treated with mercuric acetate in acetic acid. The oxidation products were allowed to react with aqueous sodium cyanide. A diastereomeric mixture of the 5'-cyanonicotine-N-d_3 (77) was the major product obtained upon purification of the crude reaction mixture on an alumina column. The 80 MHz ^{1}H-NMR spectrum confirmed that the desired product had been achieved (Figure 35). The spectrum was identical to that observed for a mixture of 44a



78 22-d_2 77

Figure 34. The synthetic pathway for 5'-cyanonicotine-N-d_3.

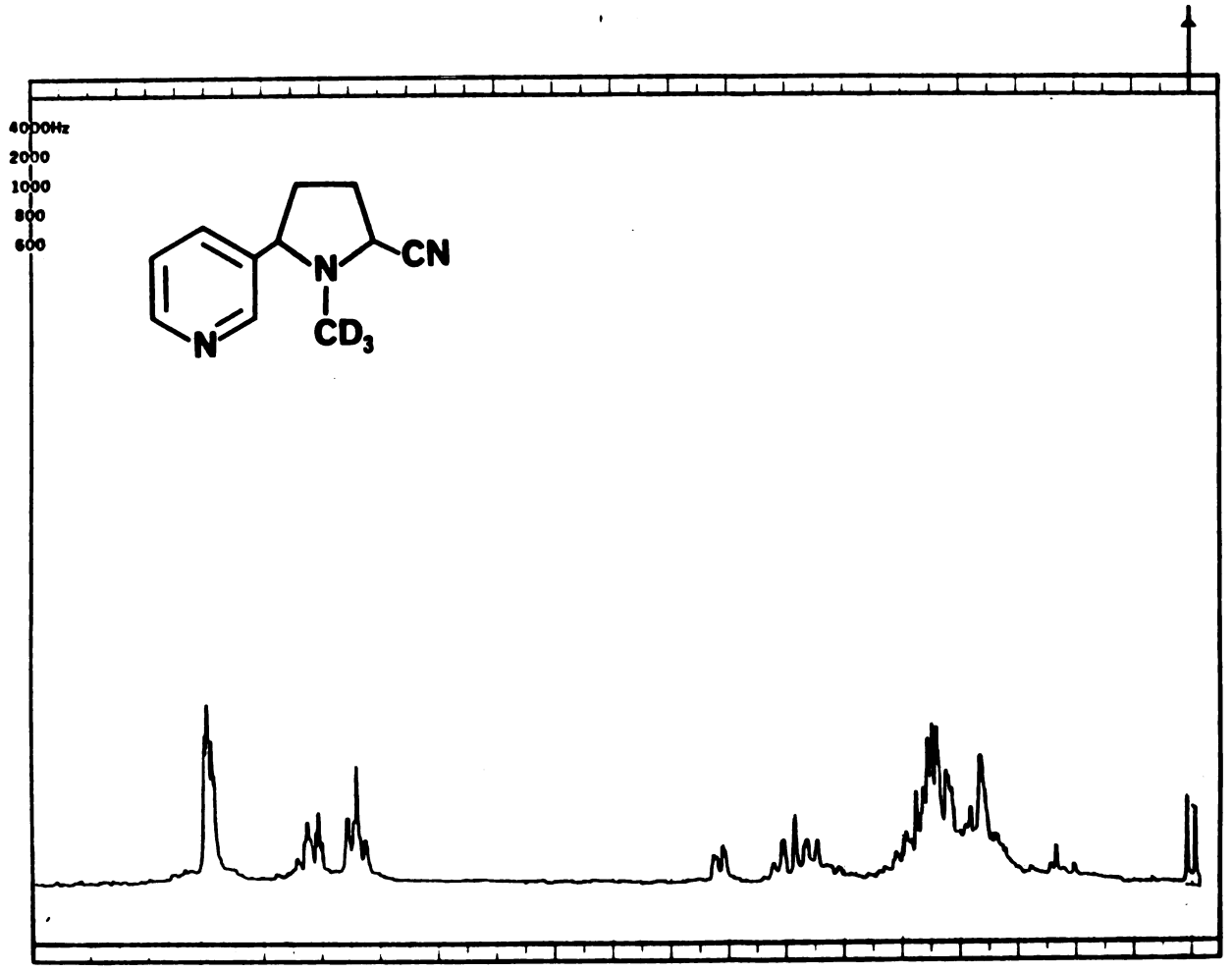
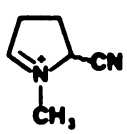


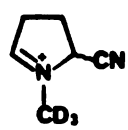
Figure 35. The 80 MHz NMR spectrum of 5'-cyanonicotine-N-d_3.

and 44b minus the N-methyl signals. Capillary GC-EIMS analysis indicated that the product was a mixture of the 5'-cyanonicotine diastereomers by the presence of two peaks which coeluted with synthetic 44a and 44b. The GC tracings of the deuterated product also indicated that they were contaminated with small amounts of nicotine-N-d_3 and N-cyanomethylnornicotine (<5%). The EI mass spectra of 5'-cyanonicotine-d_0 and 5'-cyanonicotine-N-d_3 are presented in Figure 36. The shift in the base peak at m/z 109 by 3 mass units to m/z 112 is consistent with the presence of 3 deuterium atoms on the N-methyl group. The relative intensities are listed in Table 3. The presence of significant ion current at m/z 109, 110 and 111 in the labeled compound required corrections for their contributions to these mass ions when this standard was used to quantitate labeled and unlabeled 44a and 44b formed in the incubation mixtures of 57 and 58.

Table 3. Relative intensities of the base peak and surrounding peaks in the EI-mass spectra of the 5'-cyanonicotines-d_0 and 5'-cyanonicotines-N-d_3.



m/z 106	2.4%
m/z 107	1.0%
m/z 108	1.2%
m/z 109	100%



m/z 109	1.8%
m/z 110	2.5%
m/z 111	8.4%
m/z 112	100%

Standard curves were generated with synthetic 5'-cyanonicotine diastereomers extracted from pH 7.4 buffer containing 1.0 mM sodium cyanide in the presence or absence of microsomal proteins. The ether extracts were analyzed by the capillary GC-EIMS methods described in Section II.C., monitoring the ion current at m/z 109, 110, 111, and 112

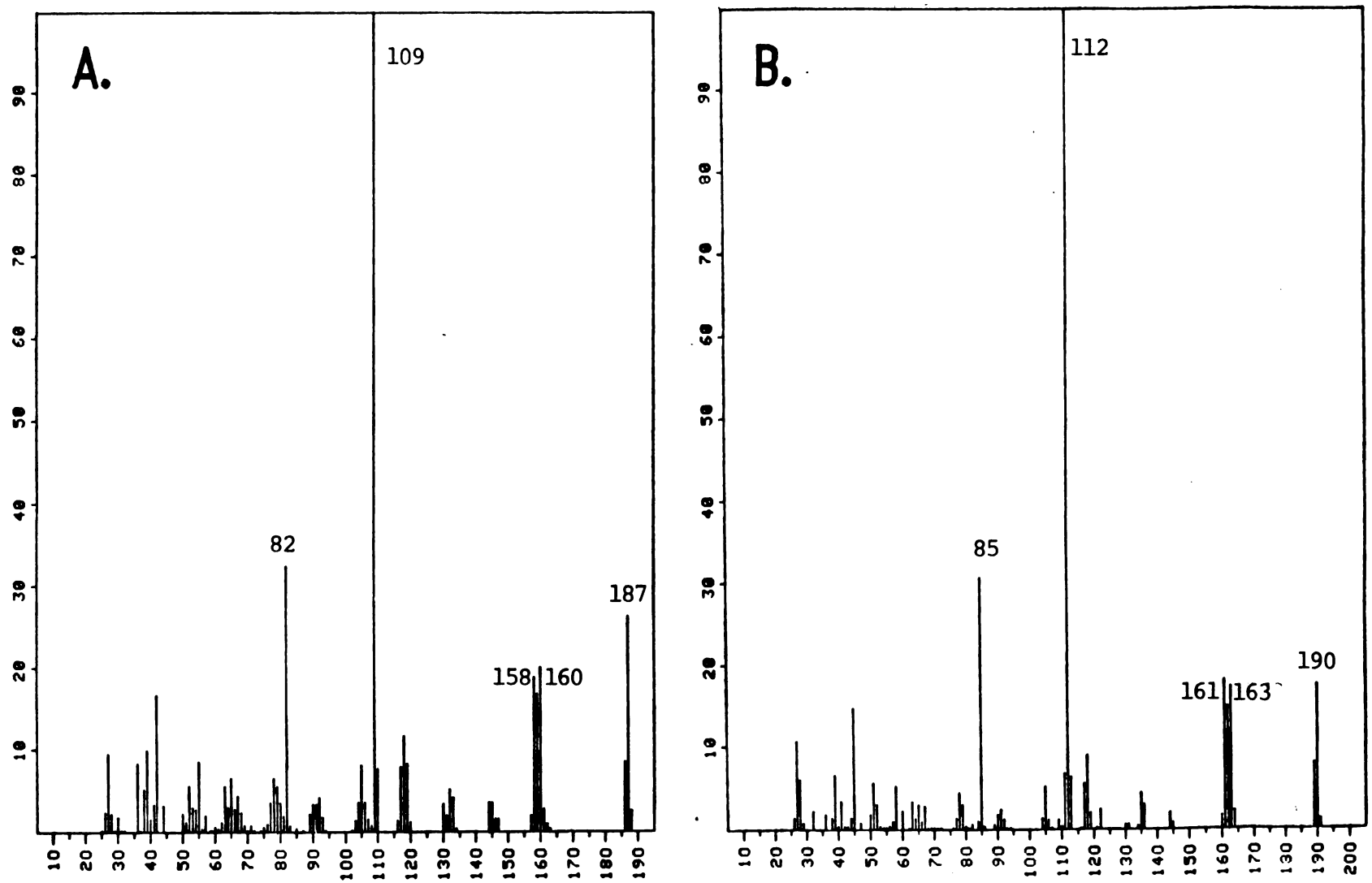
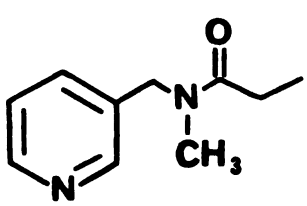


Figure 36. The EI-mass spectra of A.) (E)- and (Z)-5'-cyanonicotines and B.) 5'-cyanonicotine-N-d_3.

which correspond to the fragment in the mass spectrum of 44a and 44b which has lost the pyridine ring. The ion current for the two 5'-cyanonicotines at each mass was summed and corrected for ^{13}C-isotope contributions. The ratio of the ion currents at m/z 109 and 110 over the ion current at m/z 112 was plotted against the amount of the 5'-cyanonicotines in the sample. These data generated a linear standard curve (r=.990) which was the same in the presence or absence of microsomal proteins. Multiple injections of the same sample demonstrated a standard error of less than 3%. This internal standard was then used to quantitate the 5'-cyanonicotines formed in metabolic incubations of 57 and 58 (see section II.E.).

The disappearance of nicotine was determined by the quantitative HPLC assay developed by Mr. M. Shigenaga. This assay allowed the separation and quantitation of nicotine and the metabolites cotinine, 42, and the cyanoadducts. The isomeric cyanoamines 44a, 44b and 73 were not separated in this system so the peak which corresponded to these compounds provided a measure of the total amount of cyano adducts formed in the metabolic mixtures. Other methods such as GC analysis were required to determine what percentage of this signal was attributable to the 5'-cyanonicotines. The analysis involved the addition of the internal standard N-methyl-N-(3-pyridyl)methylpropanamide (83) and 1 M potassium carbonate solution to the microsomal incubation mixtures (1 ml) at



83

the end of the incubation period. The dichloromethane extracts were analyzed by HPLC on a silica column using an acetonitrile-1% propylamine mobile phase (Figure 37). Nicotine and its metabolites were quantitated by comparing the peak height ratios of the particular compound/internal standard to values obtained from standard curves generated with known quantities of nicotine, cotinine, 42, and the 5'-cyanonicotines.

The GC-MS assay estimated lower levels of the metabolically generated 5'-cyanonicotines than the HPLC assay (Table 4). One possible cause of these differences is that the HPLC assay does not separate the N-cyanomethyl isomer from the 5'-cyanonicotines so the value obtained by this method is for the total amount of cyanoamines formed in the incubation mixtures. GC analysis of extracts from Dutch rabbit microsomal incubations of 22, however, have demonstrated that 73 accounts for only 10% of the cyanoamines observed in these preparations (see section II.E.2). So this cyano adduct cannot fully account for the difference between the cyanoamines detected in the two analytical methods. The reasons for this discrepancy is not known.

Table 4. The amounts of α-cyanoamines formed in Dutch rabbit liver microsomal incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 in the presence of 1 mM NaCN as determined by quantitative GC-EIMS and HPLC methods.

	GC-EIMS assay		HPLC assay	
Substrate	nmoles 44a + 44b	% metabolism	nmoles CN-adducts	% metabolism
57	6.1 ± 0.2 (n=2)	23%	14.5 ± 2.5 (n=3)	54%
58	7.2 ± 0.2 (n=2)	30%	15.4 ± 2.0 (n=3)	65%

The incubations were done for 5 minutes at 37° C. The different analytical methods were performed with separate incubation mixtures. The disappearance of nicotine was determined by the quantitative HPLC assay.

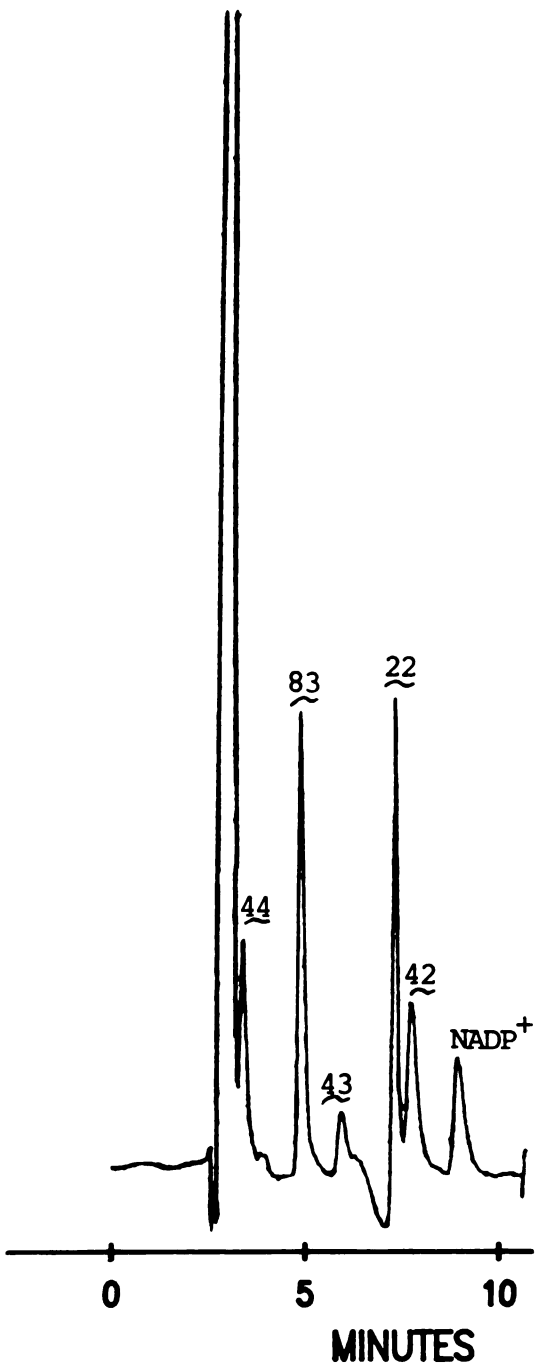
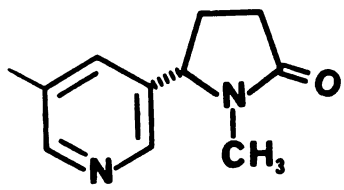


Figure 37. The HPLC tracing of a chloroform extract of a solution containing the 5'-cyanonicotines (44), N-methyl-N-(3-pyridyl)methylpropanamide (83), cotinine (43), (S)-nicotine (22), nicotine Δ-1'(5')-iminium ion (42), and $NADP^+$.

A sensitive assay also was required for the determination of the cotinine levels in the microsomal incubations of 57 and 58 to assess the quantitative importance of the further oxidation of the 5'-cyanonicotines to cotinine. The HPLC assay described above was not sensitive enough for our purposes. The analyses were performed by Dr. N. Benowitz's laboratory at San Francisco General Hospital. The internal standard 5-methylcotinine (84) was added to the incubation mixtures following the ether extractions to remove the 5'-cyanonicotines for GC-MS analysis. The aqueous solution was made basic with 1 N NaOH and extracted with toluene-isoamyl alcohol. Subsequent acid-base partitioning rendered a toluene-isoamyl solution for GC analysis on a carbowax-KOH packed column at 210° C. Standard curves were generated with synthetic cotinine.[113]



84

E. Metabolic Studies

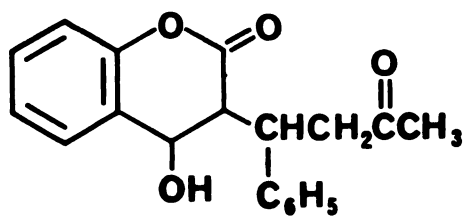
1. Introduction

The metabolic studies were carried out with magnesium sulfate and NADPH-supplemented liver microsomal preparations from several species (3 mg protein/mL) and the diastereomeric nicotines 57 and 58 (0.5 mM). The incubations were performed in the presence of sodium cyanide (1.0 mM) in order to convert the unstable metabolically generated iminium species 42 to the α-cyanoamines 44a and 44b. The deuterium content of 44a and 44b was determined by GC-EIMS selected ion monitoring techniques. These data, following corrections for the stereochemical and isotopic impurities of 57 and 58 (see Section II.C.), were used to determine the stereochemical course of this oxidation.

The deuterium composition of 44a and 44b generated during the liver microsomal incubations of 57 and 58 was used as a first approximation of the stereochemical course of the cytochrome P-450 catalyzed oxidation. While microsomes contain a wide variety of enzyme systems,[118] studies from our laboratory[30,31] and others[29] have demonstrated that the microsomal enzyme catalyzed oxidation of 22 to 42 is a cytochrome P-450 mediated process. The conversion of nicotine to cotinine by rabbit liver microsomes and soluble fraction requires NADPH and O_2 and is inhibited by carbon monoxide.[29] This reaction also is inhibited by SKF 525A, a known cytochrome P-450 inhibitor, in hamster liver 10,000xg fractions.[119] Further evidence of the involvement of the mixed function oxidase system in the 2-electron transformation of 22 to 42 is the NADPH and O_2 dependence of the formation of 44a and 44b when these studies are done in the presence of cyanide ion.[29,79] Microsomes alone catalyzed the formation of 42 in a NADPH- and O_2-dependent fashion as determined

by the detection of the 5'-cyanoamines when cyanide ion is included in the incubation mixtures.[29-31] In addition, nicotine perturbs the visible spectrum of cytochrome P-450 demonstrating that it is capable of binding at the active site of this enzyme.[120] All these studies suggest that cytochrome P-450 is involved in the α-carbon oxidation of this tertiary amine by in microsomal preparations.

These data allow us to be confident that the generated cyanoamines in the microsomal preparations are formed through a cytochrome P-450 catalyzed reaction. However, recent studies of this hemoprotein have demonstrated that it is not a single protein but a family of isozymes[118]--more than 10 isozymes have been isolated from rat and rabbit liver microsomes and characterized.[121-123] Different forms of cytochrome P-450 have also been purified from human liver microsomes.[124,125] The binding sites of these isozymes have differing but well-defined binding regions as suggested by the observed variations in the regio- and stereoselectivity in steroid hydroxylation for a number of cytochrome P-450 isozymes isolated from rats.[126] The metabolic profile of warfarin (85) is also dependent on the composition of P-450 isozymes in



85

incubation mixtures.[127] At least two types of cytochrome P-450 are believed to be responsible for the nicotine oxidase activity observed in Wistar rat liver microsomes.[128] The participation of more than one isozyme in this microsomal reaction could confound the observed stereochemical results of our experiments if these isozymes are able to cata-

lyze the oxidation of the 5'-position with varying degrees of stereoselectivity. This problem could be avoided if the reaction were studied using purified isozymes. The necessary techniques for preparing the isozymes, however, were not readily available in our laboratory so liver microsomal preparations were used as an initial approach to the study of the stereochemical course of the cytochrome P-450 catalyzed oxidation of 22 to 42.

2. Stereochemical Studies with Rodent Liver Microsomes

Preliminary studies to determine the diastereoselectivity of the C-5'-oxidation of (S)-nicotine were conducted with Dutch rabbit liver microsomes. The 5'-deutereonicotine diastereomers (0.5 mM, 2.5 mL) were incubated separately with the 100,000xg microsomal pellet obtained from untreated Dutch rabbits (3 mg protein/mL) in the presence of the required cofactors and 1 mM sodium cyanide at 37° C in pH 7.4 0.2 M phosphate buffer.[30,31] Incubations performed in the absence of NADPH served as controls. The reactions were stopped by placing the incubation mixtures on ice. Subsequent extraction with ferrous sulfate washed ether and concentration of the extracts under a nitrogen stream yielded a residue which was redissolved in dichloromethane for capillary GC-EIMS analysis.

The mass chromatograms of the ether extracts indicated the presence of unmetabolized nicotine, the diastereomeric 5'-cyanonicotines (44a and 44b) and N-cyanomethylnornicotine (73, Figure 38). Confirmation of these assignments was achieved with synthetic standards and comparison of the corresponding EI mass spectra. The N-cyanomethylnornicotine is derived from the reaction of cyanide ion with the N-methyleneiminium ion 86 generated during the oxidation of the N-methyl group of nicotine. In

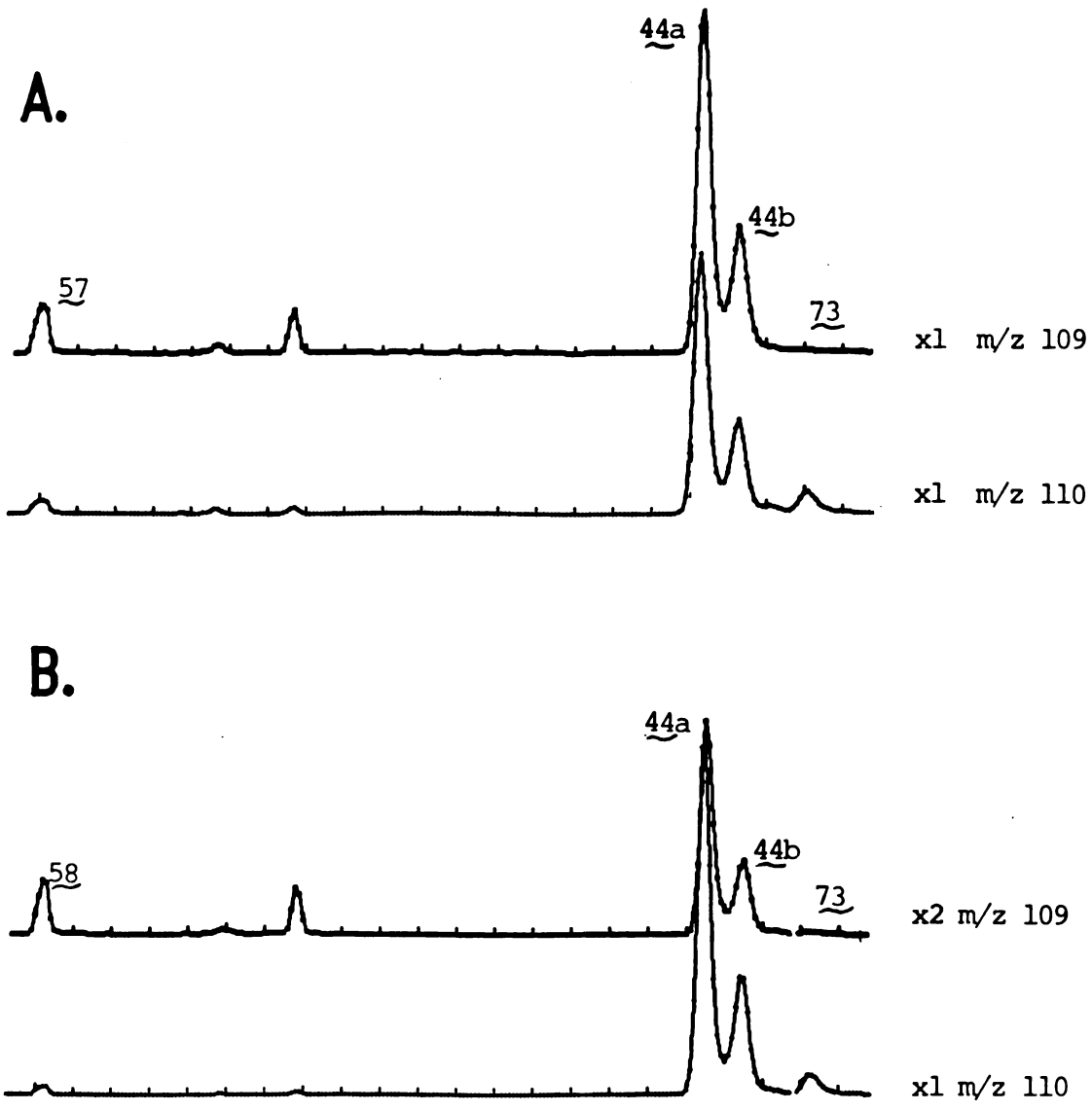
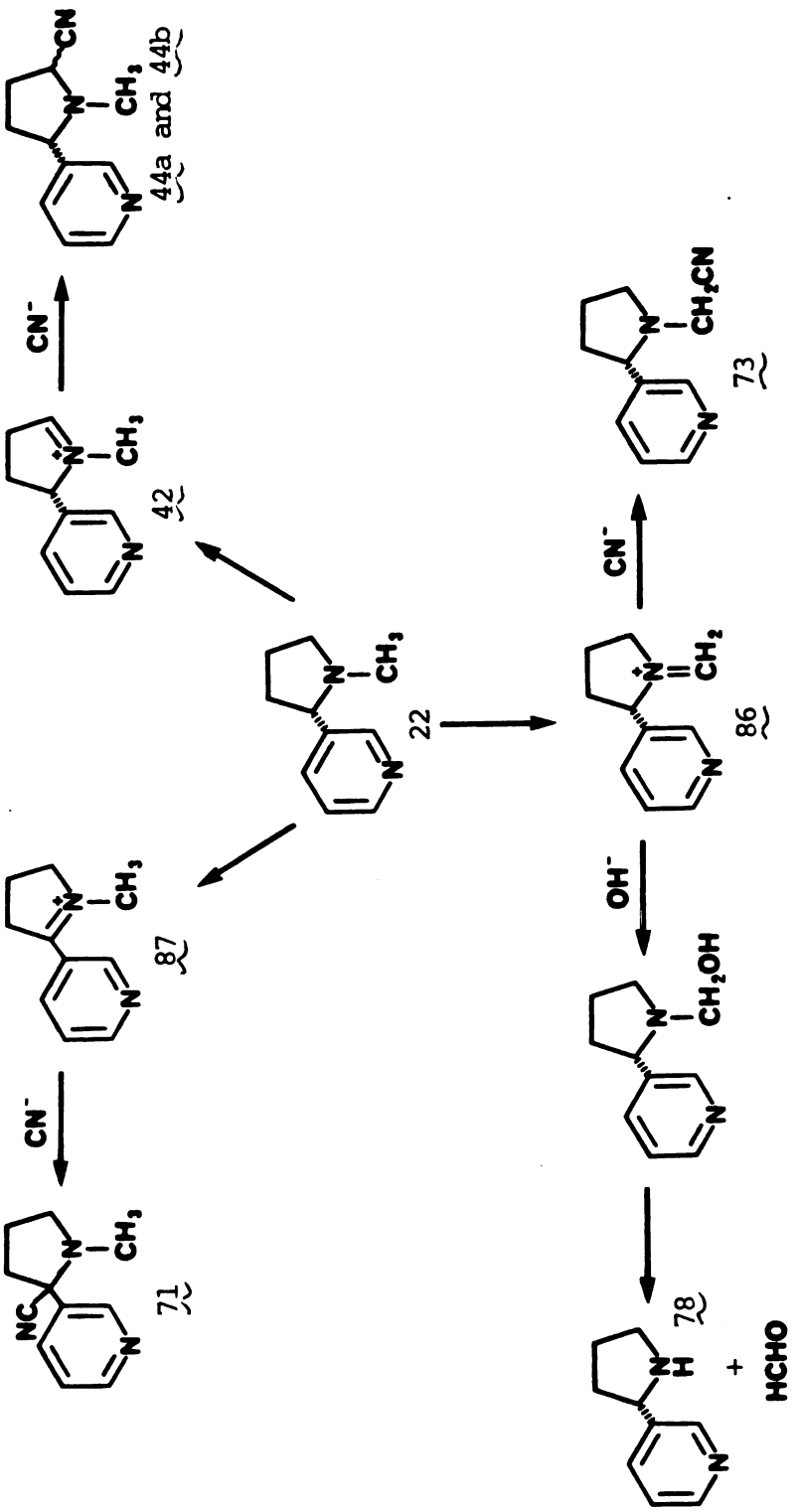


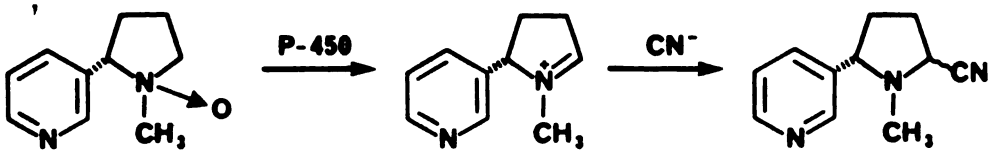
Figure 38. The mass chromatographs of the ether extracts obtained from the metabolism of the monodeutero diastereomers (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) in the presence of cyanide ion and Dutch rabbit liver microsomes supplemented with O_2 and NADPH. (S)-nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; N-cyanomethylnornicotine, 73.


CN⁻
CN⁻
CN⁻
OH⁻
+ HCHO

the absence of cyanide ion, this iminium ion undergoes hydrolysis and N-C bond cleavage to form formaldehyde and nornicotine (78), a minor metabolite of nicotine.[50,51,129] A third possible isomeric cyano adduct, 2'-cyanonicotine (71), was not detected in these preparations. This compound would be generated from the reaction of cyanide ion with nicotine $\Delta^{1'(2')}$-iminium ion (87).

The extent of nicotine metabolism was determined by the previously described HPLC assay in one Dutch rabbit liver preparation and demonstrated that 26.9 ± 0.3 nmoles of 57 and 23.6 ± 0.1 nmoles of 58 were consumed during a 5 minute incubation period (n=2). During this time 14.5 ± 2.5 nmoles of the cyanonicotines 44a, 44b and 73 were formed from 57 and 15.4 ± 2.0 nmoles were formed from 58. The α-carbon oxidation products represent 54% of the disappearance of 57 and 65% of the disappearance of 58 in these incubation mixtures. While these data are only preliminary, they are consistent with an earlier report which stated that approximately 50% of (S)-nicotine metabolism observed in rabbit liver 10,000xg homogenates was attributed to cotinine formation.[130]

We investigated the potential for nicotine-N'-oxide (88) to serve as a precursor to the iminium ion 42 since N-oxides have been proposed as discreet intermediates in microsomal α-carbon oxidations.[131] Synthetic (E)-nicotine-N'-oxide (1 mM) prepared by the methods of Craig and Purushothaman[132] was incubated with Dutch rabbit liver microsomal proteins (3 mg protein/mL) and sodium cyanide (1 mM) in pH 7.4 phosphate



88 42 44a and 44b

buffer for 15, 30, and 60 minutes at 37° C. Control experiments were performed in the absence of microsomal proteins. The incubations were extracted with ether, concentrated under nitrogen, and resuspended in n-butyl acetate (50 μL) for capillary GC analysis. Although some 44a and 44b were present, essentially no difference was observed in the amount of the 5'-cyanonicotines formed in the presence and absence of microsomal protein. This observation suggests that the formation of 42 from the N-oxide was nonenzymatic. Nicotine N'-oxide gave a cytochrome P-450 difference spectrum similar to nicotine suggesting that the compound penetrates the microsomal membrane and interacts with the active site of cytochrome P-450. There was a time-dependent increase in the amount of nicotine detected in the incubation mixtures suggesting the reduction of the N'-oxide to the tertiary amine 22. The reduction of nicotine-N'-oxide has been previously reported.[80,133] Our results demonstrate that (E)-nicotine-N'-oxide is not a competent intermediate in the formation of 42 from nicotine by cytochrome P-450.

There was approximately a 10:1 predominance of the 5'-cyano adducts over the N-cyanomethyl adduct as determined by capillary GC analysis of the metabolic extracts (Figure 39). Nguyen had reported earlier that the regioisomers were formed in roughly equal amounts.[79] This discrepancy can be explained, in part, by the method of isolation of the cyano adducts from the metabolic mixture. Nguyen performed acid-base extractions of the ether extracts of incubation mixtures prior to GC analysis. Under these conditions some, if not all, of 44a and 44b might decompose to 42 whereas N-cyanomethylnornicotine is relatively stable under these conditions. When we performed acid-base partitioning of synthetic 44a and 44b using pH 5 phosphate buffer for the acidic extraction step, only residual amounts of 44a were observed in capillary GC tracing of the

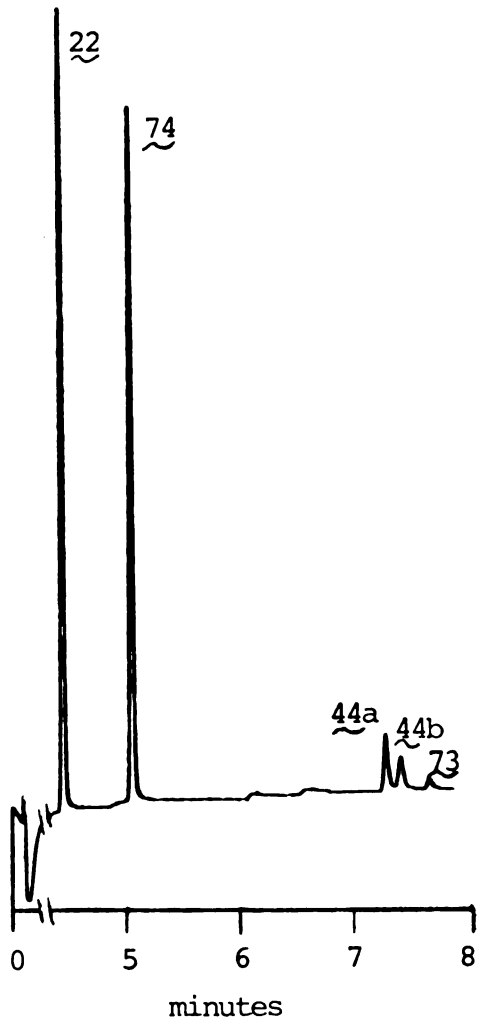
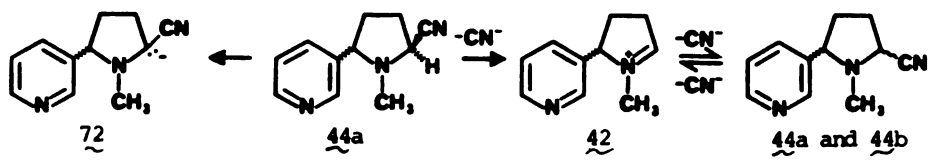


Figure 39. The capillary GC tracing of the ether extract obtained from the metabolism of (S)-nicotine in the presence of sodium cyanide (1mM) by Dutch rabbit liver microsomes supplemented with NADPH and O_2. (S)-nicotine, 22; N-ethylnornicotine, 74; (E)- and (Z)-5'-cyanonicotine, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

final extract. Since we did not subject our extracts to acid-base partitioning, our observed ratio of the 5'-cyanonicotines to N-cyanomethylnornicotine is likely to be more reliable. The predominance of oxidation at the 5'-position is consistent with reports that this pathway is the major route of nicotine metabolism in mammals.[50]

Capillary GC-EIMS analysis of the extracts led to the separation of 44a and 44b as has been observed with the synthetic standards. In all cases, the ratio of 44a to 44b was similar to that observed when synthetic 42 was allowed to react with cyanide ion at pH 7.4. This observation could indicate that the reaction of the metabolically generated iminium ion with cyanide ion occurs in solution and not at the enzyme's active site. The latter situation might have led to the selective formation of only one diastereomer since the chiral environment of the enzyme might influence the side of attack by the cyanide ion. Studies with the individual diastereomers (see Section II.C. for separation methods) have demonstrated that this second possibility cannot be ruled out since ^{1}H-NMR analysis of the cyano adducts extracted from a one hour-37° C incubation mixture of 44a in pH 7.4 phosphate buffer demonstrated that this diastereomer had epimerized to the equilibrium 44a:44b mixture of approximately 2:1. A similar reaction was observed in methanol. However, the diastereomers were stable in dichloromethane and ether. This epimerization presumably takes place through the intermediate iminium ion 42 as opposed to a resonance stabilized α-

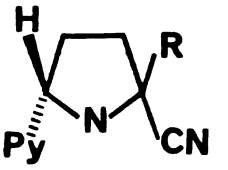


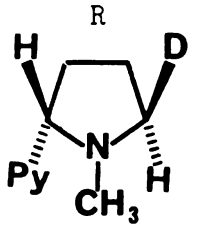
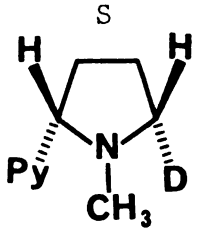
carbanion 72 since ^{1}H-NMR analysis of the cyano adducts recovered after 2 weeks in a deuterium oxide solution of potassium carbonate demonstrated that no exchange of the 5'-proton occurred. So, despite the fact that 44a and 44b are in equilibrium with 42 under the incubation conditions, we can be confident that the 5'-deuteron of the adducts formed in incubations of 57 and 58 will not be lost.

The deuterium composition of both 44a and 44b isolated from the Dutch rabbit liver microsomal incubation mixtures was identical. The values from the two diastereomeric cyano adducts were summed to determine the net retention or loss of deuterium associated with the oxidation of 57 and 58.

The stereochemical results from studies using the microsomal preparations obtained from several Dutch rabbits are presented in Table 5. Analysis of the deuterium content of the 5'-cyanonicotines isolated from experiments with 57 showed that an average of 74 $\pm$ 5% of the label was lost during the oxidation of this deutereo diastereomer whereas only 17 $\pm$ 5% was lost during the oxidation of 58. These data establish that the Dutch rabbit microsomes catalyze the oxidation of the 5'-position of 22 with the selective removal of the pro-(S) hydrogen.

Table 5. Deuterium composition of the metabolically generated 5'-cyanonicotine isolated from Dutch rabbit liver microsomal incubations of (S)-nicotine-(E)-5'-d_1 and (S)-nicotine-(Z)-5'-d_1 performed in the presence of NADPH, O_2, and sodium cyanide.*



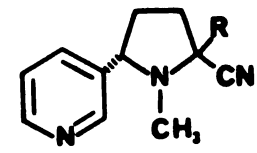
Substrate	Rabbit	% R=H	% R=D ± s.d.
	I n=16	66	34 ± 3
	II n=16	78	22 ± 1
	III n=4	70	30 ± 5
	IV n=2	72	28 ± 1
	V n=2	73	27 ± 2
	VI n=3	87	13 ± 2
	average	74	26 ± 5
	I n=16	13	87 ± 2
	II n=16	19	81 ± 2
	III n=4	11	89 ± 6
	IV n=2	21	79 ± 4
	V n=2	23	77 ± 0
	VI n=3	12	88 ± 2
	average	17	83 ± 5

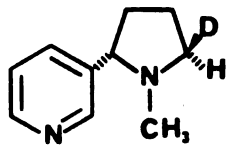
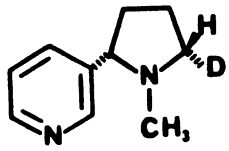
*The data have been corrected for the isotopic and stereochemical impurities of 57 and 58. See Section II.C. for details.

Storage of the microsomal preparations at -78° C did not significantly change the stereoselectivity of the oxidation. These results suggest that if more than one isozyme is involved in the oxidation, one is not selectively lost during the freezing process. All experiments conducted after this initial study were performed with microsomes that had been previously frozen.

A second series of studies considered variations in product deuterium content with time. The observed diastereoselectivity of the oxidation did not change dramatically with the length of the incubation period as demonstrated by the data in Table 6.

Table 6. Effect of the length of the incubation period on the deuterium composition of the metabolically generated 5'-cyanonicotines by Dutch rabbit microsomes (n=2).*



Substrate	Animal	Time (min)	% R=H	% R=D ± s.d.
	I	5	65	35 ± 1
		10	66	34 ± 1
		30	66	34 ± 5
	II	5	77	23 ± 1
		10	80	20 ± 0
		15	79	21 ± 1
		30	77	23 ± 1
	I	5	14	86 ± 1
		10	12	88 ± 1
		30	14	86 ± 2
	II	5	18	82 ± 0
		10	18	82 ± 1
		15	20	80 ± 0
		30	21	79 ± 1

*The data have been corrected for the isotopic and stereochemical impurities of 57 and 58. See Section II.C. for details.

The biological variability between the various preparations suggests some differences in either isozyme composition or active site requirements. Different subsets of isozymes can be selectively induced by a variety of agents.[134,135] However, our attempts to induce a Dutch rabbit by phenobarbital pretreatment were unsuccessful since the P-450 content of this preparation was lower than the untreated animals: 1.17 nmoles P-450/mg protein and 2.1 nmoles P-450/mg protein, respectively.

Interspecies differences in the diastereoselectivity of this reaction were explored using phenobarbital-pretreated New Zealand rabbit and untreated Sprague-Dawley rats. The New Zealand rabbit microsomes were obtained from Ms. K. Hoag and Mr. M. Shigenaga and the rat preparations from Dr. B. Ferraiolo. The mass chromatograms of the incubation mixtures established the presence of the diastereomeric 5'-cyanonicotines and N-cyanomethylnornicotine (Figure 40 and 41). The New Zealand rabbit liver microsomes catalyzed the formation of N-cyanomethylnornicotine to a larger extent than either the Dutch rabbit or rat. However, in all cases, 5'-cyanonicotine was the major cyano adduct formed.

The stereochemical results of these studies are presented in Table 7. The New Zealand rabbit microsomes catalyzed roughly 91-97% removal of the pro-(S) proton. This proton also was selectively removed (88-93%) in the rat microsome catalyzed reaction. In general, these two species are more selective in catalyzing the oxidation of the 5'-position than the Dutch rabbit. These interspecies differences could result from interspecies differences in isozyme composition.

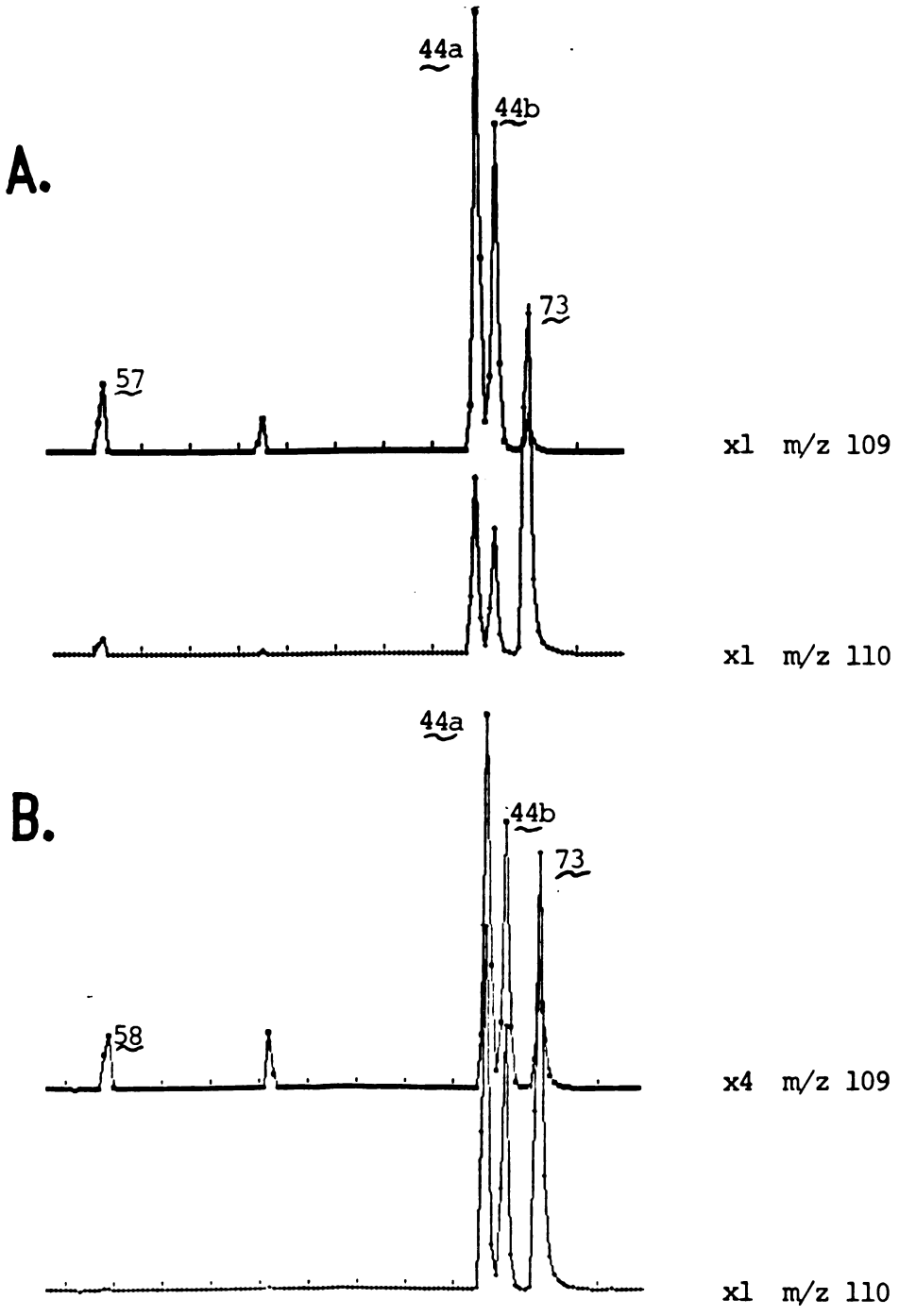


Figure 40. The mass chromatographs of the ether extracts obtained from the metabolism of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) by phenobarbital-pretreated New Zealand rabbit liver microsomes supplemented with O_2 and NADPH. (S)-nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

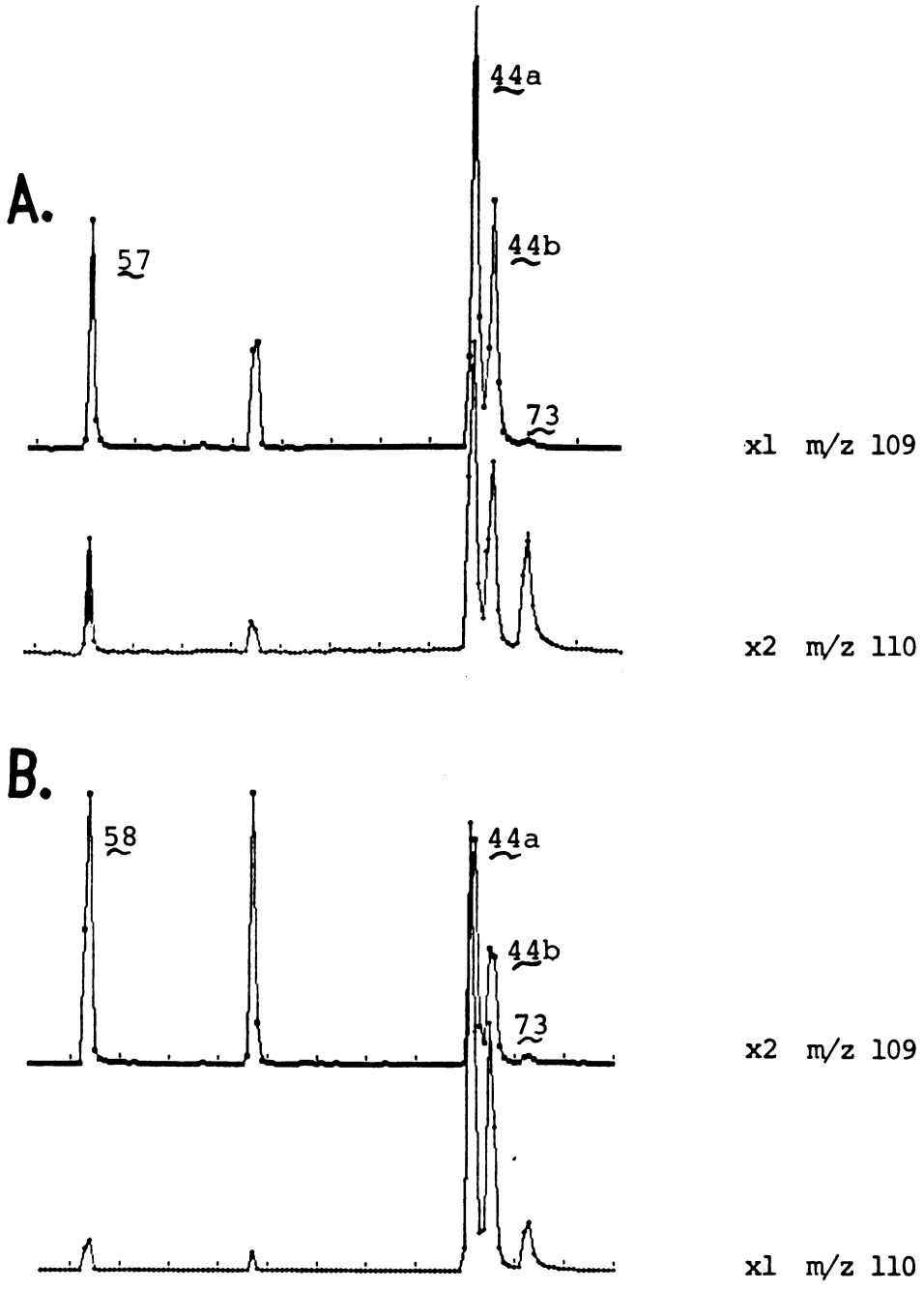
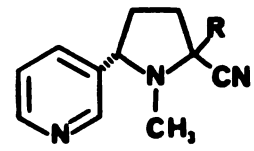


Figure 41. The mass chromagraphs of the ether extracts obtained from the metabolism of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) in the presence of sodium cyanide by Sprague-Dawley rat liver microsomes supplemented with O_2 and NADPH. (S)-nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E) and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

Table 7. Deuterium composition of the metabolically generated 5'-cyanonicotines isolated from rodent liver microsomal incubations of (S)-nicotine-(E)-5'-d_1 and (S)-nicotine-(Z)-5'-d_1 in the presence of NADPH, O_2, and sodium cyanide.*



Animal	Substrate	% R=H	% R=D ± s.d.
Rat	57	93	7 ± 1
	58	12	88 ± 0
New Zealand Rabbit			
I	57	93	7 ± 1
II		91	9 ± 3
I	58	9	91 ± 1
II		7	93 ± 1

*The data have been corrected for the isotopic and stereochemical impurities of 57 and 58. See Section II.C. for details.

3. Stereochemical Studies With Human Liver Microsomes.

With these preliminary results, we turned our attention to studies on the stereochemical course of this oxidation in human liver microsomes. Incubations of the labeled nicotines in the presence of sodium cyanide with supplemented liver microsomes from three human sources led to the isolation of almost exclusively 44a and 44b (Figure 42). Only residual amounts of N-cyanomethylnornicotine were detected in the mass chromatogram. These observations are consistent the absence of reports of nornicotine as a metabolite of nicotine in humans. These preparations displayed greater regioselectivity than any of the animal models. The adducts were absent in the control incubations lacking the pyridine nucleotide cofactor suggesting, that the α-carbon oxidation was a cytochrome P-450-mediated reaction. The importance of the α-carbon oxidation pathway in the human liver microsomal metabolism of 22 was determined using the quantitative HPLC assay described in Section II.D. In one human preparation (II), 18.5 nmoles of 22 were consumed after 15 minutes. The formation of the 5'-cyanonicotines (7.4 nmoles) accounted for 40% of the nicotine metabolized by this preparation. No cotinine or 42 was observed in this study. While these studies will require repetition in other human liver microsomal preparations to determine the importance of this finding, they allowed us to be confident that the 5'-α-carbon oxidation is a major human microsomal oxidation reaction.

In order to determine whether this reaction was stereochemically controlled, the deuterium content of the cyanoamines 44a and 44b were determined by the previously described mass spectral techniques. The results of these studies are presented in Table 8. These corrected data establish that human liver microsomes catalyze this oxidation with a

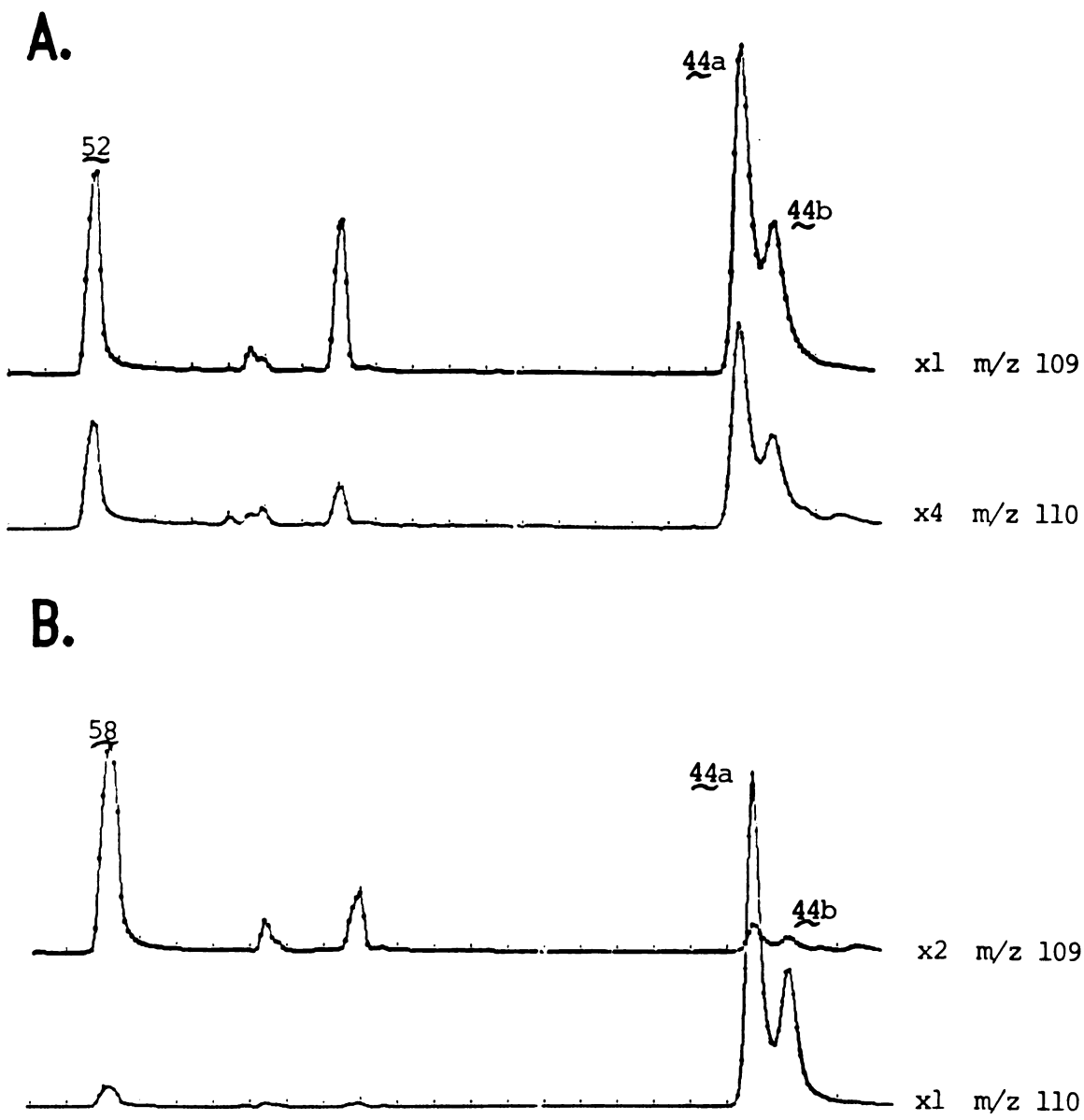
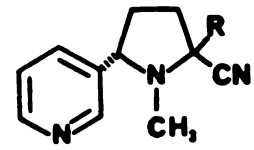
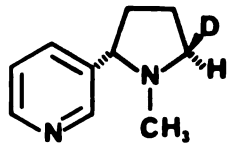
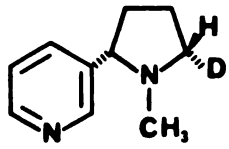


Figure 42. The mass chromatographs of ether extracts obtained from the metabolism of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-d_1 (B.) in the presence of sodium cyanide by human liver microsomes supplemented with O_2 and NADPH. (S)-nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E)- and (Z)-5'-cyanonicotine, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

high degree of stereoselectivity as demonstrated by the 90% removal of the (E)-5'-hydrogen atom of 57 and the 89% removal of the (Z)-5'-deuterium atom of 58. The stereoselectivity of these preparations is similar to that observed in the rat and phenobarbital-pretreated New Zealand rabbits. The combined regio- and stereoselectivities suggest that human cytochrome P-450 interacts with this substrate in a highly ordered complex. The mode of binding of (S)-nicotine at the active site of the enzyme will determine the direction of the oxidation. The lower extent of stereochemical control observed in experiment II may be assigned to differences in isozyme population. This explanation has been invoked by a number of investigators to explain interindividual variations in the metabolic profile of several drugs.[124,125,136] In any event, the general agreement between all of the microsomal preparations that the pro-(S) hydrogen atom is lost in this process suggests that this molecule interacts with the various cytochrome P-450s in a similar manner.

Table 8. The deuterium composition of the 5'-cyanonicotines isolated from incubations of (S)-nicotine-(E)-5'-d_1 and (S)-nicotine-(Z)-5'-d_1 with human liver microsomes in the presence of O_2, NADPH, and sodium cyanide.*

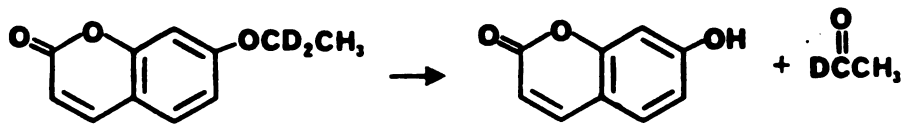


Substrate		% R=H	% R=D ± s.d.
	I	97	3 ± 1
	II	76	24 ± 5
	III	97	3 ± 6
	average	90	10 ± 10
	I	8	92 ± 1
	II	16	84 ± 6
	III	9	91 ± 3
	average	11	89 ± 4

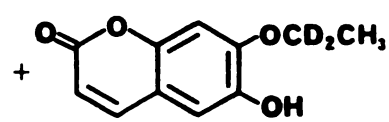
*The data have been corrected for the isotopic and stereochemical impurities of 57 and 58. See Section II.C. for details.

4. Kinetic Isotope Effect Studies

Since the oxidation of the 5'-position of 57 and 58 involves the cleavage of a C-H or C-D bond, deuterium isotope effects might influence the course of the reaction. Modest primary inter- and intramolecular isotope effects have been observed for the α-carbon oxidation of tertiary amines in studies with either reconstituted cytochrome P-450 or microsomal preparations.[19,20,60,61,73,74] Isotope effects may be detected in at least three ways. First, primary deuterium isotope effects, which could potentially influence the stereochemical course of this oxidation, might be detected if the oxidation of both 57 and 58 proceeded with the selective loss of hydrogen (vs. deuterium). Secondly, the replacement of the stereoselectively removed pro-(S) 5'-proton with a deuteron could slow the overall rate of oxidation of 57 relative to 58. This phenomenon would be exhibited by a decreased rate of formation of the 5'-cyanonicotines in incubations of 57. A third way isotope effects can be detected is via "metabolic switches" in which the extent of oxidation at a non-deuterated site in the molecule increases due to the decreased rate of oxidation of the deuterated site. This phenomenon has been observed in the studies with 7-dideuteroethoxycoumarin (89). The presence of the α-deuteron slows the rate of N-



89 90



91

deethylation to 7-hydroxycoumarin (90) by a factor of 2 while enhancing the levels of 6-hydroxy-7-dideuterocoumarin (91) production by a factor of 5.[137] Metabolic switching results in either the formation of new metabolites or in increases in the amounts of already existing metabolites. Since (S)-nicotine possesses three α-carbon atoms, it is possible that the presence of the label at the 5'-position may cause the oxidation to shift to another carbon atom. This phenomenon would be detected by the increased formation of N-cyanomethylnornicotine or the detection of 2'-cyanonicotine. In order for metabolic shifting to occur, the molecule must be able to alter its binding mode at the active site to allow oxidation of the other positions.[72]

There is also the potential for observing secondary isotope effects in our studies since a deuterium atom remains on the carbon atom undergoing oxidation. The presence of the label can increase or decrease the rate of the reaction if the rate determining step involves a change in hydridization at the substituted carbon atom. These effects are smaller than primary kinetic isotope effects, usually ranging between 0.7 and 1.5.[138] So if the rate determining step in the oxidation of 22 to 42 involves a change in hybridization of the 5'-carbon atom, α-secondary kinetic isotope effects may be observed. The formation of iminum ions from tertiary amines involves a number of steps that have hybridization changes. So it is likely that secondary as well as primary kinetic isotope effects may contribute to the overall reaction.

The observation that the (E)-5'-proton of 57 is removed to essentially the same degree as the (E)-5'-deuteron of 58 in the human microsomal incubations (see Table 8) establishes that primary deuterium isotope effects do not alter the stereochemical course of the oxidation of these substrates by these preparations. In fact, there is a slight

preference for hydrogen retention in these reactions suggesting an inverse isotope effect. This isotope effect suggests that a sp^2 to sp^3 hybridization change at the 5'-carbon atom takes place in a rate determining step.[138] The reaction of cyanide ion with the iminium ion is one possible step where the presence of an α-deuterium atom could affect the rate of product formation.

Although the stereoselectivity of this reaction as catalyzed by human microsomes is not influenced by primary isotope effects, the rate of product formation from 57 relative to 58 might be slowed if the C-H bond breakage was partially rate determining in the overall enzymatic process. This possibility was investigated in two ways. First, an approximation of the relative rate of oxidation of 57 and 58 to the cyano adducts 44a and 44b was obtained through the determination of the deuterium content of these products in incubations of 50:50 mixtures of the diastereomeric deutereonicotines. Since the stereochemical course of the oxidation is the same, half of the products of the oxidation of this mixture should be labeled if the two substrates are oxidized at the same rate. An enrichment of labeled 44a and 44b in these incubation mixtures should be indicative of a primary intermolecular isotope effect. The ratio of the labeled to unlabeled 5'-cyanonicotines in such a study was found to be 1.1 ± 0.0 (n=2). This result suggests that there is little effect of deuterium substitution in the pro-(S) 5'-position on the relative rate of product formation.

A second approach to this question was to determine the amount of the 5'-cyanonicotines formed from separate incubations of 57 and 58. Essentially equal amounts of the diastereomeric cyano adducts 44a and 44b were formed in incubations of both 57 or 58 (Table 9), confirming the absence of deuterium isotope effects in the rate of product formation in the human liver microsomal oxidation of 57 and 58.

Table 9. The amount of the 5'-cyanonicotine diastereomers formed in human liver microsomal incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 in the presence of NADPH, O_2, and sodium cyanide (n=2).

		ug 44a and 44b formed	57/58
Human II	57	3.0 ± 0.2	
	58	3.0 ± 0.1	1.0
Human III	57	5.1 ± 0.7	
	58	5.4 ± 0	1.1

The incubations were done for 15 mins. at 37° C. The amounts of 44a and 44b were determined using the internal standard 5'-cyanonicotine-N-d_3.

A third approach considered the deuterium content of the 5'-cyanonicotines formed during the competitive oxidation of unlabeled nicotine and nicotine-5',5'-d_2. This experiment provides a measure of the relative rates of C-H vs C-D bond breakage in the overall process. Since the stereochemical course was not influenced by isotope effects, the unlabeled nicotine represents 58 and nicotine-5',5'-d_2 represents 57. This approach, however, neglects the influence of any α-secondary isotope effects on the bond breakage step. This study was used only as a first approximation of intermolecular isotope effects. The deuterium composition of the oxidation products was determined by GC-EIMS analysis

of the trapped α-cyanoamines 44a and 44b. The rate of oxidation of the unlabeled material relative to the labeled substrate to 44a and 44b was determined by taking the ratio of the ion current at m/z 109 to the ion current at m/z 110 for the cyanoamines isolated from these incubation mixtures after correcting for the ^{13}C-isotope contribution of the mass ion 109 to mass ion 110. This ratio was corrected for the deuterium composition of the starting material by dividing it by the starting ratio of 22-d_0 to 22-5',5'-d_2 (1.02 ± 0.04 or 1.35 ± 0.08). As shown in Table 10, an average inverse isotope effect of 0.825 ± 0.37 was observed in one human microsomal preparation. While the data are only preliminary, they are suggestive of an inverse isotope effect and suggest that there may be a sp^2 to sp^3 conversion in a rate determining step involved with formation of 44a and 44b. One possible source of this isotope effect is in the reaction of cyanide ion with the metabolically generated iminium species 42.

Table 10. Intermolecular isotope effects determined by incubating a mixture of (S)-nicotine and (S)-nicotine-5',5'-d_2 with a human liver microsomal preparation.

Time	k_H/k_D
5 min	0.784 ± 0.120 (n=4)
15 min	0.818 ± 0.041 (n=2)
30 min	0.874 ± 0.015 (n=2)
average	0.825 ± 0.037

The incubations were conducted in the presence of NADPH, O_2, and 1 mM sodium cyanide at 37° C.

Deuterium isotope effects also lead to metabolic shifting if the enzyme has access to other oxidizable sites on the molecule. Evidence for the occurrence of metabolic shifting includes the increase in formation of another metabolite or formation of a new metabolite. The human liver microsomes catalyzed the oxidation of 22 highly regioselectively at the 5'-position as indicated by the detection of primarily 44a and 44b in the incubation mixture. Only trace amounts of N-cyanomethylnornicotine were observed. No significant differences were observed in the relative amounts of these metabolites present in the GC-EIMS tracings of human liver microsomal incubations of 57 and 58 (see Figure 42). The similarity in metabolic profile of 57 and 58 suggests that the site of oxidation in human liver preparations is unaffected by the configuration of the 5'-carbon atom.

The Sprague-Dawley rat and phenobarbital-pretreated New Zealand rabbit microsomal preparations were similar to the human microsomes in that the stereochemical course of the oxidation was not influenced by isotope effects (see Table 7). The Dutch rabbit preparations, on the other hand, demonstrated a slight preference for hydrogen versus deuterium atom abstraction in some of the preparations (see Table 5) indicating that the presence of a deuterium atom in the pro-(S) 5'-position alters the stereochemical course of the oxidation to a small degree.

The role of deuterium isotope effects in the Dutch rabbit liver microsomes was further investigated. Incubations of a 50:50 mixture of 57 and 58 with two Dutch rabbit liver microsomal preparations were conducted to see if the rate of 44a and 44b production from 57 and 58 was influenced by hydrogen isotope effects. The 5'-cyanonicotines isolated from these mixtures were enriched in deuterium suggesting that C-H

bond breakage proceeded faster than C-D bond breakage in the 5'-α-oxidation. The ratio of labeled to unlabeled product in these studies following ^{13}C-satellite corrections was 1.74 ± 0.4 as summarized in Table 11. These values suggest a primary intermolecular isotope effect for the Dutch rabbit liver microsomal 5'-α-carbon oxidation of 22.

Table 11. The ratio of labeled to unlabeled 5'-cyanonicotines formed in incubations of 50:50 nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 by Dutch rabbit liver microsomes in the presence of NADPH, O_2, and sodium cyanide.*

Rabbit	(44a-d_1 + 44b-d_1)/(44a-d_0 + 44b-d_0)
1	2.2 ± 0.3
2	1.4 ± 0.1
average	1.7 ± 0.4

*The data have been corrected for the ^{13}C-satellite contribution of m/z 109 to m/z 110 in the mass spectrum of 44a and 44b.

The possibility of isotope effects on the rate of 44a and 44b formation from 57 and 58 catalyzed by Dutch rabbit microsomes was also investigated by quantitating the amounts of the cyanoamines formed in separate incubations of 57 and 58 using the internal standard 5'-cyano-nicotine-N-d_3. The data are presented in Table 12. While the absolute amounts of 44a and 44b determined by this analytical assay are suspect, the relative amounts formed in the various incubations mixtures are trustworthy and demonstrate that more 5'-cyano adducts are formed in the incubations of 58 (14.6 nmoles) than in those of 57 (11.2 nmoles) after 5 minutes. Since the starting concentrations of the deutereo nicotine diastereomers were almost the same ([57]/[58]=0.97 as determined by

quantitative HPLC analysis), a ratio of the amount of 44a and 44b generated from 58 to the amount of 44a and 44b generated from 57 can be taken as an approximation of the isotope effect for the oxidation of the 5'-position. The ratio, 1.30, is consistent with the values obtained from the studies performed with a 50:50 mixture of 57 and 58 (see Table 12). These data are indicative of C-H bond breakage in a rate-determining step in the 5'-α-carbon oxidation of A by Dutch rabbit liver microsomal cytochrome P-450.

Table 12. The amounts of the 5'-cyanonicotines formed from nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 in one Dutch rabbit liver microsomal preparation (n=2).

Substrate	nmoles 44a and 44b	58/57
57	11.2 ± 0.8	1.3
58	14.6 ± 0.3	

The incubations were performed in the presence of NADPH, O_2, and 1 mM sodium cyanide for 5 minutes at 37° C. The amounts of 44a and 44b were determined by using the internal standard 5'-cyanonicotine-N-d_3.

Intermolecular isotope effects were also determined for the Dutch rabbit liver microsomes by measuring the deuterium content of the 5'-cyanonicotines generated from a 1:1 mixture of 22-d_0 and 22-5',5'-d_2 incubated in the presence of sodium cyanide and NADPH. After making corrections for the ^{13}C-satellite contribution of m/z 109 to 110 and for the isotope composition of the starting material, the ratio of unlabeled to labeled 5'-cyanonicotines indicated that the reaction had proceeded with no isotope effect (k_H/k_D=1.01 ± 0.03, Table 13). Nguyen also found no intermolecular isotope effect for the oxidation of 22 and 22-5',5'-d_2.[79] The observation of no isotope effect in these studies contrast

Table 13. Intermolecular isotope effects determined by incubating a mixture of (S)-nicotine and (S)-nicotine-5',5'-d_2 with Dutch rabbit liver microsomes in the presence of NADPH, O_2, and sodium cyanide.

Rabbit	k_H/k_D
I	0.96 ± 0.01
II	1.03 ± 0.01
III	1.04 ± 0.01
average	1.01 ± 0.03

with the small isotope effect observed in the studies with 57 and 58. The reason for this small difference is not clear.

As in the human liver microsomal studies, the metabolic profile of the Dutch rabbit liver microsomal incubations of 57 and 58 were essentially the same (see Figure 39). The similarity in the relative amounts of N-methyl and 5'-cyano adducts for both 57 and 58 strongly suggests that metabolic switching does not occur in these reactions.

5. The Importance of the Further Oxidation of the 5'-Cyanonicotines to Cotinine

A second concern with respect to our stereochemical conclusions was that the 5'-cyanonicotine diastereomers may be further metabolized to cotinine in a process with a large isotope effect. This possibility was investigated in order to determine its influence on the observed deuterium content of the cyanoamines. Since the microsomes were washed, only traces of soluble oxidases capable of catalyzing the oxidation of the iminium ion to cotinine should remain. Furthermore, such residual activity should be inhibited by the presence of cyanide ion.[53] However, the cyanoamines may serve as substrates for cytochrome P-450 as has been

observed for other α-aminonitriles.[82,83] In our mass spectral assay, cotinine would not be observed since it does not give a major fragment at m/z 109, 110 or 111. In addition, cotinine is not readily soluble in ether so it would not be present in our extracts. This metabolite is extractable with dichloromethane and GC analysis of dichloromethane extracts of the human liver incubation mixtures demonstrated low levels of cotinine. Quantitative determination of the cotinine by the method of Jacob and co-workers[113] demonstrated that only about 5% of the 5'-cyanonicotines were further converted to cotinine (Table 14).

Table 14. The levels of cotinine detected in human liver microsomal incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 in the presence of NADPH, O_2, and sodium cyanide (n=2).

		44a + 44b (nmoles)	43 (nmoles)	% conversion of 44a + 44b to 43
Human I	57	n.d.	0.80 ± 0.07	
	58	n.d.	0.84 ± 0.01	
Human II	57	16.0 ± 0.1	0.32 ± 0.03	2.0%
	58	16.0 ± 0.1	0.31 ± 0.05	1.9%
Human III	57	27.3 ± 3.7	1.2 ± 0.2	4.2%
	58	28.9 ± 0	1.2 ± 0	4.0%

The incubations were done for 15 minutes at 37° C. The amounts of 44a and 44b were determined using 5'-cyanonicotine-N-d_3. Cotinine analysis were determined by the method of Jacob et al.

If this oxidation were accompanied by a primary isotope effect, it may influence the observed ratio of labeled to unlabeled α-cyanoamines in the stereochemical studies. A primary isotope effect would lead to a slower oxidation of 44a-5'-d_1 and 44b-5'-d_1 which would lead to a larger

percentage of deuterium retention in the trapped intermediates. As was observed with the 5'-cyanonicotines, essentially equal amounts of cotinine were formed in these incubations with both 57 and 58 as substrates (Table 14). These data suggest that an isotope effect does not operate in the oxidation of the 5'-cyanonicotines to cotinine.

The effect of this oxidation pathway on the observed deuterium content of the cyanoamines was determined by measuring the deuterium content of the cyanoamines (0.05 mM) before and after incubation with human liver microsomes in the presence of the required cofactors and 1.0 mM sodium cyanide. Controls were conducted in which the cyanoamines were incubated in the absence of the NADPH regenerating system and also in the absence of microsomes. The data are summarized in Table 15. These results suggest that the further oxidation of the diastereomeric 5'-cyanonicotines is dependent on NADPH and does not significantly affect the observed ratio of labeled to unlabeled 5'-cyanonicotines despite the fact that a significant amount of the starting material is oxidized to cotinine.

Table 15. The effect of oxidation of the 5'-cyanonicotine diastereomers to cotinine by human liver microsomes on the deuterium content of the cyanoamines.

Conditions (min)	m/z 110/109	Cotinine ng/ml	%Conversion to 43
30-NADPH	0.788 ± 0.072	463.4 ± 5.1	5.27%
5	0.904 ± 0.003	732.6 ± 11.2	8.3%
10	0.878 ± 0.081	1024.9 ± 34.5	11.6%
15	0.758 ± 0.008	1258.6 ± 46	14.3%
30	0.881 ± 0.012	509.6 ± 35	17.2%
30-mics	0.795 ± 0.015		

The incubations were performed in the presence of 1 mM sodium cyanide at 37 °C.

The importance of the further oxidation of 44a and 44b to cotinine also was determined for the Dutch rabbit liver microsomal studies. Approximately 7% of the 5'-cyanonicotines was further oxidized to cotinine after 5 minutes in one Dutch rabbit liver preparation (Table 16). The amount of cotinine and 44a and 44b formed in incubations of 22 and 22-5',5'-d_2 were also determined for two different Dutch rabbit microsomal preparations (Table 16). These data suggest that the further oxidation of 44a and 44b to cotinine is a minor reaction in these preparations.

Table 16. The levels of cotinine and 5'-cyanonicotines detected in Dutch rabbit liver microsomal incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 in the presence of NADPH, O_2, and sodium cyanide.

Rabbit	Substrate	time (min)	44a and 44b (nmoles)	43 (nmoles)	% conversion of 44a and 44b to 43
V n=2	57	5	8.05 ± 0.6	0.62 ± 0.04	7.2%
V n=2	58	5	12.1 ± 0.3	0.72 ± 0.05	5.6%
V n=3	22:22-d_2	5	9.1 ± 0.8	0.73 ± 0.06	7.4%
IV n=2	22:22-d_2	30	28.9 ± 0.6	4.4 ± 0.2	13.2%

A comparison of the relative amounts of cotinine and 5'-cyanonicotines in the incubation mixtures of 57 and 58 revealed a small isotope effect in the oxidation of 5'-cyanonicotine to cotinine by the Dutch rabbit liver microsomes. While there was slightly more cotinine formed in incubations containing 57 than 58, a larger percentage of the 5'-cyanonicotines was converted to cotinine in the incubations containing 58 than those containing 57. Since 58 produces more unlabeled 44a and 44b than 57, these observations suggest that the unlabeled cyanonamines

are more rapidly oxidized to cotinine than the labeled compounds, consistent with a primary isotope effect in this oxidative pathway.

The further oxidation of a 1:1 mixture of unlabeled and 5'-deutero-5'-cyanonicotines to cotinine by Dutch rabbit microsomes had no effect on the deuterium composition of the α-cyanoamines as demonstrated by the data presented in Table 17. Thus, the slight tendency of deuterium retention in the diastereoselectivity exhibited by Dutch rabbit microsomes is most likely due to a small isotope effect in the first 2-electron oxidation of 22 and not in the subsequent transformation of the 5'-cyanonicotines to cotinine. Determination of the magnitude of this isotope effect would require a detailed kinetic analysis of the oxidation of 57 and 58. In addition, one would need to study the kinetics with the tritiated analogs and apply the methods of Northrup[139] to determine the intrinsic isotope effect in this oxidation. Most important to our studies is that, despite the observation of these deuterium effects and the observation of the further oxidation of the 5'-cyanonicotines to cotinine, these phenomena do not influence the diastereoselectivity observed in this enzymatic reaction.

Table 17. The effect of further oxidation of the 5'-cyanonicotines to cotinine on the deuterium content of these cyanoamines by Dutch rabbit microsomes (n=2).

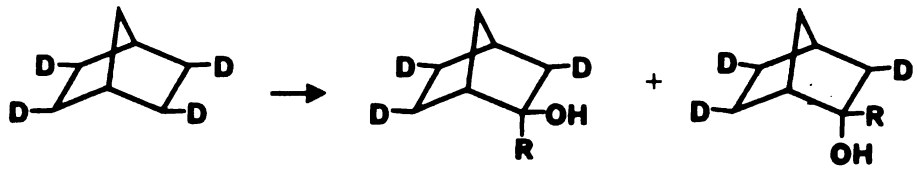
Conditions	m/z 110/109	Cotinine ng/ml	% Conversion
30-mics	0.926 ± 0.037	49.3 ± 3.9	0.7%
30-NADPH	0.981 ± 0.002	377.1 ± 11.9	4.3%
5+NADPH	0.922 ± 0.014	964.8 ± 11.2	11.2%
10+NADPH	0.917 ± 0.024	1013 ± 17.3	11.5%
15+NADPH	1.02 ± 0.058	1837 ± 120	20.9%
30+NADPH	0.894 ± 0.022	1871 ± 273.0	21.3%

The incubations were done in the presence of 1 mM sodium cyanide at 37° C.

G. Preliminary Conclusions

These studies demonstrate that the cytochrome P-450 catalyzed oxidation of (S)-nicotine to the iminium ion 42 (or an isoelectronic species) proceeds with the selective loss of the (E)-5'-hydrogen atom observed in human and rodent liver microsomal incubations containing 57 or 58. The reaction proceeds with approximately 90% loss of the (E)-5°-proton/deuteron of either 57 or 58. The diastereoselectivity of this biotransformation was not influenced by deuterium isotope effects since essentially the same amount of deuteron loss occurs during the oxidation of 57 as proton loss during the oxidation of 58. Furthermore, the presence of deuterium in the pro-(S) 5'-position did not result in metabolic shifting in the human studies to any other α-carbon atoms since the diastereomeric 44a and 44b were the only cyano adducts observed. The exclusive formation of 44a and 44b was independent of the configuration at the 5'-position. In addition, there were small amounts of the 4-electron oxidation product cotinine detected in the incubation mixtures. However, these levels were low and this reaction was shown to have little influence on the observed ratio of labeled to unlabeled 44a and 44b in the stereochemical studies.

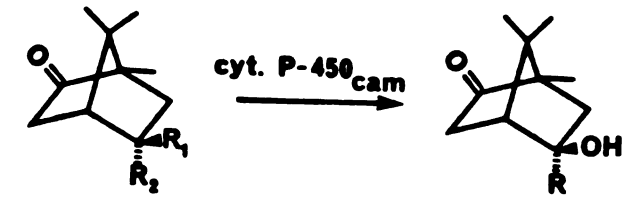
The demonstration that the oxidation of (S)-nicotine proceeds with stereoselective abstraction of the pro-(S) hydrogen atom suggests that the mode of binding at the enzyme's active site is an important determinant in the stereochemical course of this oxidation. This report contrasts to observations described for the oxidation of norbornane by reconstituted rat P-450[62] and camphor by $P\text{-}450_{cam}$.[66] The exo-hydroxylation of norbornane-exo,exo,exo,exo-2,3,5,6-d_4 (48) by reconstituted rat liver cytochrome P-450 proceeded with 75% removal of the exo-hydrogen



48 49 R=H or D R=H or D

atom in the oxidation to exo-norbornanol (49).[62] This reaction, however, was accompanied by a large isotope effect as evidenced by the shift in the exo to endo product ratio from 3.4:1 to 0.76:1 upon deuterium substitution of the exo-position. This observation suggests that this molecule is positioned such that the oxidizing agent at the active site has access to both the endo and exo hydrogen atoms. Under normal conditions, the reaction occurs selectively at the exo site; however, it will proceed at the endo site as a result of deuterium substitution of the 5'-exo-position.

The removal of the exo and endo-hydrogen atom during the 5-exo-hydroxylation of camphor by P-450$_{cam}$ could be influenced by deuterium substitution. Studies with camphor-5-exo-d_1 (56a) and camphor-5-endo-d_1 (56b) demonstrated that the choice of which hydrogen atom was removed was determined by deuterium isotope effects and not stereochemical effects. These observations suggest that the species responsible for the proton abstraction had access to both protons.[66]



56a R_1=D; R_2=H
56b R_1=H; R_2=D

55 R=H or D

Our observations indicate that either there is no significant isotope effect in the oxidation of the 5'-position of (S)-nicotine or that the stereochemical and regiochemical course of the reaction is determined upon the binding of 22 to the active site of cytochrome P-450. Furthermore, since the 5'-hydrogen atoms are diastereotopically related, they will probably react with oxidizing agents through different transition states. This might mask the intrinsic isotope effect of the reaction.[77]

The variations between the individual preparations suggest that the stereoselectivity of the enzymatic reaction is not just controlled by pure chemical differences between the pro-(R) and pro-(S) 5'-protons. The small inter- and intraspecies differences in the selectivity of the reaction may be due to variations in isozyme composition of the preparations and in the active site requirements for the various isozymes responsible for nicotine oxidase activity. One would have to study the stereo- and regioselectivity with purified enzymes from both species in order to distinguish between these two possibilities.

Not much is known about the structural characteristics of the active site of membrane-bound cytochrome P-450. Jerina *et al.* have mapped out a stereochemical model for the active site of cytochrome P-450c as a result of their studies on the enantiomeric composition of polycyclic aromatic hydrocarbon metabolites.[140] These studies, along with the stereo- and regioselectivity observed in steroid[126] and warfarin[127] oxidation reactions by a number of liver cytochrome P-450 isozyme, suggest that the cytochrome P-450 isozymes have well-defined active sites. X-ray crystal structures of $P\text{-}450_{cam}$ in the presence of camphor have shown that this substrate binds such that the 5-exo-position is closest to the heme-iron.[141] This manner of binding can

explain the stereospecificity of oxygen addition but not the lack of selectivity in hydrogen abstraction. This difference in stereoselectivity of the two reactions has led to the proposal that the moiety responsible for hydrogen abstraction is different from that which adds the oxygen atom.[66]

The similarity in the amount of diastereoselectivity of the reaction exhibited by microsomes obtained from a number of mammalian species suggests that the loss of the pro-(S) hydrogen atom is a general characteristic of the cytochrome P-450 isozymes responsible for this reaction. Consistent with the observation that the less hindered proton is lost during this reaction is the proposal that the molecule binds at the active site with the bulky pyridine group pointed away from the planar porphyrin ring of the mono-oxygenase. In this scenario, the pro-(S) 5'-position is most accessible to the proposed iron-oxene species. The observed variations in stereoselectivity may then be explained, in part, by slight variations in the nature of binding of 22 at the active site that change the acessibility of the pro-(R) 5'-hydrogen atom.

Chapter III

Stereochemical Studies of the α-Carbon Oxidation of (S)-Nicotine by Model Systems

A. Background.

In the previous section we described studies demonstrating that the cytochrome P-450 catalyzed oxidation of the 5'-position of (S)-nicotine proceeds with stereoselective abstraction of the pro-(S) proton. The high degree of regio- and stereoselectivity exhibited by this enzymatic reaction suggests that the process is strongly controlled by the enzyme. In order to understand more fully the events taking place at the enzyme's active site, we examined the stereochemical course of the α-carbon oxidation of (S)-nicotine by a variety of model oxidizing systems.

The models were chosen on the basis of their ability to probe various aspects of the cytochrome P-450 catalytic mechanism. Many investigators have studied this family of enzymes. There is a general agreement that the stiochiometry of the overall reaction is as follows:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$$

The activation of dioxygen to a reactive species, such as the electron deficient oxene (:Ö:), is a major feature of the reaction. The current view, as outined in Figure 43, involves the binding of the substrate to a hydrophobic site in the region of the oxidized (Fe^{3+}) iron-containing heme group.[17] The iron is then reduced to Fe^{2+} in a step catalyzed by the NADPH-requiring cytochrome P-450 reductase. This reduced form of cytochrome P-450 accepts dioxygen as an axial ligand and a second electron is transferred to this system either from the reductase or some-

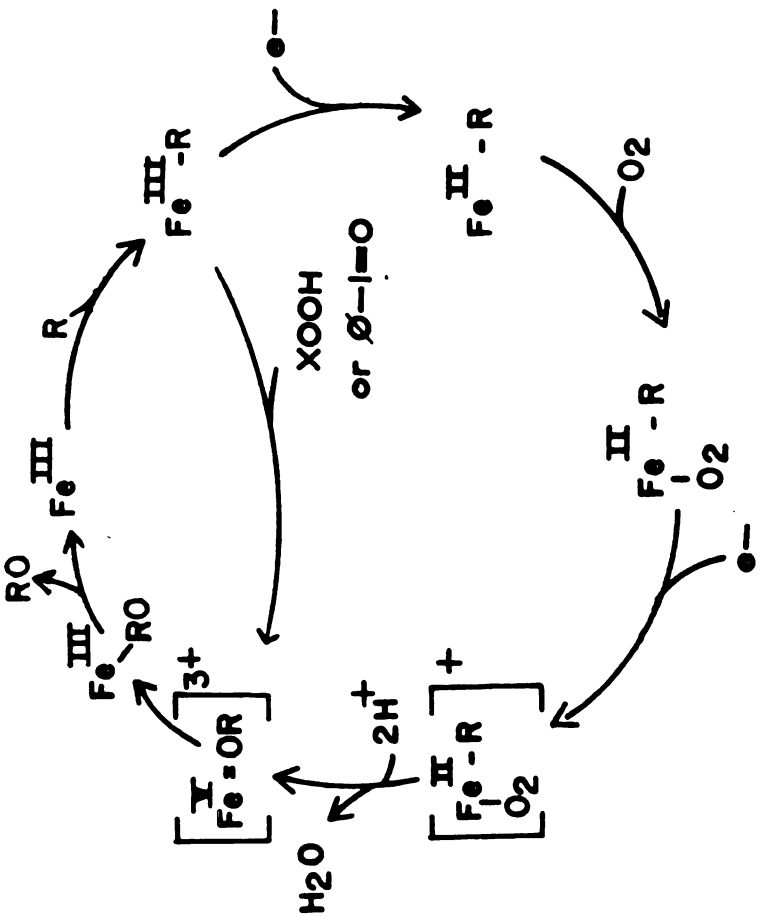


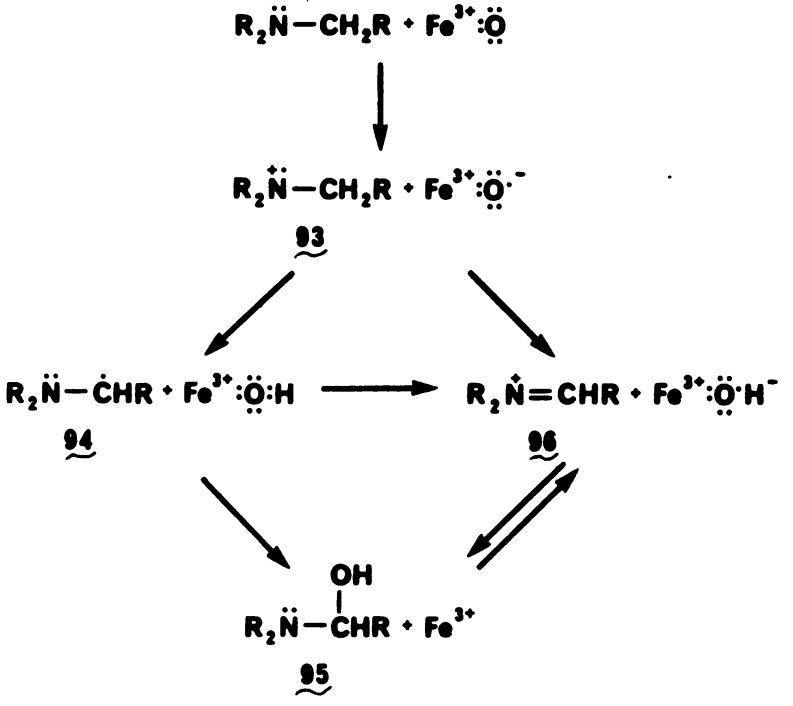
Figure 43. The proposed pathway for the cytochrome P-450 catalytic cycle.[17]

times from cytochrome b_5. The reduction generates a species that may undergo oxygen-oxygen bond cleavage to produce one mole of water and an active oxygen species. The active oxygen species is believed to be a formal perferryl oxygen complex, i.e. $Fe^{V}=O$ or $(FeO)^{3+}$. This is the proposed species responsible for oxidation of the substrate.[17]

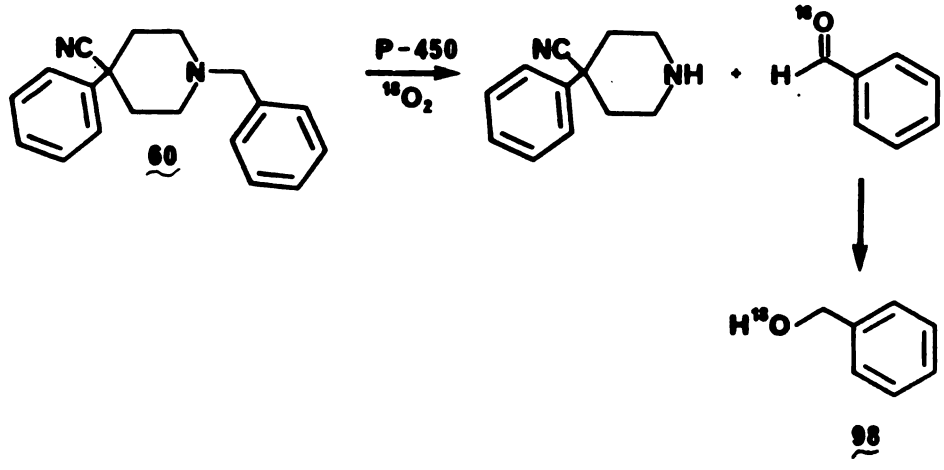
Cytochrome P-450 also catalyzes the oxidative metabolism of substrates in the absence of NADPH and dioxygen when organic oxidants are present in the incubation mixtures. These compounds include peroxides, peracids and compounds bearing a single oxygen atom such as iodosobenzene and a number of N-oxides.[17] They form a reactive iron-oxo species directly, bypassing activation steps required by O_2.

The precise chemical mechanism for substrate oxidation is unknown. Intramolecular isotope effect studies have revealed that primary kinetic isotope effects of P-450 mediated aliphatic,[62,142] and benzylic[70] hydroxylations and O-dealkylations[67,71,72] are greater than 7. These data have been used as evidence for a rate determining hydrogen atom abstraction step in the oxidation of such structural types resulting in the direct formation of a carbon centered radical. Similar studies of α-carbon oxidation reactions of tertiary amines have yielded significantly smaller deuterium isotope effects ($k_H/k_D<3$ as discussed in Section II.1)[19,20,61,73,74] suggesting that this reaction type proceeds via a different mechanism. Mechanisms involving intermediary N-oxide formation,[18] oxene insertion,[12] intermediate aminium radical formation[12,19-22] and stabilization of the transition state by the nitrogen atom's lone electron pair[73] have been proposed.

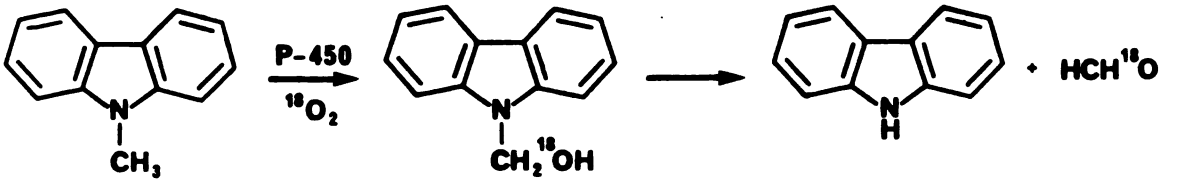
The majority of the evidence as reviewed below supports an initial transfer of one electron from the substrate's nitrogen atom to the active oxygen species in the activated P-450 complex resulting in the formation of a bound superoxide radical anion (92) and an amine radical cation (93). The subsequent fate of the aminium radical is unknown. One possibility involves loss of a proton to form a neutral radical (94). The α-amino-alkyl radical then can either combine with the enzyme-bound oxygen moiety to form the carbinolamine 95 or it can lose another electron to generate the iminium ion 96. Alternatively, the aminium radical 93 can lose a hydrogen atom directly to generate the iminium species 96 in a single step. The iminium ion can either be released directly or combine with the hydroxide ion generated during the reaction and be released as the corresponding carbinolamine 95. The carbinolamine then could be in rapid equilibrium with the iminium ion in solution.



Support for the enzymatic formation of carbinolamines has been sought with $^{18}O_2$ studies. The instability of the intermediate carbinolamines makes it difficult to determine the source of oxygen since these compounds rapidly decompose to the corresponding carbonyl and secondary amine products. Subsequent exchange of the carbonyl oxygen with water results in the loss of the ^{18}O-label. McMahon et al. circumvented this problem by reducing the benzaldehyde (97) formed during the microsomal oxidation of 1-benzyl-4-cyano-4-phenylpiperidine (60) to benzyl alcohol (98) by alcohol dehydrogenase.[74] When the incubations were done in an $^{18}O_2$ atmosphere, 78% of the alcohol was labeled with ^{18}O. This experiment demonstrated that the oxygen source was molecular oxygen and supports the formation of a carbinolamine prior to substrate release from the enzyme's active site.

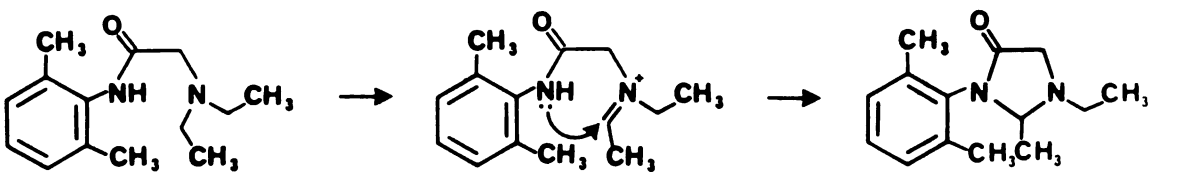


^{18}O-Incorporation also has been observed when the carbinolamine intermediate is stable enough to be isolated and examined directly. For example, the arylamine N-methylcarbazole (99) is oxidized by cytochrome P-450 to N-hydroxymethylcarbazole (100). Experiments with $^{18}O_2$ demonstrated that the oxygen in the product carbinolamine is derived from dioxygen by the incorporation of >95% ^{18}O in the metabolite.[143] In addition, no labeled product was observed when the reaction was supported by NADPH and O_2[143,144] or a number of peracids in $H_2{}^{18}O$.[59,144] These studies suggest that carbinolamines are important intermediates in the enzymatic reaction of these weakly basic amines. Nevertheless, they do not rule out the initial formation of an iminium ion at the active site which then reacts with the hydroxyl ion generated from dioxygen prior to substrate release.



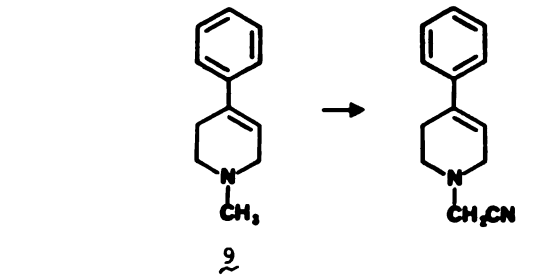
99 100

Evidence for a role of iminium ions in α-carbon oxidations has been derived from a number of experiments. The isolation and characterization of the intramolecular cyclization product 17 from microsomal incubations of lidocaine (16) suggests the intermediacy of the iminium ion

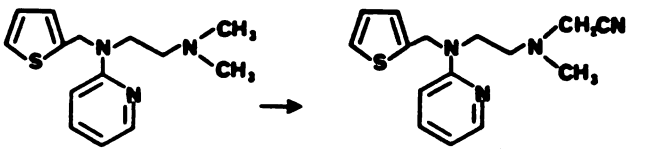


16 18 17

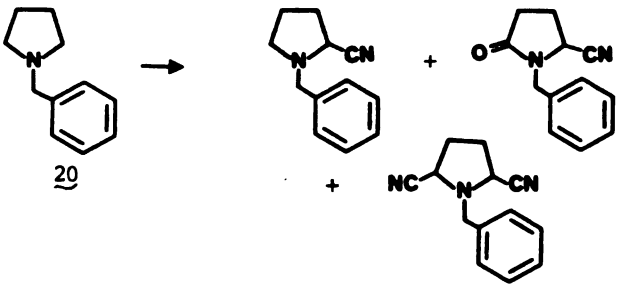
18.[27] Indirect evidence for the formation of iminium ions during the oxidative metabolism of MPTP (9),[32] methapyrilene (19),[26] 1-benzylpyrrolidine (20),[27] phencyclidine (21),[28] and (S)-nicotine (22)[29-31] has been provided through the characterization of the corresponding α-cyanoamines isolated from microsomal incubations containing the required



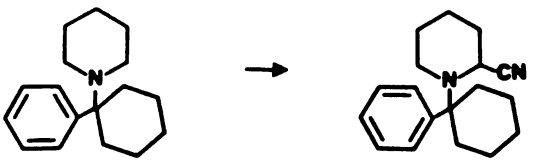
9



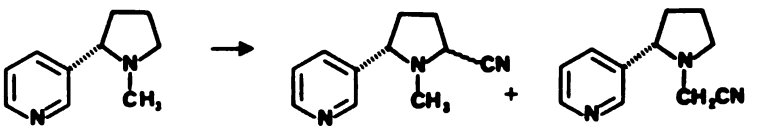
19



20

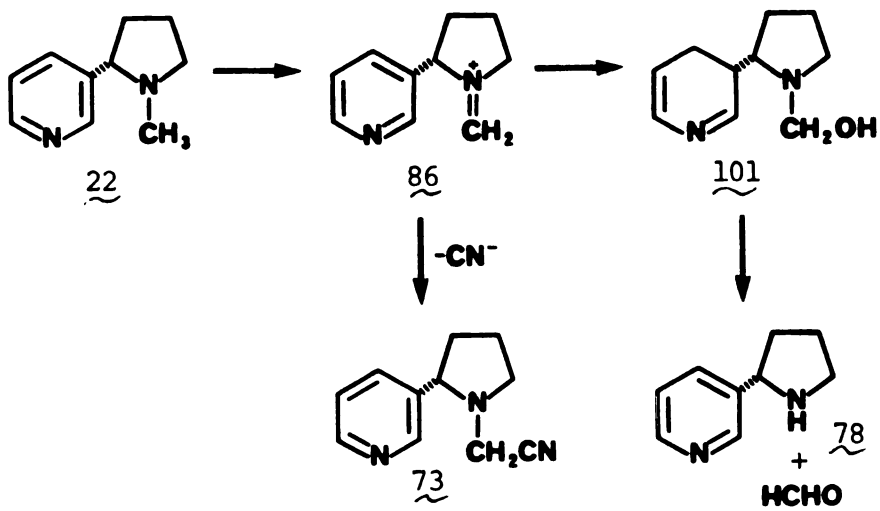


21

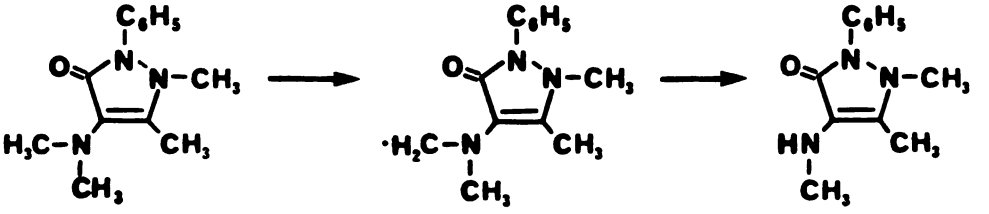


22

cofactors and sodium cyanide. Deuterium labeling studies have demonstrated that the N-methyleneiminium ion (86) is a discrete intermediate in the N-demethylation of 22 to nornicotine (78) and formaldehyde.[30] While the iminium ion will be in rapid equilibrium with N-hydroxymethylnornicotine (101), one might expect that the carbinolamine might more readily decompose to the dealkylation products if it is directly released as such from the enyzme.

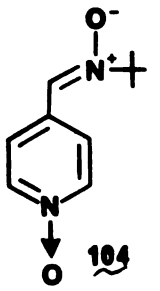


The involvement of free radicals in the α-carbon oxidation of amine substrates has been derived from studies with aryl amines. The ability of these compounds to stabilize free radical intermediates has allowed the detection of these reactive intermediates. A stable aminopyrine free radical (102) was detected both spectrophotometrically and by electron paramagnetic resonance (EPR) spectroscopy during the cumene hydroperoxide-supported N-demethylation of aminopyrine (103) by phenobarbital-pretreated rat liver microsomes.[22] The role of cytochrome P-450 in this process was demonstrated by the inhibition of radical formation by the cytochrome P-450 inhibitor metyrapone and by known cytochrome P-450 substrates.[22] This same radical is formed during the electrochemical oxidation of aminopyrine,[145] a reaction generally believed to proceed via initial one electron oxidation of the nitrogen atom.[146] This similarity supports a common mechanism for these two processes.



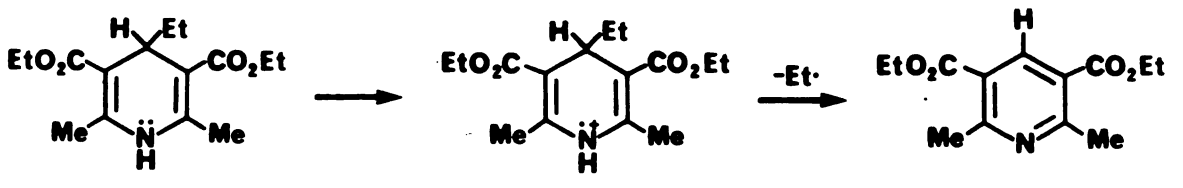
103 102

Further evidence for a one-electron oxidation sequence in the biotransformation of nitrogen-containing compounds was provided by Augusto and co-workers in their investigations of the suicide inactivation of cytochrome P-450 by a number of 4-substituted-3,5-bis(ethoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridine substrates.[147] Spin trapping studies



104

with α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (104) established that an ethyl radical was generated during the cytochrome P-450 mediated oxidation of 4-ethyl-3,5-bis(ethoxycarbonyl)-2,6-dimethyl-1,4-dihydropyridine (105). The formation of this radical implies an initial abstraction of one electron from the nitrogen atom in this reaction sequence.[147]



105

The intermediacy of aminium radicals in the oxidative transformation of amines also has been derived from studies on the suicide inactivation of cytochrome P-450 by N-benzylcyclopropylamines.[21,148-150] Radiolabeling and structure activity studies demonstrated that the cyclopropyl group was required for the mechanism-based inactivation.[148] The observation that both cyclopropylbenzylamine (106) and (1-methylcyclopropyl)benzylamine (107) were equally able to inhibit cytochrome P-450 in a dose and time dependent fashion led to the proposal of a radical mechanism for the inhibition process.[21,149] As shown in Figure 44, the first step involves the initial removal of one electron from the nitrogen atom to form an aminium radical 108. Rapid ring opening of the

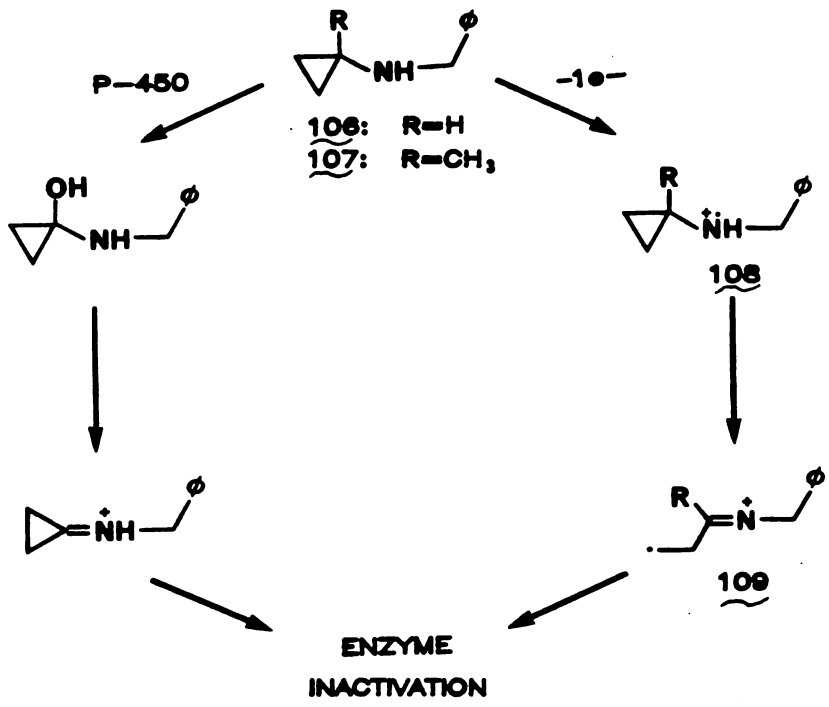
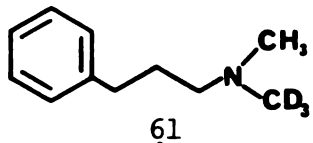


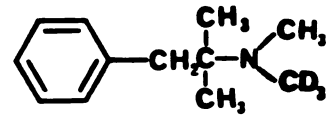
Figure 44. Proposed mechanism for the inactivation of cytochrome P-450 by N-benzylcyclopropylamines.[21,149]

cyclopropyl group at the enyzme's active site generates a highly reactive carbon-centered radical (109), which then inactivates the enzyme. While the involvement of a reactive iminium ion in the case of 106 cannot be ruled out, the equal effeciency of inactivation of 106 and 107 suggests a common mechanism. Since the methyl analog does not permit formation of an iminium species, iminium intermediates may not be involved in the inactivation step.[21,149] A recent report from the Guengerich laboratory demonstrated a high degree of correlation between the rate of inactivation of cytochrome $P\text{-}450_{PB\text{-}B}$ by a series of heteroatom-substituted cyclopropanes and the substrate's single-electron oxidation potential ($E_{\frac{1}{2}}$). These observations also support a one-electron reaction mechanism.[150]

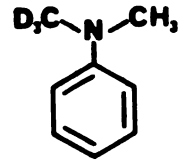
The intermediacy of amine radical cations in the cytochrome P-450 catalyzed oxidation of tertiary aliphatic and aryl amines also is suggested by the modest intramolecular isotope effects observed for these enzyme-mediated reactions: 1.3 for the N-demethylation of 1-(N-methyl-N-trideuteromethylamino)-3-phenylpropane (61) by phenobarital pretreated and untreated rats,[60] 1.45 for the same oxidation catalyzed by mouse liver microsomes,[60] 1.6-2.0 for the N-dealkylation of N-trideuteromethyl-N-methylphentermine (62a) by reconstituted cytochrome P-450[61] and 1.7-3.1 for the demethylation of N-methyl-N-trideuteromethylaniline (110) by two cytochrome P-450 isozymes.[20,59]



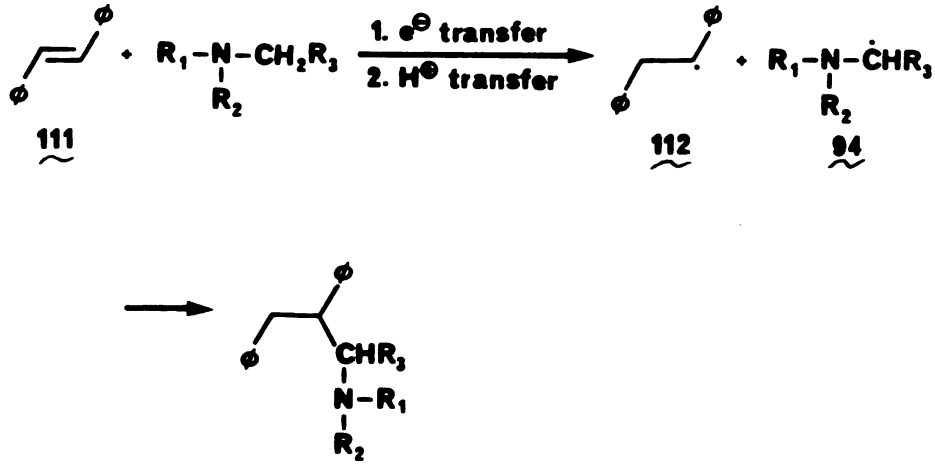
61



62a

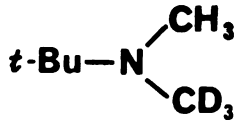


110

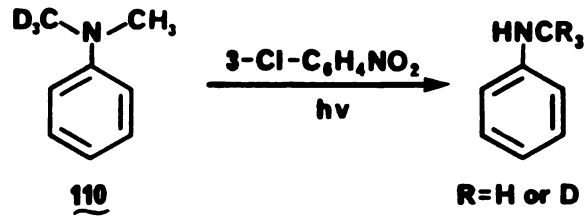


111 112 94

These values are similar to those reported for chemical processes generally believed to proceed via initial one electron abstraction from the nitrogen atom. The photochemical reaction of tertiary amines with singlet trans-stilbene (111) involves electron transfer from the amine to stilbene.[151] A proton then is lost to generate a carbon-centered radical (94) that combines with the stilbene radical (112). Chemical analysis of the addition product generated with N-methyl-N-trideuteromethyl-t-butylamine (113) demonstrated that the reaction occurred preferentially at the unlabeled carbon atom. The product ratio revealed a kinetic isotope effect of 2.2 for the proton loss step.[151]

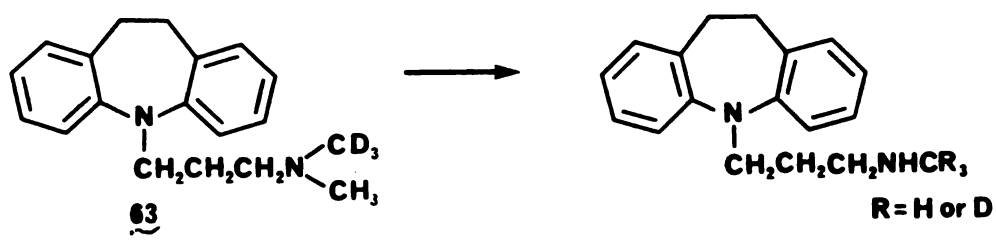


113



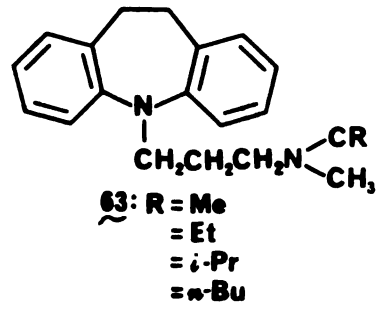
110

The 1-chloro-4-nitrobenzene sensitized photochemical oxidation of N-methyl-N-trideuteromethylaniline (110) exhibited solvent-dependent isotope effects of 3.5, 2, and 2.47 for the reaction in benzene, cyclohexane, and acetonitrile, respectively.[152] These modest values suggest that the abstraction of an electron from the nitrogen atom, not abstraction of a hydrogen atom, is the rate-determining step in the reaction. The electrochemical and microsomal catalyzed oxidation of N-methyl-N-trideuteriomethylimipramine (63) revealed small isotope effects: k_H/k_D=1.88 and 1.64, respectively.[19] These observations led Shono et al. to conclude that a common mechanism was operating in these two reactions.

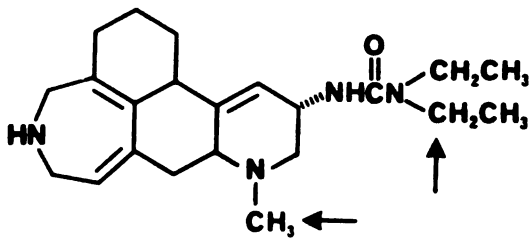


63

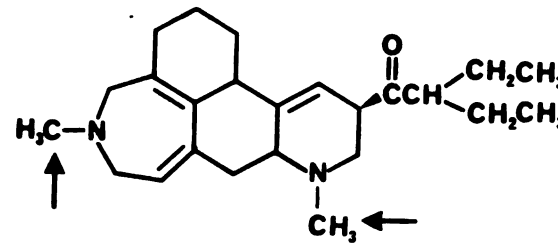
Further evidence for an electron transfer process from nitrogen in the microsomal oxidation of tertiary amines comes from the similarity between the regioselectivity of anodic and microsomal N-dealkylation reactions.[19] A comparison of the selectivity in N-demethylation versus



63: R = Me
= Et
= i-Pr
= n-Bu

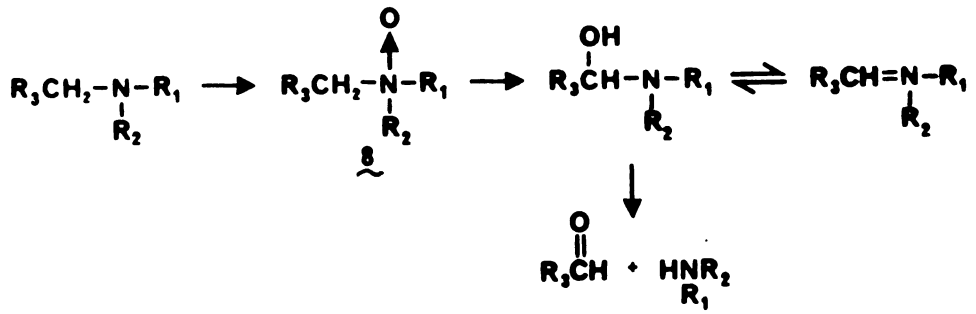


114

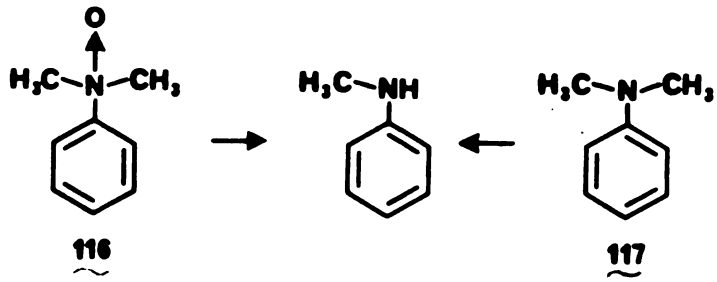


115

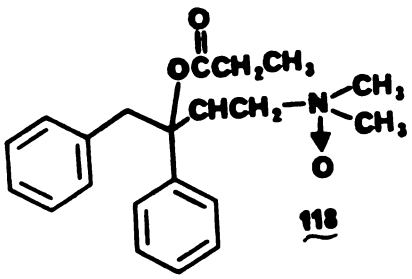
N-dealkylation of a series of N-alkyl-N-methyl derivatives of imipramine (63) demonstrated that the elimination of the methyl group was always more rapid than dealkylation of higher alkyl groups in both the electrochemical and enzymic oxidation of these compounds.[19] The degree of preferential demethylation in the microsomal reactions of a number of drugs including lisuride (114) and methysergide (115) paralleled that observed in the electrolysis experiments.[153] The agreement between the selectivity of these two oxidation processes is consistent with a common reaction mechanism.



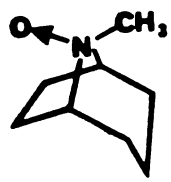
An obligatory role for N-oxide intermediates (8) in oxidative N-demethylation reactions of tertiary amines was proposed by Ziegler and Pettit in the mid 1960's.[131] This hypothesis was based on the observation that N,N-dimethylaniline N-oxide (116) was demethylated several times more rapidly in rat and pig liver microsomes than N,N-dimethylaniline (117) itself.[154] The N-oxides, which are formed in a flavin-dependent N-oxidase catalyzed reaction, undergo demethylation by cytochrome P-450.[131] An enzyme system capable of carrying out the N-dealkylation of a number of N,N-dialkylarylamine N-oxides has been characterized and has similar properties to cytochrome P-450.[155,156] However, the generality of this mechanism has been questioned.[157]



The majority of available evidence argues against the intermediacy of N-oxides in the α-carbon oxidation of tertiary aliphatic amines.[158] Several tertiary amine N-oxides are poor substrates for the dealkylation pathway. For instance, levopropoxyphene N-oxide (118) was not demethylated *in vitro*.[159] Furthermore, the N-demethylation of cocaine N-oxide

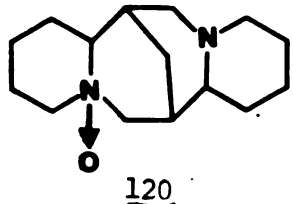


118

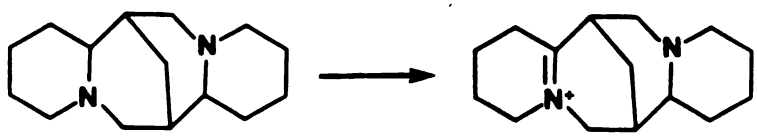


119

(119) was only a minor *in vivo* pathway in rats.[160] The polarity of these compounds may prevent them from interacting with the membrane-bound enzyme. However, a recent study demonstrated that sparteine N-oxide (120) was unable to generate the iminium ion (121) observed during the microsomal oxidation of sparteine (122) despite the ability of the N-oxide to perturb the visible spectrum of microsomal P-450.[161] Further

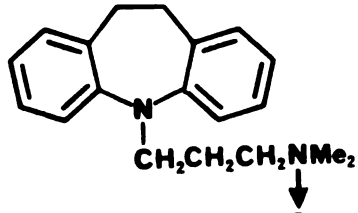


120



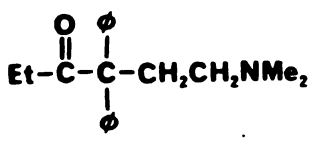
122 121

evidence that N-oxidation is a minor pathway in *in vitro* α-carbon oxidations is the observation that impramine-N-oxide (123) is reduced much

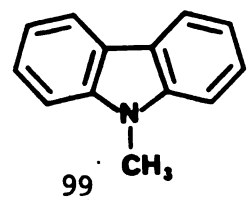


123

faster than it is dealkylated.[162] Furthermore, the N-oxidase inhibitor dithiothreitol prevents guinea pig microsomal normethadone (124) N-oxidation without affecting the overall rate of demethylation.[163] Additionally, there are amines such as methylcarbazole (99) that cannot undergo N-oxidation but are N-dealkylated in supplemented liver microsomes.[164] All these findings suggest that N-oxidation is not an obligatory pathway for the N-dealkylation of tertiary amines. However, they do not explicitly rule out the formation of an intermediate N-oxide at the active site that is N-dealkylated prior to release from the enzyme.



124



99

We have employed the monodeutereo diastereomers 57 and 58 to determine the stereochemical course of the oxidation of 22 by a number of model systems to gain more information about the cytochrome P-450 catalyzed reaction. Iodosobenzene and cumene hydroperoxide were substituted for NADPH and dioxygen to probe the molecular mechanism of oxidation. The tetraphenylporphyrin-iodosobenzene system and hemoprotein models including hemoglobin, horseradish peroxidase, and chloroperoxidase were used to determine the role of the environment of the heme iron in determining the stereochemical course of the enzymic process. Finally, the chemical mechanism of the cytochrome P-450 mediated reaction was explored by comparing the stereochemical course of nicotine oxidation in several chemical systems generally thought to proceed through aminium

radical intermediates with the data obtained from the enzyme studies. The information gained from all the models was the used to draw conclusions about how cytochrome P-450 directs the regio- and diastereoselectivity of (S)-nicotine oxidation.

B. Iodosobenzene- and Cumene Hydroperoxide-supported Cytochrome P-450 Catalyzed Oxidation of (S)-Nicotine.

1. Background.

In the catalytic cycle of cytochrome P-450, dioxygen is activated to a reactive oxygen species capable of carrying out oxidation reactions. Much controversy has surrounded the nature of this species. Dioxygen is believed to be reduced to a peroxide-related intermediate 125 at the heme active site which then undergoes O-O bond cleavage to generate the active oxygen species.[17] The O-O bond splitting can undergo one of at least two pathways as shown in Figure 45. The first, an "oxenoid" mechanism, involves heterolytic cleavage of the O-O bond (pathway A, Figure 45). This mechanism results in oxygen atom donation to iron(III) to generate a ferryl complex 126.[17,165] Alternatively, the peroxide bond can undergo homolytic cleavage in a "quasi-Fenton" reaction to generate an oxy radical 127 and a ferric hydroxide complex 128. In carbon hydroxylation reactions, the oxy radical has been proposed to abstract a hydrogen atom from the substrate. The resulting carbon radical 129 then recombines with the ferric oxy-heme complex to generate the product and the oxidized enzyme.[17,165]

Oxygen Activation: $S^- Fe^{2+}$ O-O $\longrightarrow$ $S^- Fe^{3+}$ O-O^- (125)

Pathway A: $S^- Fe^{3+}$-O-O-R' + RH $\xrightarrow[\text{cleavage}]{\text{hetereolytic}}$ $S^- Fe^{3+}$:Ö: (126) + RH + R'O^-

$\longrightarrow$ $S^- Fe^{3+}$ + ROH

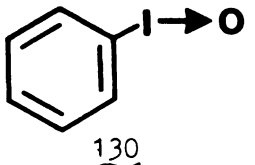
Pathway B: $S^- Fe^{3+}$-O-O-R' $\xrightarrow[\text{cleavage}]{\text{homolytic}}$ S$^\bullet$ Fe^{3+}:Ö:$^-$ (127) + $^\bullet$OR' (128) + RH

R'O$^\bullet$ + RH $\longrightarrow$ R$^\bullet$ + R'OH (129)

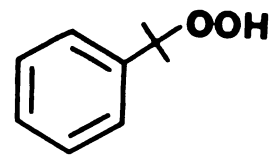
S$^\bullet$$Fe^{3+}$:Ö:$H^-$ $\longrightarrow$ S^-Fe^{3+} + ROH

Figure 45. Two possible pathways for O-O bond cleavage.[17]

The discovery that a number of exogenous oxidants can replace NADPH, the reductase, and dioxygen in a large number of oxidation reactions[17,69,166-168] has provided an experimental tool to investigate the mechanism of oxygen cleavage in the complicated NADPH/O_2-supported cytochrome P-450 reaction.[17,165] The single-oxygen oxidants such as iodosobenzene (130) and N-oxides must undergo heterolytic cleavage of the hetereoatom-oxygen bond in order to yield an iron-reactive oxygen



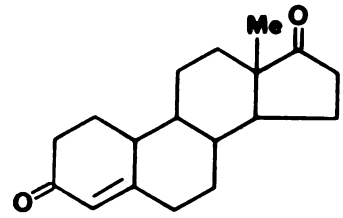
130



131

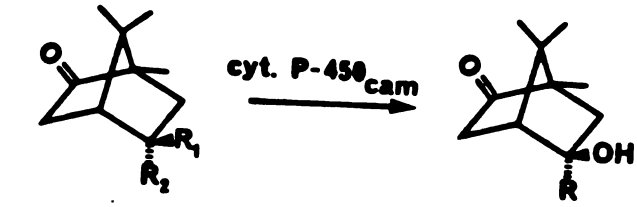
species such as 126.[17] Therefore, they are believed to mimic the heterolytic O-O cleavage mechanism.[17,165] The peracid oxidants such as cumene hydroperoxide (131), on the other hand, can undergo either heterolytic or homolytic cleavage.[17,165] Homolytic cleavage results in the formation of an oxidant radical 127 (pathway B, Figure 45). Since the homolytic mechanism predicts that this radical abstracts a hydrogen atom from the substrate, one might expect to see some oxidant-dependent variations in reaction characteristics if the O-O bond undergoes cleavage to generate the oxidizing agent. If a hetereolytic mechanism is operative, the same oxidizing species, formally the perferryl complex (126), is formed independent of the oxidant. Consequently, one would predict that the cytochrome P-450 catalyzed reaction would not vary with the chemical nature of the oxidant.

Conflicting evidence exists concerning mechanisms of forming the oxidizing species in the mono-oxygenase reactions supported by the various types of exogenous oxidants and NADPH/O_2. The reader can refer to a recent review for a more in-depth discussion of the data.[165] Support for a common oxygen cleavage mechanism for NADPH/O_2 and cumene hydroperoxide comes from studies on aromatic hydroxylation reactions. The observation that the NIH shift occurs in the peroxide-dependent oxidation as well as in NADPH/O_2-dependent reactions suggests a mechanism which leads to a common oxidizing agent.[169] A series of iodoaryl derivatives gave product ratios for the hydroxylation of androstenedione



132

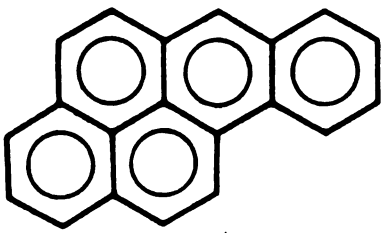
(132) similar to those observed in the NADPH/O_2, suggesting that iodosobenzene and O_2/NADPH lead to the same activated oxygen intermediate.[170] The substitution of cumene hydroperoxide or iodosobenzene for NADPH and O_2 in the P-450$_{cam}$ catalyzed 5-exo-hydroxylation of 5-exo-d_1-camphor (56a) and 5-endo-d_1-camphor (56b) did not significantly alter the deuterium composition of the product alcohol (55), implying that the activated form of the enzyme was the same for all three oxidants.[66] Further evidence for a common mechanisms for the different oxidants is the EPR and optical spectroscopic evidence for similar enzymatic intermediates for all classes of oxidants.[171]



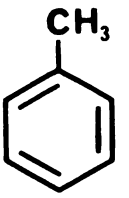
56a R_1=D; R_2=H
56b R_1=H; R_2=D

55 R=H or D

A large body of evidence, however, suggests that the active oxygen intermediate varies with the nature of the oxidant. Arguing against a common oxidizing agent is the principal formation of quinones, not epoxides, during the cumene hydroperoxide-dependent oxidation of benzo(a)-pyrene (4).[172,173] A number of oxidants, including NADPH/O_2, cumene



4



133

hydroperoxide, ethyl hydroperoxide, and iodosobenzene, gave oxidant-dependent product distributions for the hydroxylation of methylcyclohexane (133) by purified P-450$_{LM2}$.[174] Furthermore, no predictable relationship between microsomal N-demethylation reactions supported by NADPH/O_2 and those supported by hydrogen peroxide were found by Estabrook et al.[175] These variations in reactions mediated by various oxidants, in addition to other experimental evidence, suggest that these reactions do not proceed through a uniform reaction mechanism involving a common intermediate.

The results given below summarize our investigations on the effects of substituting the oxidants cumene hydroperoxide and iodosobenzene for NADPH and O_2 on the diastereoselectivity of proton/deuteron abstraction in the Dutch rabbit liver microsomal catalyzed 5'-α-carbon oxidation of 57 and 58.

2. Results.

In order to determine the effect of the exogenous oxidants iodosobenzene and cumene hydroperoxide on the stereochemical course of this oxidation, these compounds were examined in Dutch rabbit liver microsomal incubations in the absence of NADPH. Initial studies were performed with the monodeuterated nicotine diastereomers in the presence of Dutch rabbit liver microsomes (6 mg protein/ml), cumene hydroperoxide or iodosobenzene (1 mM) and sodium cyanide (1 mM) for 30 minutes at 37° C.

Later incubations were performed with other rabbit preparations (3 mg protein/ml) for 5 minutes. Controls were performed in the absence of microsomes or oxidant. The ether extracts were analyzed by GC-EIMS in the selected ion monitoring mode to determine the deuterium content of the generated α-cyanoamines.

Both oxidants supported the oxidation of the 5'- and N-methyl carbon atoms of 22 as indicated by the detection of the 5'-cyanonicotines and N-cyanomethylnornicotine in the mass chromatographs (see Figure 46 and 47). Only residual amounts of these adducts were observed when either the microsomal systems or the oxidant was excluded from the incubation mixture, suggesting that the oxidation of 22 by the exogenous oxidant was mediated by cytochrome P-450. There was preferential formation of the 5'-cyanonicotines with a product ratio of 9:1 for 44a and 44b to N-cyanomethylnornicotine. No significant change in the regioselectivity of the enzymatic reaction was observed when either cumene hydroperoxide or iodosobenzene was substituted for NADPH and O_2 in this reaction.

The diastereoselectivity of these oxidations was determined by measuring the deuterium composition of the 5'-cyano adducts generated in incubations of either 57 or 58. The data obtained after making the appropriate corrections for the stereochemical and isotopic impurities of the substrates are summarized in Table 18. The results from the NADPH- and O_2-supported oxidations are included for comparative purposes. In general, the cumene hydroperoxide-supported reactions were similar to the NADPH/O_2-dependent reactions. The pro-(S) proton was removed to almost the same extent in both of these reactions. Preparations V and VIII displayed more selectivity for the removal of

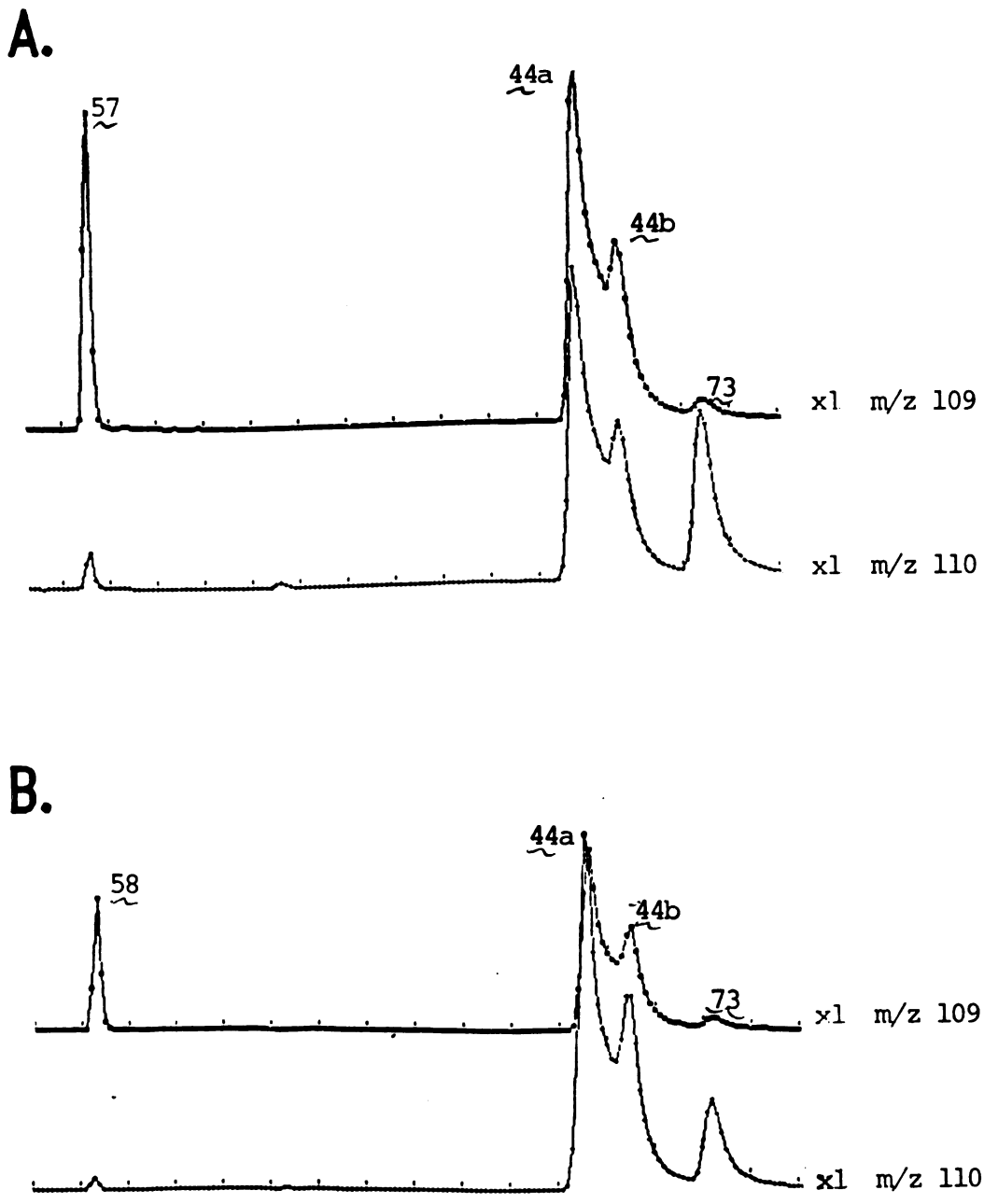


Figure 46. The mass chromatographs for the ether extract obtained from the metabolism of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) in the presence of sodium cyanide by Dutch rabbit liver microsomes supplemented with iodosobenzene. (S)-Nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

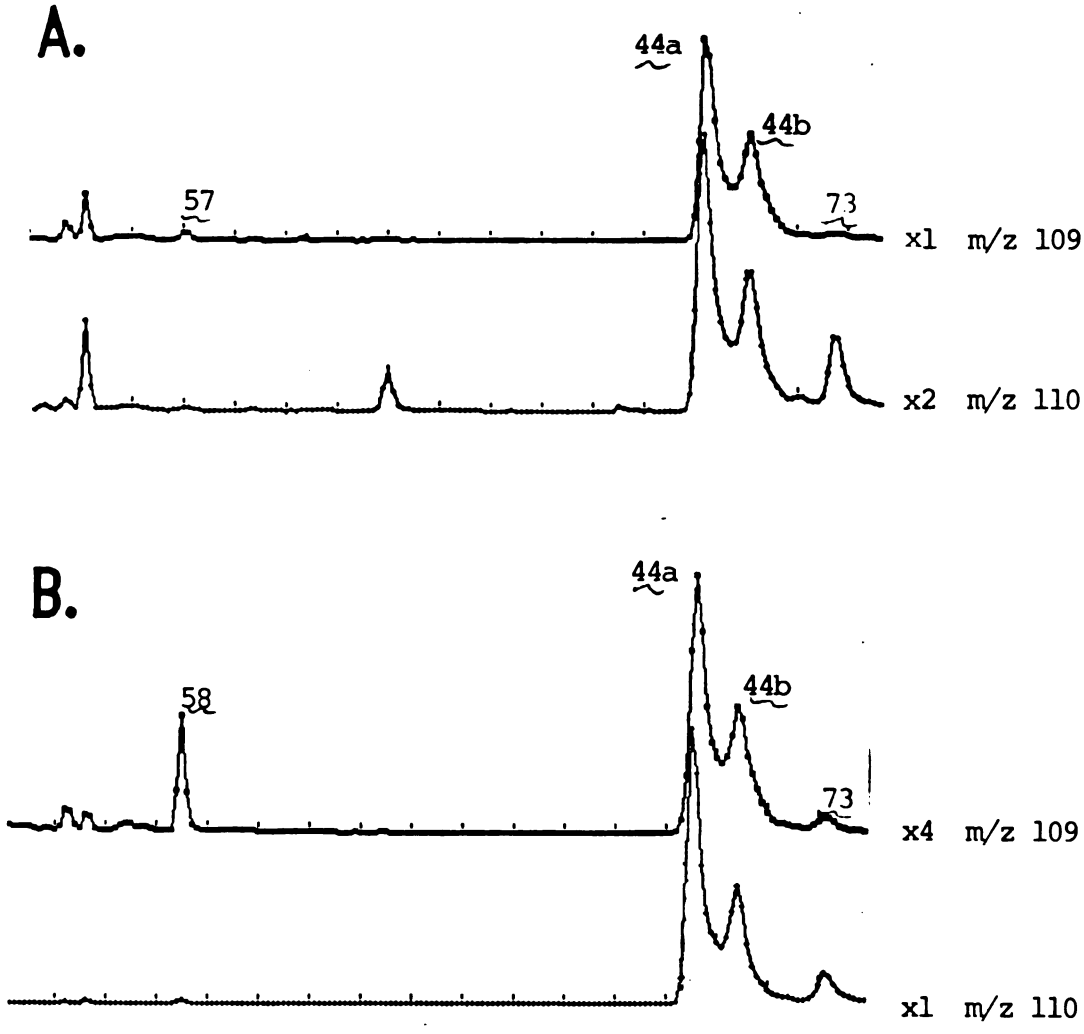
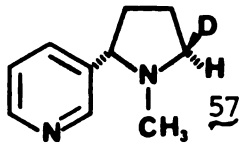
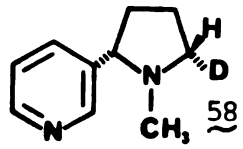


Figure 47. The mass chromatographs of the ether extracts obtained from the metabolism of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) in the presence of sodium cyanide by Dutch rabbit liver microsomes supplemented with cumene hydroperoxide. (S)-Nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E)- and (Z)-5'-cyanonicotine, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

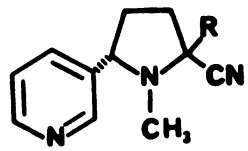


57



58

Table 18. The deuterium composition of the 5'-cyanonicotines isolated from incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 with Dutch rabbit microsomal preparations* in the presence of 1 mM sodium cyanide using various oxygen sources.



Rabbit	Substrate	Oxidant	Time (min)	% R=H	% R=D ± s.d.
V	57	$NADPH/O_2$	15	73	27 ± 2
	58	$NADPH/O_2$	15	23	77
	57	CHP	30	76	24 ± 1
	58	CHP	30	14	86 ± 0
	57	IB	30	63	37 ± 3
	58	IB	30	25	75 ± 2
VI	57	$NADPH/O_2$	5	80	20 ± 3
	58	$NADPH/O_2$	5	12	88 ± 2
	57	CHP	5	83	17 ± 2
	58	CHP	5	11	89 ± 1
	57	IB	5	55	45 ± 0
	58	IB	5	21	79 ± 1
VII	57	$NADPH/O_2$	5	74	26 ± 1
	58	$NADPH/O_2$	5	13	87 ± 1
	57	CHP	5	75	25 ± 1
	58	CHP	5	9	91 ± 2
	57	IB	5	55	45 ± 2
	58	IB	5	24	76 ± 1
VIII	57	$NADPH/O_2$	5	67	33 ± 1
	58	$NADPH/O_2$	5	21	79 ± 1
	57	CHP	5	77	23 ± 1
	58	CHP	5	15	85 ± 0
	57	IB	5	46	54 ± 3
	58	IB	5	27	73 ± 3

IB=iodosobenzene
CHP=cumene hydroperoxide
*The data have been corrected as described in Section II.C.

---

the pro-(S) hydrogen atom in the cumene hydroperoxide-supported reaction than the NADPH/O_2-dependent oxidation. The incubations utilizing iodosobenzene as the oxidant were less selective although the pro-(S) proton still was lost preferentially.

These oxidants can reveal isotope effects which may be hidden in NADPH/O_2 reactions since they circumvent the rate-determining dioxygen activation steps.[17] In one preparation (VI) quantitative estimations of the 5'-cyanonicotines, using 5'-cyanonicotine-N-d_3 as the internal standard, were obtained. The data are summarized in Table 19. These data, although of a preliminary nature, suggest that the oxidation of 57 to 44a and 44b versus 58 to 44a and 44b is accompanied by an isotope effect of 1.3 in the NADPH/O_2-dependent reaction. A somewhat larger value of 1.65 was obtained for the iodosobenzene-supported oxidation whereas no isotope effect was observed when cumene hydroperoxide was used as the oxidant. The small variations in isotope effects between the various oxidants can be explained, in part, by oxidant-dependent changes in the rate-determining step in the overall enzymatic reaction such as the formation of the activated oxygenated enzyme intermediate or the breakage of the C-H bond. These experiments should be repeated in order to determine their reproducibility.

Table 19. The amounts of the 5'-cyanonicotines formed in Dutch rabbit liver microsomal incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 in the presence of 1 mM sodium cyanide with various oxidants.

Oxidant	Substrate	nmoles of 44a and 44b	58/57
NADPH, O_2	57	11.2 ± 0.8	1.3
	58	14.6 ± 0.3	
CHP	57	17.5 ± 2.8	0.96
	58	16.8 ± 1.7	
IB	57	8.21 ± 0.46	1.64
	58	13.5 ± 0.2	

The incubations were performed at 37° C for 5 minutes.

CHP=cumene hydroperoxide
IB=iodosobenzene
* The ratio of the starting concentrations of 57/58 is 0.97.

The competitive oxidation of a 1:1 mixture of 22 and 22-5',5'-d_2 also was examined in a number of Dutch rabbit liver microsomal preparations using the various oxidants to determine the intermolecular isotope effect for the 5'-α-carbon oxidation. The deuterium content of the trapped cyanoamines was determined by GC-EIMS analysis. The ion current pertaining to 44a and 44b at m/z 110 was corrected for the ^{13}C-satellite contribution from the ion m/z 109 to m/z 110 and the ratio of m/z 109 to m/z 110 was take as a measure of the kinetic isotope effect for the 5'-α-carbon oxidation of 22. The results from several Dutch rabbit liver preparations are show in Table 20. These data demonstrate that only small intermolecular isotope effects accompany the oxidation of 22 in reactions supported by cumene hydroperoxide or iodosobenzene. These

hydrogen-isotope studies suggest that the cleavage of the C-H bond has little or no influence on the overall enzymatic process regardless of the oxidant employed.

Table 20. Intermolecular deuterium isotope effects observed for the 5'-α-carbon oxidation of (S)-nicotine in Dutch rabbit liver microsomes supplemented with various oxidants.

Oxidant	k_H/k_D	Average
NADPH, O_2	0.96 ± 0.01, 1.03 ± 0.01, 1.04 ± 0.01	1.01
CHP	1.40 ± 0.5, 1.10 ± 0.1, 1.03 ± 0.15	1.20
IB	1.21 ± 0.24, 1.07 ± 0.03, 1.14 ± 0.07	1.14

Determined using a 1:1 mixture of 22 and 22-5',5'-d_2.

The extent of further oxidation of the 5'-cyanonicotines in the cumene hydroperoxide and iodosobenzene fortified incubations was determined by estimating the amounts of 44a and 44b produced in incubations of 1:1 mixture of 22-d_0:22-5',5'-d_2. The results (see Table 21) suggest that less than 10% of the 5'-cyanonicotines were further oxidized to cotinine. This oxidative pathway was less significant in the cumene hydroperoxide- and iodosobenzene-supported reactions than in the NADPH/O_2-dependent reactions. These observations suggest that these oxidants are less able to support the oxidation of 44a and 44b to cotinine than the NADPH/O_2 system.

Table 21. The amounts of the 5'-cyanonicotines formed during the oxidation of (S)-nicotine by Dutch rabbit liver microsomes in the presence of 1 mM sodium cyanide and various oxidants.

oxidant	time (min)	nmoles 44a and 44b	nmoles 43	% conversion
-oxidant	5	0	0.28 ± 0.09	
NADPH, O_2	5	9.1 ± 0.8	0.73 ± 0.06	7.4%
IB	5	9.6 ± 1.2	0.38 ± 0.02	3.8%
CHP	5	14.0 ± 1	0.49 ± 0.09	3.4%
-oxidant	30	0	0.68 ± 0.17	
NADPH/O_2	30	28.9 ± 0.6	4.45 ± 0	13.3%
IB	30	18.6 ± 1.7	1.54 ± 0.1	7.6%
CHP	30	12.9 ± 0.06	1.52 ± 0.03	10.5%

As reviewed in Section III.B.1, it is controversial whether the iodosobenzene-, cumene hydroperoxide-, and NADPH/O_2-supported cytochrome P-450 catalyzed reactions proceed through a common active oxygen species. We have observed slight differences in the diastereoselectivity but not the regioselectivity of the NADPH/O_2-, iodosobenzene-, and cumene hydroperoxide-supported cytochrome P-450 catalyzed oxidation of 22. Possible sources for the observed variations could be due to different isozymes,[173] nonuniformity of the reactive oxygen species generated by these agents,[172-174] or simply from the presence of oxygen donor fragments at the active site.[69] It is notable, however, that the pro-(S) proton is preferentially removed in all cases, suggesting that the diastereoselectivity of the reaction is determined by the manner in which (S)-nicotine binds at the active site and not by the nature of the oxidant.

C. Iodosobenzene-Tetraphenylporphyrin Catalyzed Oxidation of (S)-Nicotine.

1. Background.

Metalloporphyrin catalysts have been widely used to investigate the catalytic mechanism of mono-oxygenase mediated reactions.[176] Since the protein components of the cytochrome P-450 system are directly involved in the delivery of O_2 and electrons for the dioxygen activation cycle, only chemically reactive compounds such as anilines are oxidized in systems consisting of an synthetic iron-porphyrin with excess amounts of a thiol and a base in the presence of oxygen.[176] These porphyrin catalysts, however, are capable of oxidizing substrates in the presence of oxygen donors such as peroxides,[176] iodosoarenes,[176] and N-oxides.[177,178] Comparative studies on the characteristics of hydroxylation reactions mediated by synthetic porphyrin-iodosylarene model systems in organic solvents have revealed remarkable similarities to those catalyzed by the mono-oxygenase.[167,176,179] The reactive oxidant generated in the reaction between tetraphenylporphinatoiron(III) chloride (Fe^{III}TPPCl) and iodosylbenzene is believed to be very similar to that present in the enzymatic process,[179] particularly when dioxygen and NADPH are replaced by exogenous oxidants such as iodosobenzene.[180] The majority of these studies have centered on investigations of mechanisms of aliphatic[69,179] and aromatic hydroxylation,[180] and alkene epoxidation.[69,180-182] Fe^{III}TPPCl in the presence of iodosobenzene also has been shown to catalyze the N-demethylation of dimethylaniline.[177] A comparison of the stereochemical course of the 5'-α-carbon oxidation of 22 by this model system to that of cytochrome P-450 should help in elucidating factors involved in directing the enzymic process. Unlike the cytochrome P-450 catalyzed reactions, these studies were carried out

in organic solvents since the catalyst was not readily soluble in aqueous systems. However, studies on the aliphatic and aromatic hydroxylation reactions by these systems have demonstrated that they closely resemble the enzymatic system.[179,180]

2. Results

The diastereoselectivity of the tetraphenylporphyrin-iodosobenzene mediated oxidation of nicotine was investigated using 57 and 58. Reaction conditions initially were investigated using unlabeled nicotine. In the stereochemical studies the bistartrate salts of 57 and 58 were converted to the free base since the salts were not soluble in any system that would solubilize the catalyst. A 2:1:0.2 molar ratio of nicotine:iodosobenzene:catalyst was stirred under argon in a number of organic solvents. Reaction mixtures of nicotine with iodosobenzene and nicotine with tetraphenylporphyrin were used as controls. After 45-60 minutes, an aqueous solution of sodium cyanide (pH 6) was added to trap any iminium ions as the α-cyanoamines. When the reaction was examined in dichloromethane at 25° C only unreacted nicotine was recovered as determined by GC analysis of the crude reaction mixture. When dichloromethane-methanol (4:1) or dry ethanol was used as the solvent, both 5'-cyanonicotines and N-cyanomethylnornicotine were formed (see Figure 48). The product ratio of the 5'-cyano diastereomers to N-cyanomethylnornicotine was 2.1 ± 0.5. The extent of 5'-carbon oxidation was approximately 1% as estimated from the GC tracings. No detectable levels of nornicotine or 2'-cyanonicotine were observed. No product formation was observed in the controls.

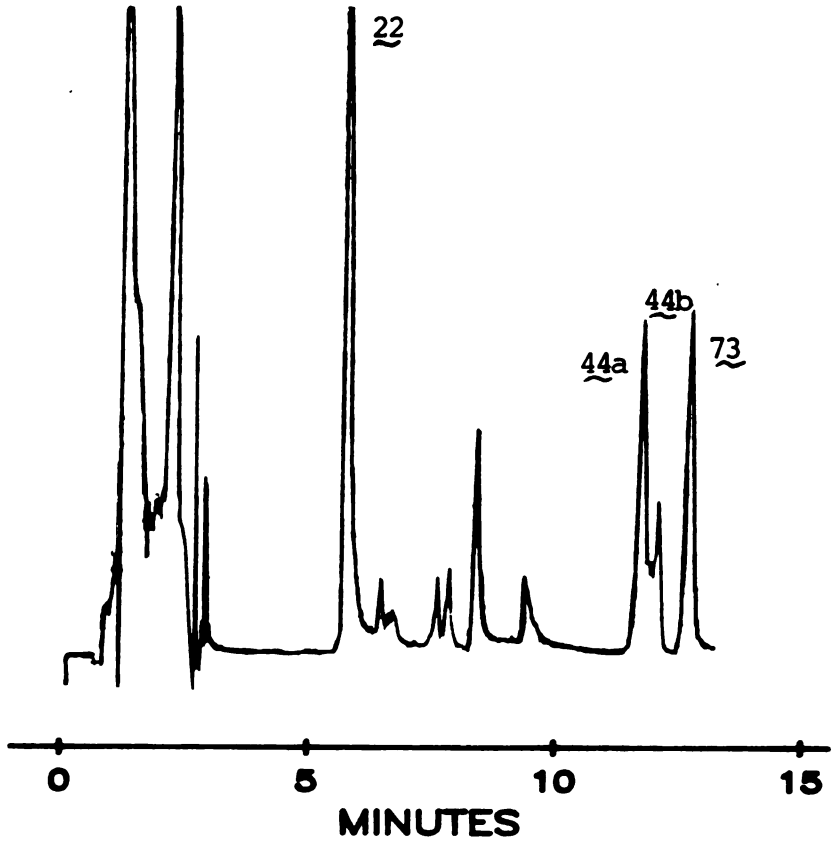


Figure 48. The capillary GC tracing of the dichloromethane extract obtained form the iodosobenzene-tetraphenylporphyrin catalyzed oxidation of (S)-nicotine. (S)-Nicotine, 22; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

The reaction of nicotine-N'-oxides with tetraphenylprophyrin in dry ethanol under argon was also unsuccessful in generating iminium ions as determined by the absence of significant amounts of 44a and 44b in the ether extracts of the reaction mixtures. The GC tracing of the reaction mixture was the same as the controls that were done in the absence of the catalyst. These results suggest that (E)-nicotine-N'-oxide in these porphyrin systems does not generate iminium ions.

The stereochemical studies were conducted in dichloromethane-methanol (4:1) and the deuterium content of the cyano adducts was determined by GC-EIMS to determine the deuterium content of the generated cyanoamines. The mass chromatographs confirmed the formation of the 5'-cyanonicotines and N-cyanomethylnornicotine (Figure 49). Only low levels of 2'-cyanonicotine were observed in the mass chromatographs of these reaction mixtures. When 57 was the substrate, 56 ± 2% of the cyanoadducts were represented as the 5'-cyano diastereomers as opposed to 65 ± 5% of those generated from 58. These data suggest that the presence of deuterium in the pro-(S) 5'-position does not cause significant "metabolic shifting" due to an isotope effect.[137]

The deuterium compositions of the 5'-cyanonicotines generated from 57 and 58 are shown in Table 22. The oxidation of 57 showed a slight preference for the abstraction of the (E)-5'-deuteron whereas the oxidation of 58 proceeded with the selective loss of the (E)-5'-hydrogen. This oxidation demonstrates a small degree of selectivity for the removal of the pro-(S) 5'-proton of 22 as was observed for the cytochrome P-450 catalyzed oxidation when iodosobenzene was the oxidant.

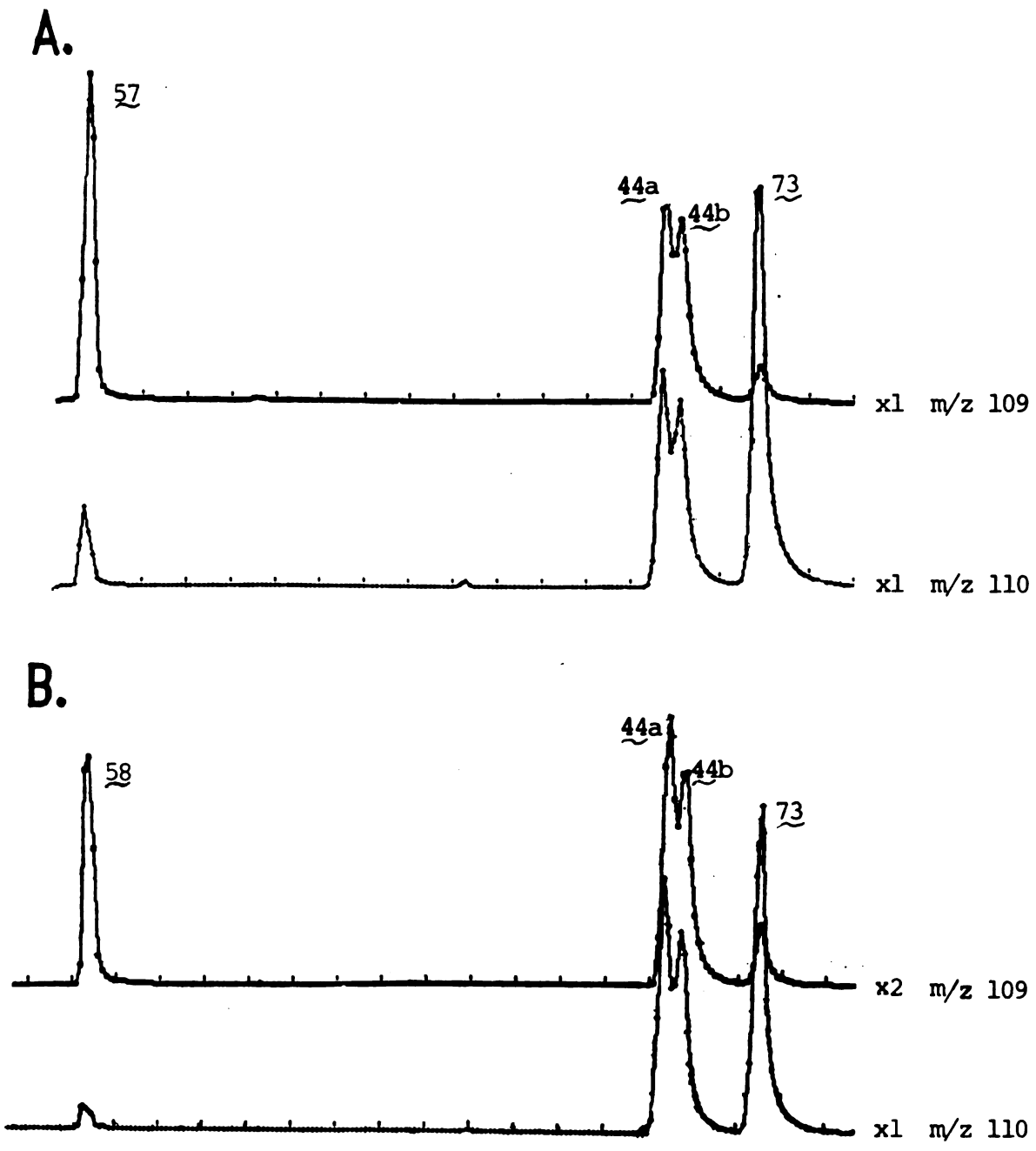
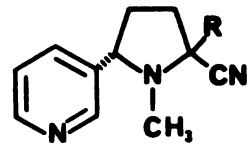
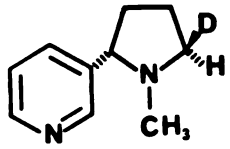
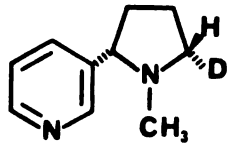


Figure 49. The mass chromatographs of the dichloromethane extracts obtained from the iodosobenzene-tetraphenylporphyrin catalyzed oxidation of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.). (S)-Nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E)- and (Z)-5'-cyanonicotine, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

Table 22. The deuterium composition of the 5'-cyanonicotines isolated from the iodosobenzene-tetraphenylporphyrin catalyzed oxidation of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 (n=3).*



Substrate	% R=H	% R=D ± s.d.
	55	45 ± 1
	32	68 ± 1

*The data have been corrected for the isotopic and stereochemical impurities of 57 and 58 (see Section II.C).

The role of isotope effects in this process was examined by determining the deuterium content of the cyanoamines formed in the competitive oxidation of 22-d_0 and 22-5',5'-d_2. The ion currents at m/z 109, 110 and 111 were measured, ^{13}C-isotope corrections were made and the ratio of ion current at 109/110 for the 5'-cyanonicotines was used as a measure of the kinetic isotope effect for the 5'-α-carbon oxidation. Since the presence of the label in the 5'-position might shift the oxidation to the N-methyl position, we also examined the deuterium content of the N-cyanomethylnornicotine formed in these studies. The deuterium composition was determined by measuring the ion current at m/z 109 for the unlabeled adduct and m/z 110 for the dideuterated adduct.

If the presence of the deuterium label in the 5'-position has no effect on the rate of N-methyl oxidation, the deuterium composition of the N-cyanomethylnornicotine should be equal to the deuterium composition of the starting material. The ion current at m/z 109/111 was used to determine the effect of the 5'-label on the deuterium composition of the N-cyanomethylnornicotine formed in these studies. No isotope effect was observed for the oxidation of the 5'-position. Furthermore, no enrichment of label in the N-cyanomethyl adduct was observed, suggesting that the presence of deuterium in the 5'-position does not cause the oxidation to shift to the N-methyl group. The absence of kinetic isotope effects suggests that another step, such as the reaction of the catalyst with isodosobenzene is the rate-determining step in this reaction.[183]

In general, the Fe^{III}TTPCl-iodosobenzene catalyzed oxidation of 22 resembles the cytochrome P-450/iodosobenzene mediated process. The pro-(S) 5'-proton is preferentially removed by both systems. The synthetic porphyrin reaction, however, is considerably less selective than the NADPH/O_2-dependent microsomal oxidation. Nevertheless, the observation that the pro-(S) proton was selectively lost in all the microsomal oxidations and in the synthetic porphyrin systems suggests that the planar porphyrin ring influences the stereoselectivity of the reaction.

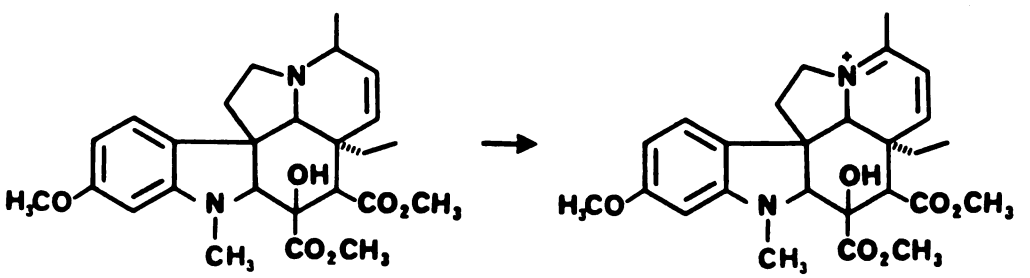
The metalloporphyrin/iodosobenzene-catalyzed reaction was less regioselective than that observed in either human or rabbit microsomes independent of the oxygen source, implying that the protein environment of the enyzme plays a role in directing the regioselectivity of the reaction. However, the porphyrin system must have some effect on the reactions since 2'-cyanonicotine was an insignificant product in both systems.

Both the enzymatic and synthetic porphyrin systems did not exhibit "metabolic switching" to the N-methyl group as a result of isotope effects when the 5'-α-position was labeled with deuterium. These observations suggest either that the reaction is unaccompanied by a significant isotope effect or the regiochemical course of the oxidation is determined upon binding of the molecule to the catalyst.

D. Peroxidase Catalyzed Oxidation of (S)-Nicotine.

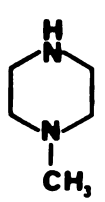
1. Background.

The influence of the heme environment on the stereochemical course of the cytochrome P-450 catalyzed 5'-α-carbon oxidation of 22 was explored further with several hemoprotein models. A number of hemoproteins including horseradish peroxidase (HRP; EC 1.11.1.7, donor hydrogen peroxide oxidoreductase), chloroperoxidase (EC 1.11.1.10; chloride: hydrogen peroxide oxidoreductase) and methemoglobin are able to catalyze the peroxide supported N-demethylation of a variety of tertiary arylamines.[20,184-188] Recently, three reports have demonstrated the ability of these hemoproteins to oxidize aliphatic tertiary amines as well. The cyclic allylamine vindoline (134) is converted to the iminium ion 135 by

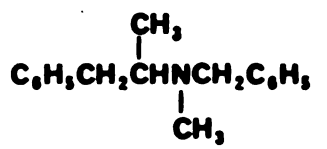


134 135

horseradish peroxidase and hydrogen peroxide.[189] Methemoglobin, in the presence of hydrogen peroxide, catalyzed the N-demethylation of N-methylpiperazine (136).[190] A system consisting of hemoglobin, NADPH, and cytochrome P-450 reductase is able to catalyze the N-demethylation



136



137

of benzphetamine (137).[191] The ability of these hemoproteins to catalyze the oxidations of tertiary amines like cytochrome P-450 suggests that cytochrome P-450 and the peroxidases oxidize the substrates via related reaction pathways.

The enzyme intermediates in the chloroperoxidase and horseradish peroxidase mediated processes have been well-characterized. The enzymes react with peroxides to form an alcohol and compound I, formally a two-electron oxidized form of the enzyme which is represented as an oxygenated Fe^{4+} species with a porphyrin π-cation radical. The substrate then reduces compound I by one electron to yield compound II and a substrate radical. A second one electron reduction by the substrate regenerates the native enzyme.[192] While these intermediates have been well-characterized in the horseradish peroxidase and chloroperoxidase, the iron-oxygen intermediates in the peroxidase activity of methemoglobin remain unclear. A distinct enzyme intermediate equivalent to compound I has not been detected in these reactions. Studies with the closely related myoglobin demonstrated that these hemoproteins lead almost directly to the one-electron oxidized form of the enzyme (similar to compound II).[193,194] How this one-electron equivalent species carries out two electron oxidation reactions remains unclear.

The chemical mechanism for the α-carbon oxidation reactions mediated by the various peroxidases is unknown. Several radical mechanisms have been proposed[20] and are shown in Figure 50. Pathway A is similar to that postulated for the cytochrome P-450 catalyzed oxidation of amines and involves initial one-electron abstraction from the nitrogen's lone pair by the hemoprotein's iron-oxo species to generate an aminium radical 93. The aminium radical can then undergo one of several routes shown in Figure 50 to yield the N-dealkylated product

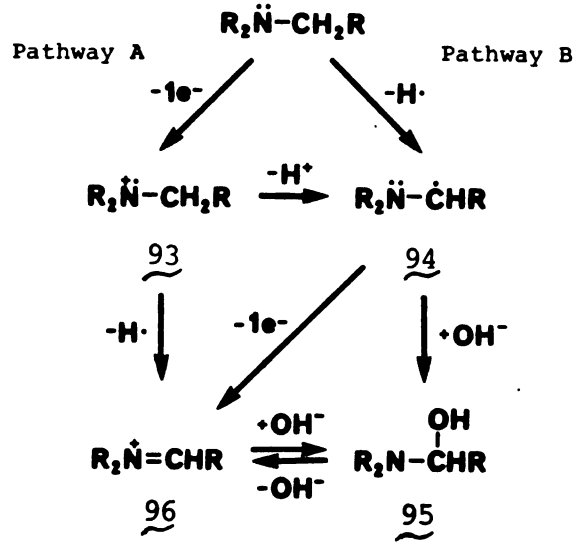
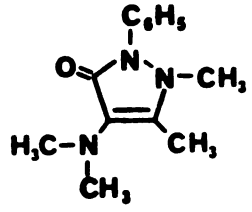


Figure 50. Possible mechanisms of α-carbon oxidation of tertiary amines by peroxidases.[20]

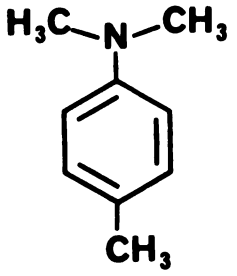
(see Section III.A. for a more in-depth discussion). Alternatively, the enzyme can directly abstract a hydrogen atom from the amine to form the neutral α-aminoalkyl radical 94 (Pathway B, Figure 50). This carbon-centered radical can either recombine with the oxygen to form a carbinolamine (95) or yield a second electron to generate an iminum ion 96.[20]

Several studies lend support to a radical mechanism for peroxidase catalyzed N-dealkylation reactions. EPR studies demonstrated radical formation in the N-demethylation of aminopyrine (103) by a number of hemoproteins.[186] A cation radical was detected by EPR spectroscopy in

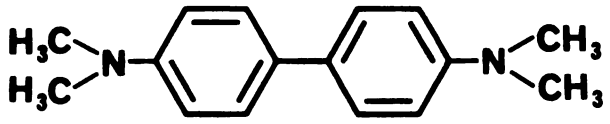


103

the horseradish peroxidase catalyzed N-demethylation of N,N-dimethyl-p-toluidine (138) by hydrogen peroxide.[188] Radical intermediates in the horseradish peroxidase catalyzed N-demethylation of N,N-dimethylaniline were suggested by the isolation and characterization of the radical dimerization product N,N,N',N'-tetramethylbenzidine (139) from incubation mixtures.[188] Further evidence for radical intermediates in the α-carbon oxidations catalyzed by horseradish peroxidase is the one electron reduction of compound I to compound II upon addition of vindoline to solutions containing horseradish peroxidase and hydrogen peroxide.[189]

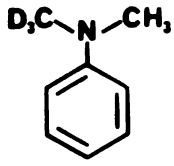


138



139

Intramolecular isotope effects in the N-demethylation of N-methyl-N-trideuteromethylaniline (110) by ethyl peroxide in a reaction catalyzed by chloroperoxidase revealed a modest intrinsic isotope effect (k_H/k_D=2.55).[20] The magnitude of this isotope effect is similar to that observed for the NADPH/O_2-supported cytochrome P-450$_{PB}$ catalyzed reaction (k_H/k_D=1.78)[20] and is consistent with a reaction mechanism where the rate-determining step is aminium radical formation.[20] In contrast,



110

the hydrogen peroxide-supported horseradish peroxidase and hemoglobin catalyzed N-demethylation of 110 exhibited large intramolecular isotope effects of 10.1 and 9.33, respectively.[20] The large isotope effects associated with the latter reactions have lead to the proposal that the horseradish peroxidase and methemoglobin reactions proceed by direct hydrogen abstraction from the amine.[20]

The fifth ligand to the heme-iron has been proposed to be the cause of the dramatic differences in the isotope effects observed in the N-demethylation of 110 by the two sets of hemoproteins.[20] Chloroperoxidase and cytochrome P-450, the two enzymes systems which exhibited small intramolecular isotope effects for the reaction have a thiolate anion serving as the fifth ligand.[195] Horseradish peroxidase and methemoglobin, on the other hand, both displayed large isotope effects in the oxidation reaction and have histidine as the fifth ligand.[192,196] Thus, the fifth ligand to the heme may influence the chemical mechanism of these reactions.[20]

In summary, hemoproteins such as methemoglobin, chloroperoxidase, and horseradish peroxidase can catalyze α-carbon oxidations of tertiary amines via radical mechanisms. The mechanisms proposed for the demethylation of N,N-dimethylaniline by cytochrome P-450 and chloroperoxidase differ from that proposed for methemoglobin and horseradish peroxidase. The differences have been attributed to variations in the heme iron's fifth ligand.

We decided to explore the stereochemistry of the 5'-α-carbon oxidation of 57 and 58 by these hemoproteins since a comparison of the results obtained in these studies to those observed in the cytochrome P-

450 catalyzed reaction may allow us to model the role of the active site in directing the stereochemical course of the cytochrome P-450 mediated process.

2. Methemoglobin Catalyzed Oxidation of (S)-Nicotine.

The methemoglobin catalyzed oxidation of nicotine was first investigated with unlabeled nicotine. The incubations contained nicotine (10 mM), hydrogen peroxide (0.6 mM) and bovine methemoglobin (0.64 mg/mL) in pH 7.4 0.2 M sodium phosphate buffer. The reactions were run at 37° C for one hour. The mixtures were cooled on ice prior to the addition of sodium cyanide to convert any iminium ions to the corresponding α-cyanoamines. Inclusion of cyanide ion during the incubation period would have inhibited the enzymatic activity of this hemoprotein.[197] The ether extracts were concentrated under N_2 and resuspended in n-butyl acetate for capillary GC analysis.

The GC tracings displayed unreacted nicotine, 2'-cyanonicotine, the diastereomeric 5'-cyanonicotines, trace levels of N-cyanomethylnornicotine and an unidentified metabolite that eluted about 1 minute after 22 (Figure 51). Nornicotine was not detected. The 5'-cyano isomers 44a and 44b were present in 2-3 times greater quantities than 2'-cyanonicotine. When the incubations were examined with the labeled compounds at 30' C for 30 minutes and extracted with CH_2Cl_2, the ratio of the 5'-cyanonicotines to 2'-cyanonicotine was 9:1 with only trace amounts of the N-cyanomethyl adduct and nornicotine. The extent of 5'-α-carbon oxidation was estimated from the GC tracing to be approximately 5%. Small amounts of cotinine were also observed in the dichloromethane extracts. In all cases, the 5'-position was the major site of oxidation.

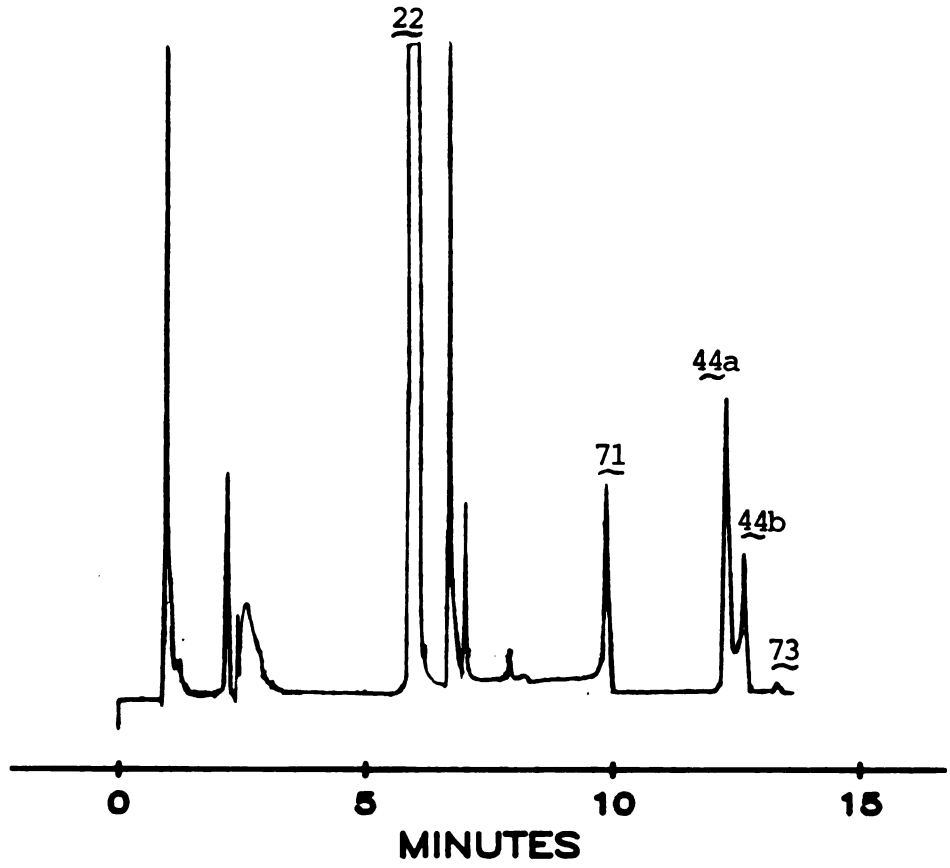
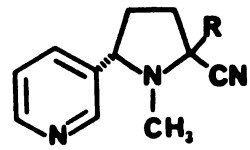
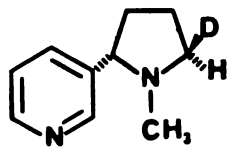
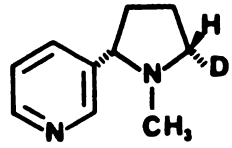


Figure 51. The GC tracing of the dichloromethane extract obtained from the oxidation of (S)-nicotine by hydrogen peroxide catalyzed by methemoglobin. (S)-Nicotine, 22; 2'-cyanonicotine, 71; (E)- and (Z)-5'-cyanonicotine, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

The labeled compounds 57 and 58 were used to determine if the 5'-α-carbon oxidation was under stereochemical control. The mass chromatographs of the incubation mixtures are shown in Figure 52. The data displayed in Table 23 demonstrate that the 5'-cyanonicotines generated from both 57 and 58 were enriched in deuterium; 71% of the adducts isolated from incubations of 57 and 62% of the adducts isolated from incubations of 58 were labeled. The selective retention of the label during the oxidation of both 57 and 58 indicates that the course of the reaction at the 5'-position is controlled by deuterium isotope and not stereochemical effects. The hydrogen peroxide-supported methemoglobin catalyzed reaction contrasts dramatically with the cytochrome P-450 mediated oxidation where the (E)-5'-proton/deuteron is selectively lost from the monodeuterated nicotine diastereomers.

Table 23. Deuterium composition of the 5'-cyanonicotines isolated from incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 with hydrogen peroxide and methemoglobin following the addition of sodium cyanide (n=3).*



Substrate	% R=H	% R=D ± s.d.
	29	71 ± 1
	38	62 ± 1

*The data has been corrected for the isotopic and stereochemical impurities of 57 and 58 (see Section II.C for details).

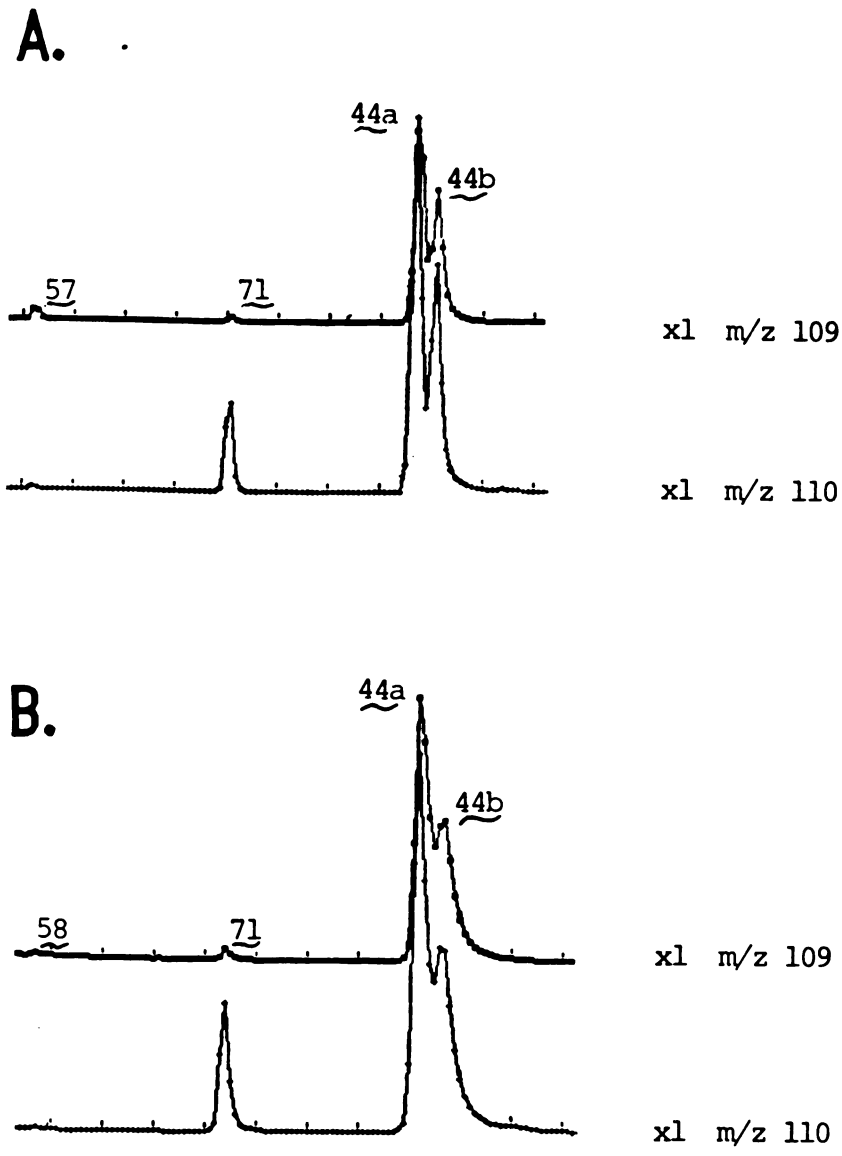


Figure 52. The mass chromatographs of the dichloromethane extracts obtained from the oxidation of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) by hydrogen peroxide by methemoglobin. (S)-Nicotine-(E)-5'-d_1, 57; (S)-Nicotine-(Z)-5'-d_1, 58; 2'-cyanonicotine, 71; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively.

Although not strictly an intramolecular isotope effect since the 5'-protons are diastereotopically related, the ratio of the labeled to unlabeled 5'-cyanonicotines generated from 57 and 58 reveal an isotope effect of 2.4 and 1.63, respectively. No intermolecular isotope effect was observed for the 5'-α-carbon oxidation of nicotine when a 4:3 mixture of 22-d_0 and 22-5',5'-d_2 was incubated with methemoglobin and hydrogen peroxide. However, the ratio of unlabeled to labeled 2'-cyanonicotine was 0.8 ± 0.1 (n=3). This enhanced deuterium content in the 2-cyano isomer suggests that the presence of the label in the 5'-position shunts the oxidation to the 2'-position.

The stereochemical studies demonstrated that the methemoglobin catalyzed 5'-α-carbon oxidation of 22 is not under stereochemical control but is directed by deuterium isotope effects. These results differ greatly from the cytochrome P-450 catalyzed oxidation and suggest that the methemoglobin-catalyzed process may proceed through a mechanism different from the cytochrome P-450 catalyzed reaction. Intramolecular isotope effect studies with N-methyl-N-trideuteromethylaniline support this proposal since the methemoglobin-ethyl peroxide reaction proceeded with a large kinetic isotope effect (k_H/k_D=9.33) whereas the cytochrome $P\text{-}450_{PB}$ catalyzed reaction was accompanied by only a modest one (k_H/k_D=1.78).[20] The influence of a kinetic isotope effect in the choice between the pro-(R) and pro-(S) 5'-hydrogen atoms of nicotine indicates that C-H bond breakage occurs in a rate determining step. It is difficult to make mechanistic interpretations from this observed isotope effect because of the diastereotopic relationship of the 5'-hydrogen atoms.

The lack of stereochemical control in the methemoglobin reaction suggests that 22 either is only loosely bound at the active site or is bound in such a manner that both hydrogen atoms are accessible to the abstracting species. The first possibility seems unlikely since NMR studies have demonstrated that compounds known to bind to the heme iron of hemoglobin and myoglobin[198] have restricted movement inside the heme pocket.[199] Another methemoglobin catalyzed oxidation, the epoxidation of styrene, occurs with the loss of the alkene's stereochemistry.[197] Ortiz de Montellano and Catalano argue that this oxidation occurs outside the heme pocket since the congested active site would be expected to strongly influence the stereochemical course of this reaction.[197] They suggest that the reaction is mediated by an amino acid radical such as a tyrosine radical at the outer protein surface. Such a radical might account for the loss of one electron equivalent in the oxygenation of the hemoprotein by peroxide (see section III.D.1.).[193] Consistent with this proposal is the detection of protein radicals by EPR spectroscopy when methemoglobin and myoglobin are allowed to react with hydrogen peroxide.[200,201] This hypothesis would explain the absence of stereochemical control in the oxidation of the C-5'-position of 22.

3. Horseradish Peroxidase Catalyzed Oxidation of (S)-Nicotine.

The stereochemical course of the horseradish peroxidase catalyzed oxidation of 22 was determined following the procedure described by Sariaslani et al. for the oxidation of vindoline.[189] Initial studies were performed with unlabeled nicotine to determine if this aliphatic tertiary amine is a substrate for this enzyme. Nicotine (0.85 mM) was incubated with horseradish peroxidase (Type IV, 0.1 mg protein/mL) and hydrogen peroxide (0.136 mM) in pH 6.8 phosphate buffer (0.1 M) for 60

minutes. While some studies were conducted at 37° C as in the microsomal studies, others were conducted at 25° C since most reactions with horseradish peroxidase are conducted at 25' C.[20,189] The reaction profile was unaffected by the temperature. Incubations of nicotine either in the absence of hydrogen peroxide or in the absence of the hemoprotein were used as controls. The reaction was stopped by cooling on ice. Sodium cyanide was added to the mixture to convert any iminium ion to the corresponding cyanoamines since the incubations were done in the absence of sodium cyanide to avoid inhibiting the enzyme. The incubation mixtures were extracted with ether and the ether extracts were concentrated under nitrogen and redissolved in n-butyl acetate for capillary GC analysis.

The capillary GC tracing obtained using the complete system indicated the formation primarily of the diastereomeric 5'-cyanonicotines and a small amount of 2'-cyanonicotine (Figure 53). Only trace amounts of N-cyanomethylnornicotine were formed. However, nornicotine was detected indicating that oxidation of the N-methyl group occurred during the incubation period but that the intermediate was not trapped due to the late addition of sodium cyanide. The metabolites were identified by comparing GC retention times with synthetic standards. Low levels of the oxidation products were observed in the controls; N-demethylation was the most significant pathway in these incubations. When the complete system was present, the amount of the 5'-cyanonicotines increased by a factor of 100, suggesting that the oxidation of the 5'-position required both the enzyme and hydrogen peroxide.

The relative amounts of these oxidation products were determined by comparing GC peak areas. Since the detector was a nitrogen/phosphorus detector, corrections were required because nornicotine contains only

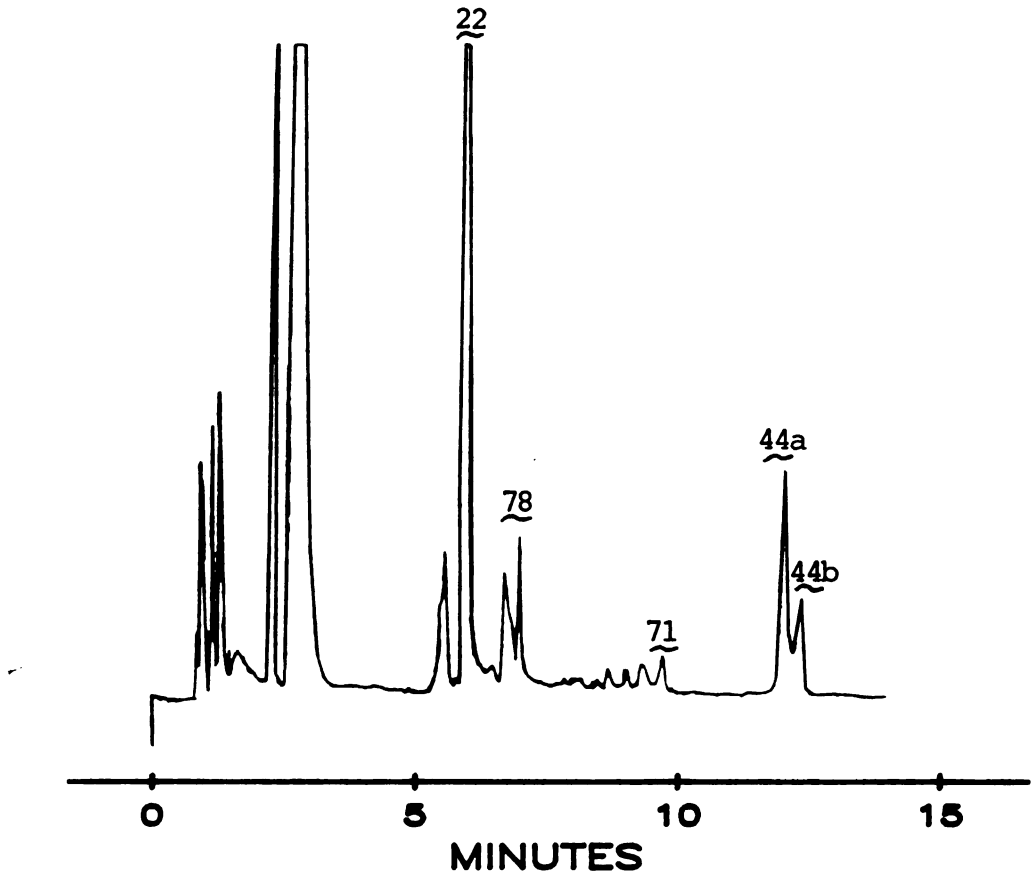


Figure 53. The capillary GC tracing of the ether extract obtained from the oxidation of (S)-nicotine by hydrogen peroxide catalyzed by horseradish peroxidase followed by the addition of sodium cyanide. (S)-Nicotine, 22; nornicotine, 78; 2'-cyanonicotine, 71; and (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively.

two nitrogen atoms whereas the cyanoadducts contain 3. A comparison of the peak areas for the 5'-cyanonicotines, nornicotine and 2'-cyanonicotine indicated a product ratio of approximately 9:4.5:1, respectively, demonstrating that the horseradish peroxidase catalyzed reaction proceeds preferentially at the 5'-carbon atom. The extent of oxidation at the 5'-position was approximately 2% as estimated from the GC tracings.

We then used the well-established redox characteristics of the peroxidase to confirm that the reaction was mediated by the oxygenated enzyme and to obtain evidence that the oxidation proceeded through a radical process. It is well-known that horseradish peroxidase reacts with hydrogen peroxide to generate HRP-I, a porphyrin iron (4^+) species that contains a stabilized radical cation.[202,203] This intermediate absorbs visible light at 410 nm. When the enzyme is in this redox state, it catalyzes one electron oxidations of substrates to generate the products and the reduced form of the enyzme, HRP-II. The production of HRP-II can be followed by an increase in absorbance at 418 nm. A second electron transfer regenerates the native enzyme which absorbs at 403 nm.[189]

We investigated the formation of these intermediates in the hydrogen peroxide-horseradish peroxidase catalyzed oxidation of (S)-nicotine. The spectroscopy was performed by Mr. M. Shigenaga (Figure 54). HRP-I was generated upon addition of hydrogen peroxide (9.4 μM) to a pH 6.8 buffer solution of horseradish peroxidase (4.7 μM) as indicated by the shift in the absorption maximum from 403 nm to 410 nm. Nicotine (4 mM) then was added to the solution and the maximal absorption changed from 410 to 418 nm. This shift is consistent with the generation of HRP-II. This species was distinguished from HRP-III, another redox form

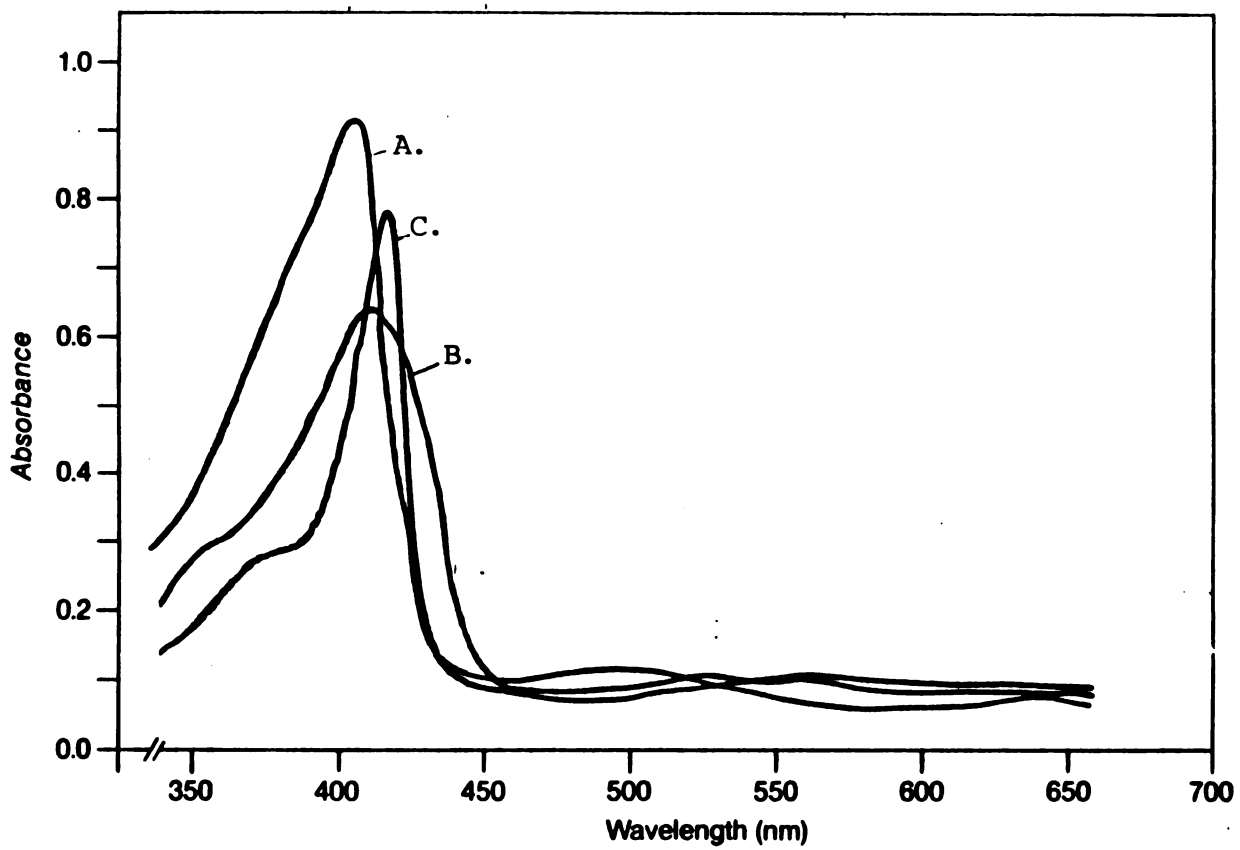


Figure 54. The visible spectra of A.) horseradish peroxidase (4.7 μM); B.) horseradish peroxidase plus hydrogen peroxide (9.4 μM); and C.) horseradish peroxidase, hydrogen peroxide, and (S)-nicotine (4mM).

considered to be either a oxygen-ferroperoxidase or superoxide-ferriperoxidase[192] that also absorbs at 418 nm, by examining the visible spectrum at higher wavelengths. We observed absorbances at 527 and 555 nm as expected for HRP-II.[192] HRP-III absorbs light at 545, 583 and 673 nm.[189] The formation of HRP II in the presence of 22 is consistent with a one electron transfer step from 22 to HRP-I. The one electron reduction of HRP-I to HRP-II can result either from an electron transfer from the nitrogen lone pair of electrons to form an aminum radical or from the abstraction of a hydrogen atom from the α-carbon atom to generate an α-aminoalkyl radical. A second one-electron oxidation of these compounds would lead to the iminium product 42. While we cannot distinguish between these two mechanisms, this experiment demonstrates that the oxidation is mediated by the enzyme in two one-electron steps since HRP-II is generated from HRP-I upon addition of nicotine.

The diastereomers 57 and 58 were incubated with horseradish peroxidase at 25° C and 37° C for 30 minutes to determine the stereochemical course of this oxidation. Following the addition of sodium cyanide, the mixtures were extracted with either ether or dichloromethane. The extracts were concentrated under a N_2 stream and redissolved in CH_2Cl_2 for GC-EIMS selected ion monitoring analysis (see Figure 55 for the mass chromatographs of the dichloromethane extracts). The results of this study are presented in Table 24. The reaction at the 5'-position was not under stereochemical control since the 5'-cyanoadducts isolated from incubations of either 57 or 58 were enriched with the deuterium label. There is a slight preference for selective removal for the pro-(R) proton as suggested by the smaller amount of deuterium retention during the oxidation of 58. Overall, a significant isotope effect governs the removal of the 5'-hydrogen atoms in the horseradish peroxidase catalyzed

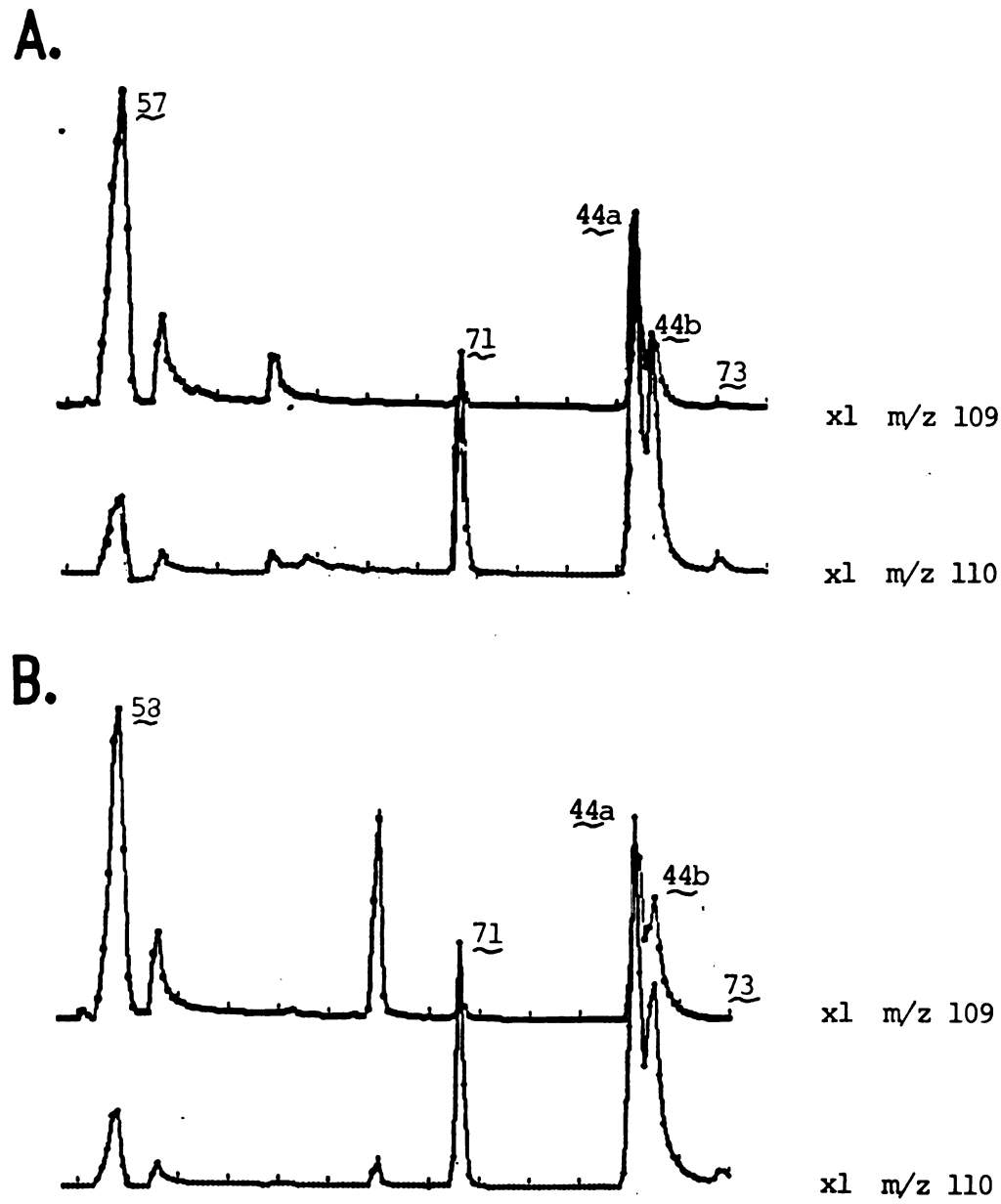
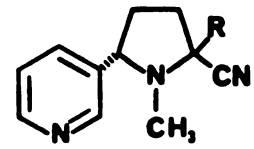
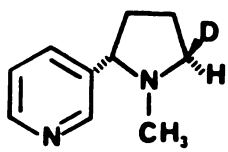
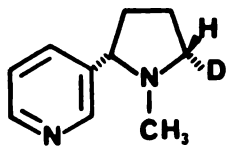


Figure 55. The mass chromatographs of the dichloromethane extracts obtained from the oxidation of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 by hydrogen peroxide catalyzed by horseradish peroxidase following the addition of sodium cyanide. (S)-Nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; 2'-cyanonicotine, 71; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

reaction. This process contrasts with the corresponding reaction catalyzed by cytochrome P-450 where the selective loss of the pro-(S) proton was relatively uninfluenced by deuterium kinetic isotope effects. This difference suggests that these two enzymic processes are quite different.

Table 24. The deuterium composition of the 5'-cyanonicotines isolated from incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 with horseradish peroxidase and hydrogen peroxide (n=3).*



Substrate	% R=H	% R=D + s.d.
	22%	78% ± 5
	32%	68% ± 7

*The data has been corrected for the stereochemical and isotopic impurities of 57 and 58 (see Section II.C).

The ratio of the deuterated to unlabeled 5'-cyano adducts generated from the monodeuterated nicotine diastereomers provide an apparent intramolecular isotope effect for this reaction. Values of 3.1 and 2.1 were obtained for 57 and 58, respectively. Mechanistic interpretations of these values are not possible due to the diastereotopic relationship

of the 5'-protons. Intermolecular isotope effects were determined for this reaction by measuring the deuterium content of the cyanoamines isolated from incubations of a 1:1 mixture of $\underset{\sim}{22}$-d_0 and $\underset{\sim}{22}$-5',5'-d_2. The ratio of the labeled to unlabeled 5'-cyanonicotines gave a kinetic isotope effect of 1.72 $\pm$ 0.34 for this reaction, demonstrating that the breakage of the 5'-C-H bond is partially rate-limiting in the overall enzymatic process.

In summary, the hydrogen peroxide-supported horseradish peroxidase catalyzed oxidation of $\underset{\sim}{22}$ proceeds preferentially at the 5'-position. The choice of the 5'-hydrogen atoms was not under stereochemical control but was primarily determined by deuterium isotope effects. The influence of deuterium on the stereochemical course of this oxidation suggests that the breakage of the 5'-C-H bond is rate-determining in the chemical oxidation. The results of this study differ significantly from the results obtained in the microsomal experiments where the 5'-α-carbon oxidation proceeded with the stereoselective removal of the pro-(S) proton/deuteron without significant deuterium isotope effects. The differences suggest that the peroxidase and cytochrome P-450 catalyzed oxidations do not proceed through the same reaction mechanism. This conclusion is supported by the observations of Miwa et al. for the N-demethylation of N-methyl-N-trideuteromethylaniline catalyzed by horseradish peroxidase and cytochrome P-450.[20] A large intramolecular isotope effect ($k_H/k_D>8$) was found for the horseradish peroxidase-mediated process whereas a modest one ($k_H/k_D=1.78$) was observed for the cytochrome P-450 process.[20] The difference in magnitude of the two values suggests that these enzymatic processes follow different reaction mechanisms.

The absence of significant diastereoselectivity in this reaction suggests that the horseradish-peroxidase catalyzed oxidation is much different than the cytochrome P-450 mediated reaction. The dissimilarity could result from different chemical mechanisms as proposed by others[20] or from a different mode of interaction between the substrate and the hemoprotein's active site. The observation that both 5'-hydrogen atoms can be removed suggests that they are equally accessible to the abstracting species.

4. Chloroperoxidase Catalyzed Oxidation of (S)-Nicotine.

The stereochemical course of the chloroperoxidase catalyzed oxidation of 22 was examined employing the horseradish peroxidase incubation conditions. Ethyl peroxide was used as the oxidant for these reactions. Either 57 or 58 (0.85 mM) was incubated with chloroperoxidase (66 µg protein/mL) and ethyl peroxide (1.7 mM) in pH 6.8 0.1 M phosphate buffer. Control incubations in the absence of ethyl peroxide, or protein were included. The reaction was stopped by placing the incubation mixture on ice and adding sodium cyanide. The dichloromethane extracts were analyzed both by GC-EIMS selected ion monitoring techniques and capillary GC linked to a nitrogen-phosphorus detector.

The mass chromatographs demonstrated that the major cyanoadducts were the diastereomeric 5'-cyanonicotines (Figure 56). 2'-Cyanonicotine was also observed. A product ratio of 4:1 for 2'-:5'-cyanonicotines was determined. The extent of conversion nicotine to the 5'-cyanonicotines was estimated to be 5% from the GC tracings. While N-cyanomethylnornicotine was not observed, GC analysis demonstrated the presence of nornicotine, demonstrating that chloroperoxidase oxidizes the N-methyl group. This metabolite was a minor metabolite, however. An unidentified

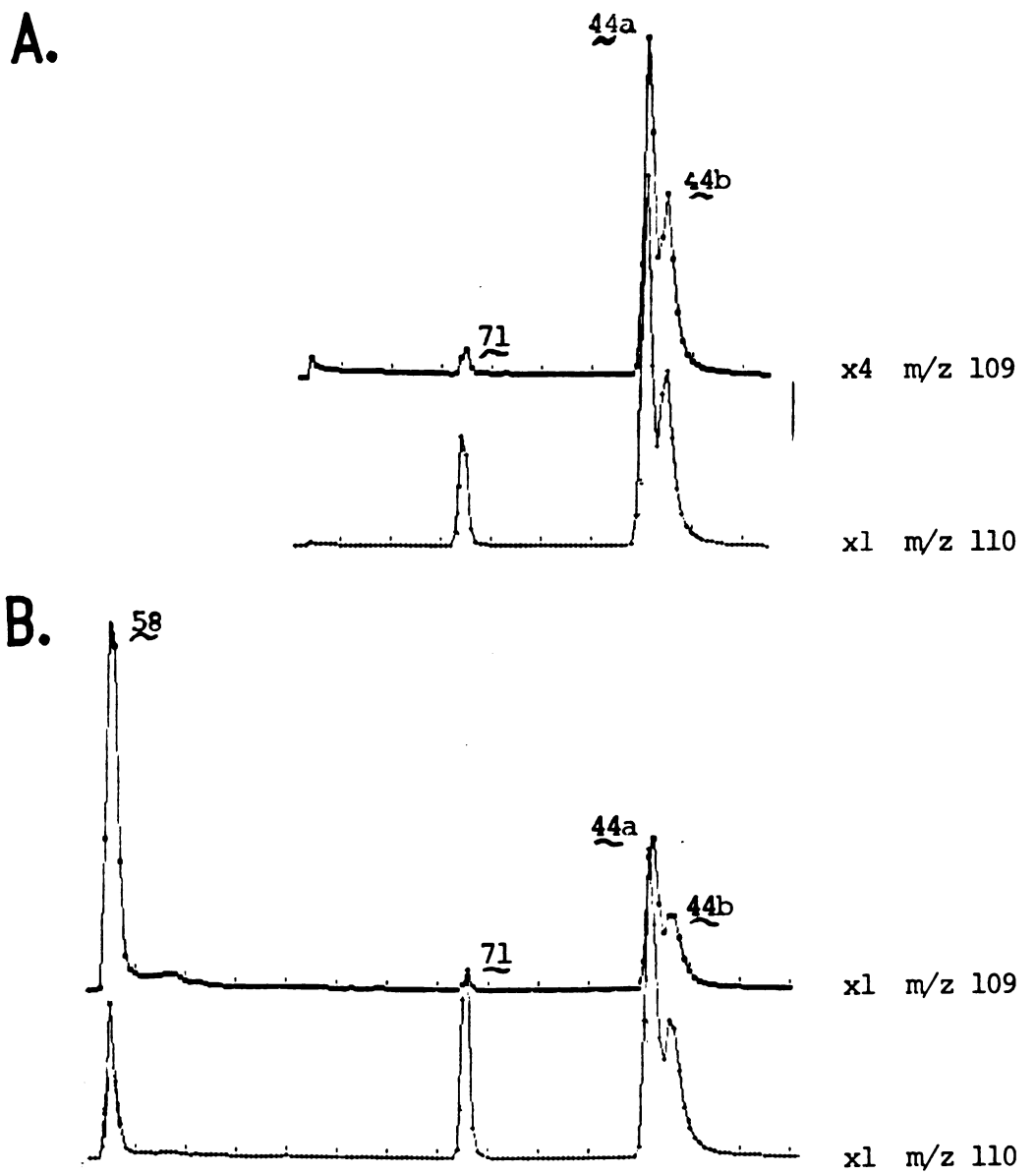
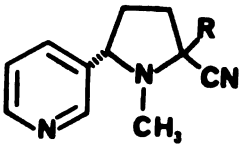


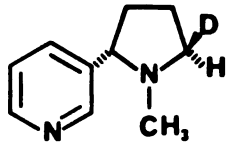
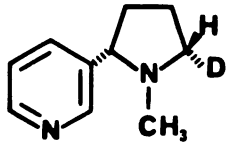
Figure 56. The mass chromatographs of the dichloromethane extracts obtained from the oxidation of the monodeutero diastereomers, (S)-nicotine-5'-d (A.) and (S)-nicotine-(Z)-5'-d (B.) by ethyl peroxide catalyzed by chloroperoxidase. (S)-Nicotine-(E)-5'-d , 57; (S)-nicotine-(Z)-5'-d , 58; 2'-cyanonicotine, 71; and (E)- and (Z)-5'-cyano-nicotines, 44a and 44b, respectively.

metabolite was observed eluting between nicotine and nornicotine. Only trace amounts of cotinine were observed. No detectable oxidation products were observed when the enzyme was excluded from the incubation mixture. Only trace levels of the products were observed in the incubations lacking ethyl peroxide. These observations demonstrate that the formation of cyanoadducts is dependent on the complete system and that the 5'-position was the major site of oxidation.

The ion currents at m/z 109 and 110 were measured to determine the stereochemical course of the chloroperoxidase-mediated reaction. The results are presented in Table 25. These data demonstrate that the catalysis of the oxidation at the 5'-position by chloroperoxidase leads to removal of 94% of the (Z)-5'-proton of 57 and 72% of the (E)-5'-proton of 58. The slight preference for removal of the (Z)-5'-hydrogen atom suggests that the reaction is under some stereochemical control. However, the choice of the 5'-hydrogen atoms is determined by deuterium isotope effects.

Table 25. The deuterium composition of the 5'-cyanonicotines isolated from incubations of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 with chloroperoxidase and ethyl peroxide (n=2).*



Substrate	% R=H	% R=D ± s.d.
	6	94 ± 0.2
	28	72 ± 0.2

*The data has been corrected for the stereochemical and isotopic impurities of 57 and 58 (see Section II.C for details).

The enhanced proton versus deuteron loss reveals a significant isotope effect for this reaction, demonstrating that the breakage of the C-H bond is a rate-determining step in the oxidation of the 5'-position. These results, like those obtained for the reaction catalyzed by the other hemoprotein models, differ significantly from the results of the cytochrome P-450 catalyzed process. The differences imply that the peroxidase and mono-oxygenase mediated reactions proceed through different mechanisms. These observations are at variance with the conclusions reached by Miwa et al. that the chloroperoxidase and cytochrome P-450 catalyzed N-demethylation of N-methyl-N-trideuteromethylaniline follow the same reaction pathway.[20] If the chloroperoxidase and cyto-

chrome P-450 catalyzed 5'-α-carbon oxidation of 22 share a common reaction mechanism, our results suggest that 22 binds very differently at the active site of chloroperoxidase than at the active site of cytochrome P-450. The molecule must be positioned such that the hydrogen abstractor is situated between the two 5'-protons.

E. Methylene Blue-Sensitized Photo-oxidation of (S)-Nicotine.

1. Background.

Next we explored the chemical mechanism of the cytochrome P-450-mediated oxidation of 22 by studying the diastereoselectivity of the 5'-α-carbon oxidation of 22 in two chemical model systems. The first reaction studied was the methylene blue (MB)-sensitized photochemical oxidation of 22. This type of reaction is believe to proceed by initial one-electron abstraction from the nitrogen atom as proposed for the cytochrome P-450 catalyzed α-carbon oxidation of tertiary amines.[204]

The mechanism proposed by Hubert-Brierre et al.[204] for the methylene blue-sensitized photo-oxidation of tertiary amines is outlined in Figure 57. The first step in the reaction involves the activation of the dye by uv light. The triplet state methylene blue (140) interacts with the amine in an electron transfer process that leads to the radical species 93 and 141. Proton loss generates the carbon-centered radical 94 which undergoes a second electron transfer to yield the reduced dye (142) and the oxidized amine as the iminium ion. Although the rates of such α-carbon oxidations are slow under anaerobic conditions, only catalytic amounts of methylene blue are required in the presence of O_2. Dioxygen is believed to reoxidize the reduced form of methylene blue as it is reduced to hydrogen peroxide.[205] Other investigators have proposed that the α-aminoalkyl radical 94 is further oxidized by oxygen to the products as is the dye-derived radical.[206,207]

Evidence for radical intermediates in methylene blue-sensitized photo-oxidations also comes from flash photolysis studies on the anaerobic photoreduction of this dye by tertiary amines.[206,208] Flash photolysis in the absence of oxygen resulted in the formation of a transient

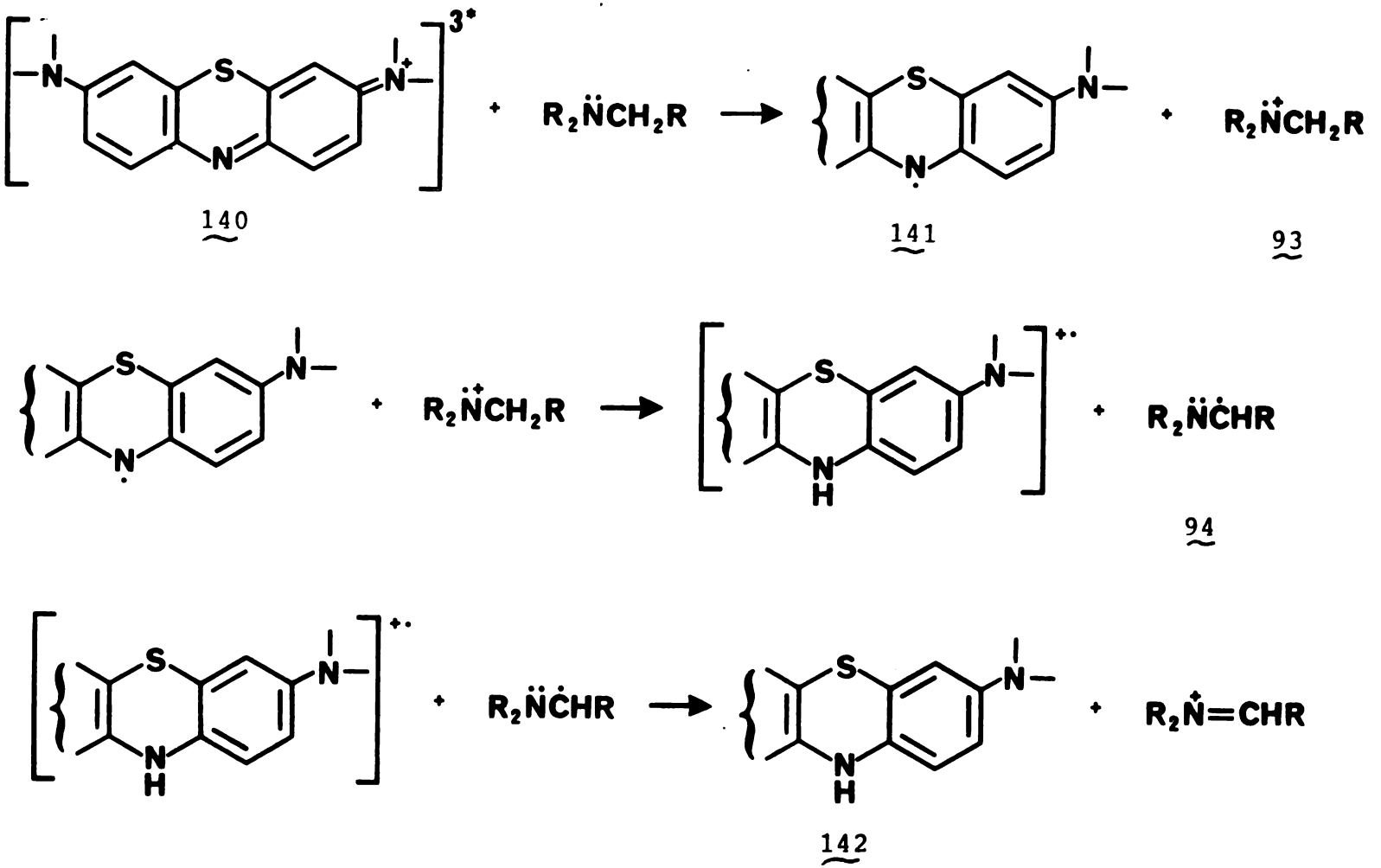
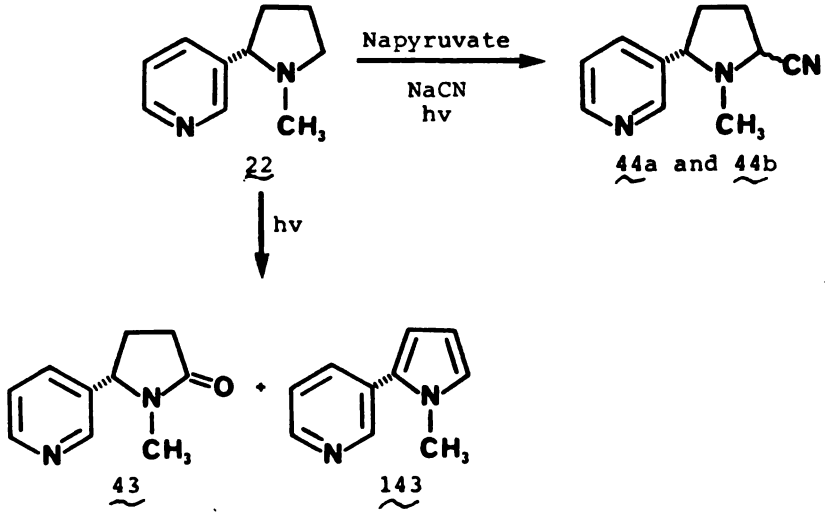


Figure 57. Proposed mechanism for the methylene blue photosensitized oxidation of tertiary amines.[204]

species (λ_{max} 410 and 430 nm). This intermediate was not observed when the amines were excluded from the reaction mixture. The identity of this species is the semiquinone of MB presumably formed by electron transfer from the amine to triplet MB (141). The reaction was then proposed to proceed via H^+ transfer from the aminium radical to the semiquinone.[208] In addition, a flash photolysis study of the photoreduction of methylene blue by a series of alkylamines demonstrated a linear relationship between the log of the rate constant for the interaction of the triplet dye with the amine and the ion potentials for the amines. This finding has been used as evidence for the involvement of a partial charge-transfer intermediate.[206]

A role for iminium ion intermediates in this process was suggested by Hubert-Brierre et al. who isolated α-cyanoamines from dye-sensitized oxidations of a number of aliphatic amines performed in the presence of sodium cyanide.[91,204,205,209] For example, irradiation of a methanolic solution of 22 containing sodium cyanide and sodium pyruvate in the presence of O_2 and methylene blue led to the formation of the 5'-cyanonicotines 44a and 44b in a 79% yield. Cotinine (43) and β-nicotyrine (143) were the major products when sodium cyanide and sodium



pyruvate were excluded from the reaction mixture (sodium pyruvate was used as a hydrogen peroxide trap and had no effect on the reaction's regioselectivity).[91] These results suggest that the iminium ion 42 was an intermediate in the photooxidation of 22 to cotinine and β-nicotyrine. Similar results were obtained for other tertiary amine alkaloids.[91,204,205]

In summary, the evidence supports a mechanism involving a one-electron transfer from the tertiary amine's nitrogen lone electron pair to the triplet state dye. The aminium radical loses a proton to generate an α-aminoalkyl radical. This carbon-centered radical can either lose another electron to the semiquinone dye to form the fully reduced dye and an iminium ion or it can be further oxidized by oxygen. This involvement of aminium radical intermediates in this process and the similar regioselectivity of the dye-sensitized oxidation of 22 and the cytochrome P-450 catalyzed oxidation of 22 led us to investigate the diastereoselectivity of the reaction with the monolabeled compounds 57 and 58.

2. Results.

The stereochemical course of the methylene blue sensitize photooxidation of 22 was investigated following the procedure described by Hubert-Brierre et al.[91] Initial studies were carried out using unlabeled nicotine as the free base and the bistartarte salt to develop conditions that would allow the investigation of this question with the bistartrate salts of 57 and 58. The results of the various coditions investigated are summarized in Table 26. Irradiation of methanolic solutions of 22 in the presence of catalytic amounts of methylene blue did not lead to product formation unless dioxygen was bubbled through

the reaction mixture. Product formation was estimated by ^{1}H-NMR analysis of chloroform extracts of the reaction mixtures. The reaction also required the free base as indicated by the lack of oxidation under acidic conditions. The stereochemical studies were performed at pH 8.8-9 instead of 11 since we observed the presence of enamine at the higher pH (see Table 26).

Table 26. Conditions for methylene blue-sensitized photo-oxidation of (S)-nicotine.

Reactant	Solvent	O_2	Results
22	methanol	-	no 44a and 44b after 4.5 hours
22	methanol	+	44a and 44b after 1.75 hours
22 bistartrate	methanol	+	no reaction, solubility problems
22 bistartrate	H_2O pH 3.6	+	no reaction after 2.5 hours
22	H_2O pH 9.4	+	44a and 44b after 2.5 hours
22 bistartrate	H_2O pH 11	+	44a and 44b plus enamine
22 bistartrate	H_2O pH 8	+	44a and 44b

Determination of reaction products made by NMR analysis of the CH_2Cl_2 extracts of the reaction mixtures.

The reaction mixtures were irradiated with a mercury lamp for one hour with O_2 bubbling through the solution. As estimated by ^{1}H-NMR analysis of the reaction mixture, there was approximately a 3:2 mixture of the 5'-cyanonicotines and nicotine after this time period (some nicotine was probably lost during the irradiation period since the samples were heated by the uv lamp and O_2 was bubbling through the mixture).

The mass chromatographs displayed the 5'-cyanonicotines and N-cyanomethylnornicotine in a ratio of 3:1 (Figure 58). No 2'-cyanonicotine was detected in these studies. Hubert-Brierre et al. reported the formation of only the 5'-cyanonicotines.[91] They ran their reactions in methanol instead of aqueous buffer.

The stereochemical results were determined by GC-EIMS analysis of the cyanoadducts 44a and 44b formed in these incubations. The data are summarized in Table 27. These data demonstrate that 85% of the deuterium of 58 is lost during this oxidation whereas 98% of the deuteron is present in the reaction products of 57, i.e., the pro-(R) proton/deuteron is selectively lost from both 57 and 58 in this process. The diastereoselectivity of this reaction contrasts dramatically with that observed in the cytochrome P-450 catalyzed reaction where the pro-(S) hydrogen/deuteron was preferentially lost.

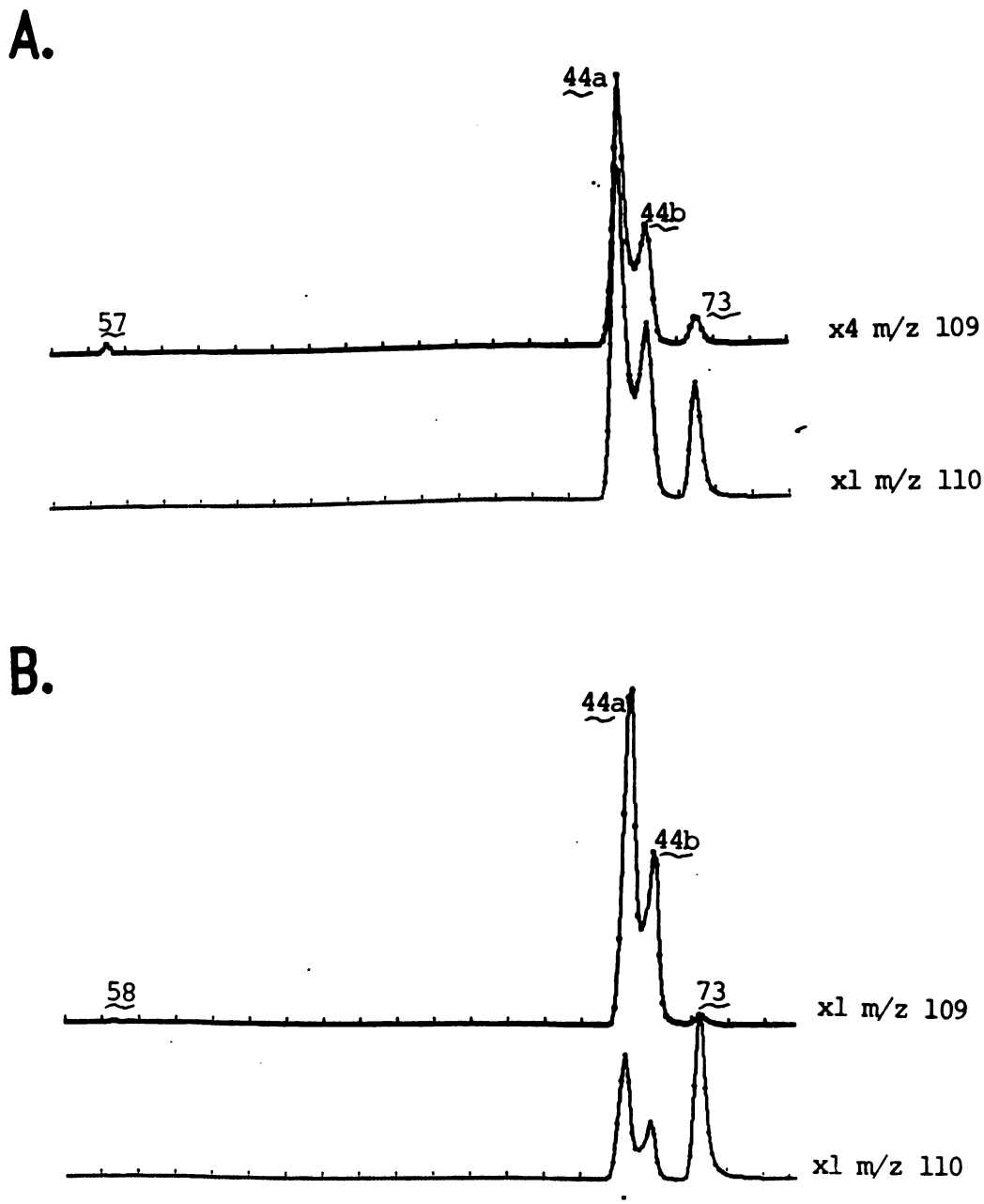
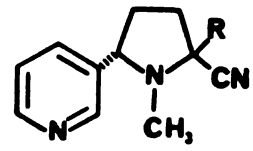
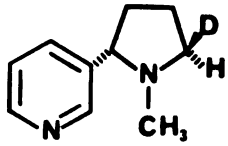
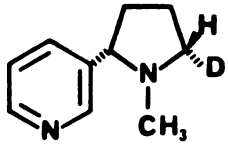


Figure 58. The mass chromatographs of the dichloromethane extracts obtained from the methylene blue-sensitized oxidation of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-(Z)-5'-d_1 (B.) in the presence of sodium pyruvate and sodium cyanide. (S)-Nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

Table 27. The deuterium composition of the 5'-cyanonicotines formed during the methylene blue-sensitized photo-oxidation of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 conducted in the presence of sodium cyanide and sodium pyruvate (n=2).



Substrate	% R=H	% R=D ± s.d.
	2	98 ± 1
	85	15 ± 2

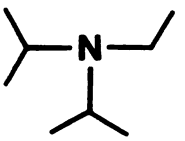
*The data has been corrected for the isotopic and stereochemical impurities of 57 and 58 (see Section II.C).

There is a tendency towards deuterium retention since the amount of proton loss in the oxidation of 57 (98%) is greater than the deuteron loss in the oxidation of 58 (85%)-suggestive of a modest deuterium isotope effect. Intermolecular isotope effects studies were conducted with a 1:1 or 2.2:1 mixture of 22-d_0 and 22-d_2. The deuterium contents of the cyano* adducts, which were determined by GC-EIMS analysis, indicated an isotope effect of 1.36 ± 0.02 (n=2). The isotope effect, in fact, may be larger since the N-cyanomethylnornicotine-5'-d_0:-d_2 ratio was 0.759 ± 0.034. The increased oxidation rate of the methyl group of 22-5',5'-d_2 versus 22-d_0 suggests that the presence of deuterium in the 5'-position shifts the oxidation to the N-methyl group to generate the

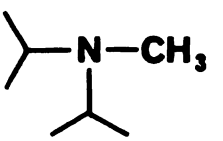
N-methylene iminium ion. No evidence for such metabolic shifting was observed with the monodeuteronicotine diastereomers. The observed intermolecular isotope effect values must be treated with caution because the conversion of starting material to the 5'-cyanonicotines was greater than 50% as determined by NMR analysis. The enrichment of deuterium in N-cyanomethylnornicotine may be due solely to a change in the deuterium composition of the starting material over the course of the reaction as unlabeled 22 is depleted. Since some nicotine probably was lost during the course of the experiment due to volatilization, it is difficult to determine precisely the extent of the reaction. A rough estimate is that about 10% of the substrate was converted to 42.

Our studies have demonstrated that the methylene blue-sensitized photooxidation of 22 proceeds with the selective loss (>85%) of the pro-(R) 5'-proton. A small isotope effect may contribute to the stereochemical course as indicated by the relative enrichment of the deuterium composition of the 5'-cyanonicotines derived from 58. Intermolecular isotope effect studies demonstrate a small isotope effect in the oxidation of the 5'-position. Mechanistic information from the intermolecular isotope effect studies is of little value due to the limited data obtained.

The selective loss of the pro-(R) proton in this photochemical oxidation may be explained in at least two ways. First, the loss of the proton following aminium radical formation is probably under stereoelectronic control. Lewis and Ho found that the regioselectivity of the photochemical oxidation of tertiary amines such as ethyldiisopropylamine (144) and methyldiisopropylamine (145) by singlet trans-stilbene was not dependent only on the relative acidity of the α-protons.[151] They argued that the loss of a proton from the aminium radical was under stereoelec-



144



145

tronic control. That is, the deprotonation proceeds in such a way that the p-orbital of the forming carbon radical must partially overlap with the half empty p-orbital of the planar aminium radical. Molecular models suggest that when the C-$H_{E-5'}$ bond of 22 is aligned with the half vacant p-orbital on the nitrogen atom, the pyridine ring and the N-methyl group are eclipsed (A, Figure 59). Thus, steric hindrance prevents maximal overlap of the orbitals when the C-$H_{E-5'}$ is removed. The interaction between the pyridine and N-methyl groups is minimal when the C-$H_{Z-5'}$ bond is broken, thus allowing maximal overlap of the p orbitals of the aminium radical and the forming carbon-centered radical (B, Figure 59). This hypothesis is consistent with the selective loss of the (Z)-5'-proton during the oxidation. This proposal also may explain, in part, the regioselectivity of the oxidation at the 5'-position since

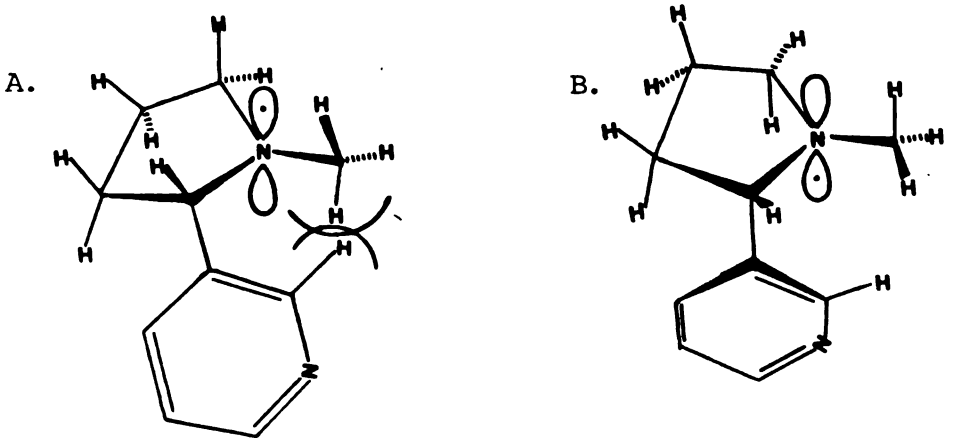


Figure 59. Stereoelectronic effects in the deprotonation of the aminium radical of (S)-nicotine.

the steric interactions between the pyridine and the methyl group also would prevent optimal overlap of the p-orbital generated when the 2'-proton is lost with the half empty aminium radical p-orbital.

π-π interactions between 22 and methylene blue may also play a role in directing the diastereo- and regioselectivity of this oxidation. A literature report of the relationship between the ease of oxidation of a series of tertiary aryl and alkylamines and the ionization potential demonstrated that arylamines were oxidized faster than predicted by their ionization potential.[206] This observation led to the proposal that π-π interactions between methylene blue and the phenyl group may enhance the oxidation rate of the arylamines over that of alkylamines by facilitating interactions between the dye and the amine.

Molecular models demonstrate that if there is a π-π interaction between the pyridine ring of 22 and the π-system of methylene blue, the pro-(R) proton will be closest to the methylene blue molecule (Figure 60). The proposed mechanism for these reactions involves an initial electron transfer from the nitrogen atom to methylene blue followed by proton transfer. If nicotine does interact with methylene blue via π-π-

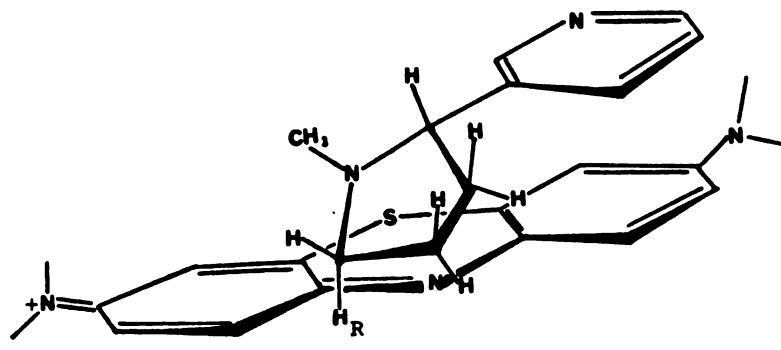


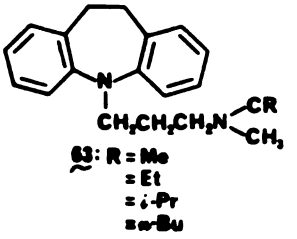
Figure 60. Proposed π-π interactions between (S)-nicotine and methylene blue.

interactions, our results are consistent with this proposal. The π-π interactions also would explain why the 2'-cyanonicotine is not formed since the 2'-proton would not be accessible for transfer to the semi-reduced dye.

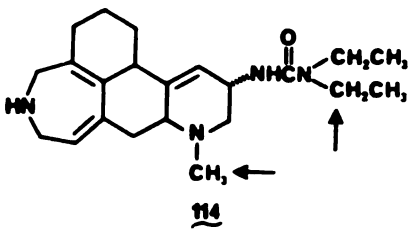
F. Electrochemical Oxidation of (S)-Nicotine.

1. Background.

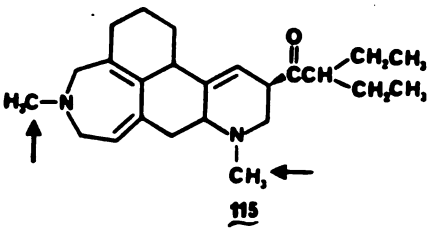
The proposal that the α-carbon oxidation reactions catalyzed by cytochrome P-450 and electrochemical oxidations of tertiary amines share a common reaction mechanism[19,153] led us to investigate the stereochemical course of the anodic oxidation of 22. This hypothesis is supported by intramolecular isotope studies with N-methyl-N-trideuteromethylimipramine that revealed that the values for these two processes were similar (K_H/k_D=1.64 and 1.88).[19] Additionally, intramolecular isotope effects for a number of microsomal α-carbon oxidation reactions also are modest (see Section III.A.).[20,59-61] Furthermore, the regioselectivity of electrochemical and cytochrome P-450-mediated processes are quite similar as revealed by studies with a series of N-methyl-N-alkylimipramine (63) derivatives[19] and a number of drugs including methysergide



63: R = Me
= Et
= i-Pr
= n-Bu



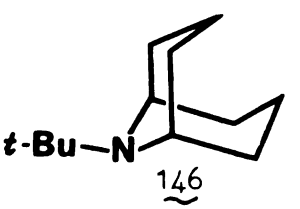
114



115

(114) and lisuride (115).[153] The anodic oxidations of tertiary amines are generally believed to proceed via initial one electron transfer from the nitrogen atom to the electrode to generate an aminium radical cation.[146] This species then loses a proton, generating an α-amino alkyl radical which then undergoes a one electron oxidation to an iminium ion.[146]

This reaction mechanism is preferred over direct abstraction an α-carbon hydrogen atom because of evidence provided from cyclic voltametric studies of aliphatic amines. Much of the evidence for aminium radical formation comes from the irreversibility of the oxidation of amines as demonstrated by cyclic voltametry.[210-213] Cyclic voltametry is an experimental method used to determine oxidation potentials (E°). A triangular potential vs. time wave is applied to a working electrode and the current passing through a solution is measured as a function of potential. The irreversibility of the oxidation of amines suggests that the species that is generated during the oxidation wave is not long lived enough to be present during the reduction cycle. The species has undergone some chemical reaction. It has been proposed that the amine substrate is oxidized by removal of 1 electron from the lone pair of the amine nitrogen. If there is an α-proton, it will be lost, generating a carbon centered radical. Since the applied potential is already above the oxidation potential of a carbon radical, this species readily yields an electron to the electrode leading to the formation of an iminium ion.[146] Aminium radicals have been detected only by cyclic voltametry when generated from compounds unable or only slowly able to lose an α-proton. A completely reversible wave has been reported for 9-tert-



146

butyl-9-azabicyclo[3.3.1]nonane (146).[146] Most aliphatic amines generate amine radical cations which are too reactive to be observed in a reduction wave.[146]

Electron transfer from the nitrogen atom to the electrode also is supported by the absence of any oxidation wave for acidic solutions at glassy-carbon electrodes. This observation, along with the pH dependence of the half peak potential ($E_{\frac{1}{2}p}$) ($E_{\frac{1}{2}p}$ decreases as pH increases, i.e., the oxidation occurs more easily) suggests that a free lone pair of electrons on the nitrogen atom is required for the oxidation to occur.[210] In addition, the peak potential of tertiary amines is also dependent on the electron withdrawing ability of the substituents. It becomes more positive with increasingly more electron-withdrawing substitutuents suggesting that the oxidation requires available nitrogen electrons.[210] Furthermore, a linear relationship was found between the first oxidation wave of a series of tertiary amines and their pK_a values.[210] These observations are consistent with the abstraction of an electron from the amino-nitrogen's lone pair of electrons.

Support for aminium radical formation followed by proton and not hydrogen atom loss comes from a number of controlled potential electrolysis studies where the oxidation products of asymmetric amines have been characterized. These studies demonstrate that the group lost in the N-dealkylation had the most acidic proton. The ease of oxidation of the resulting carbon-centered radical also plays a role in determining the regioselectivity of the oxidation[214,215] with the differences less pro-

nounced in nonaqueous media.[215] Steric factors also appear to be important in directing the regioselectivity of these reactions.[216] Thus, identification of the products formed during the electrochemical oxidation of 22 should allow us to determine the inherent reactivity of the various α-protons in a reaction proceeding via an aminium radical intermediate.

2. Results.

We employed the methods described by Chiba and Takata[216] for the electrochemical cyanation of a series of cyclic aliphatic tertiary amines to determine if the electrochemical oxidation of 22 was under regio- and stereochemical control. Initially, cyclic voltametry studies were performed on solutions of 22 in 0.2 M sodium cyanide in methanol-water (1:1) using a platinum electrode against a standard calomel electrode to determine if 22 was oxidized under these conditions. The oxidation was irreversible (see Figure 61). Two oxidation waves were observed; the first occurred at 1.1 V and the second at 1.5 V. The observation of two oxidation waves is consistent with an initial oxidation of the tertiary amine followed by further oxidation of the product. The value for the first wave is similar to those observed for tertiary amines under these conditions.[216] The second wave is presumably due to the oxidation of the cyanoadducts generated from reaction with the intermediate iminium ion. This hypothesis was proposed by Chiba and Takata.[216] An investigation of the cyclic voltagram of the nicotine cyano adducts would confirm this proposal.

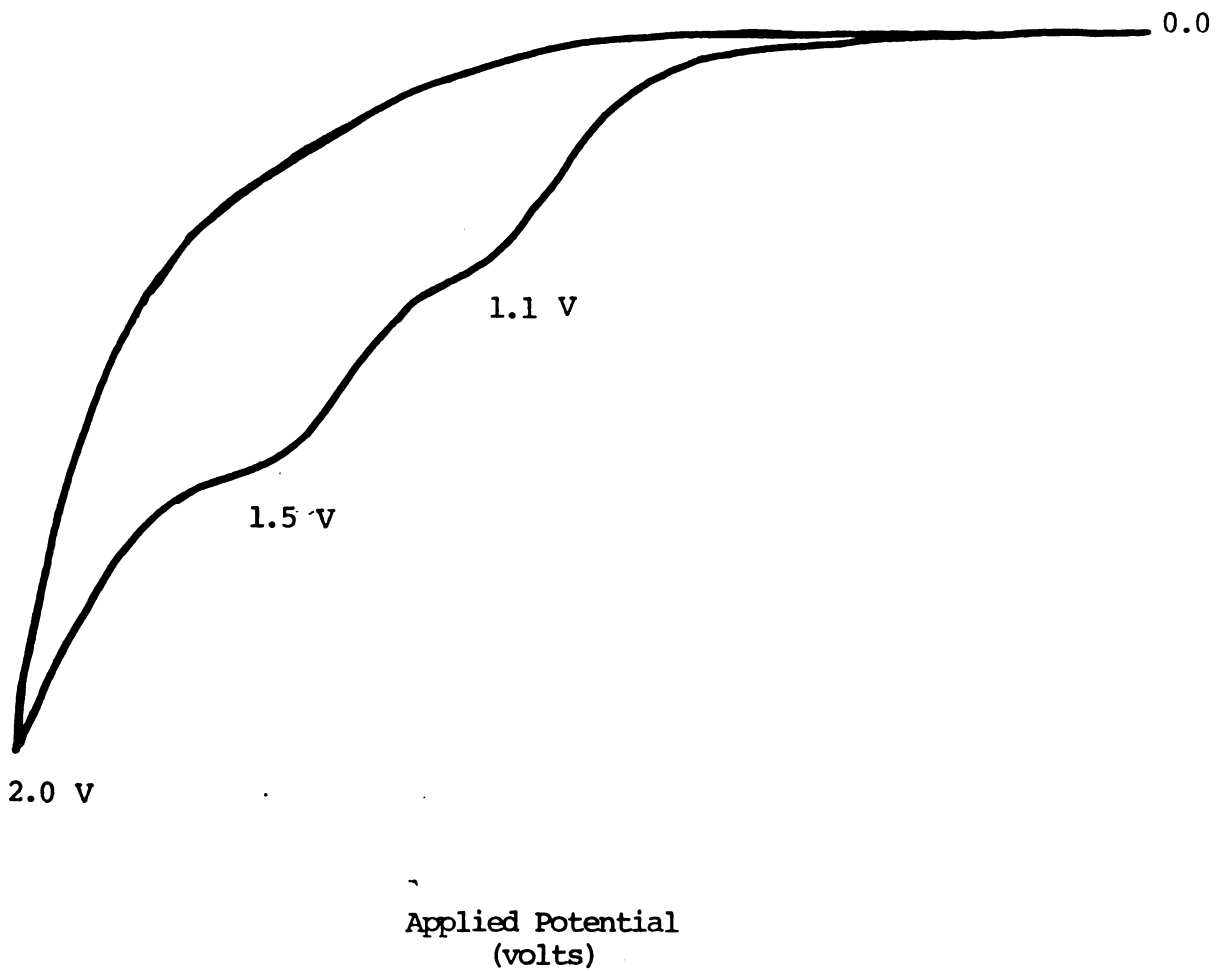


Figure 61. The cyclic voltammogram of a solution of (S)-nicotine (0.4M) in 0.2 M sodium cyanide in methanol-water (1:1) (scan rate: 200 mV/sec).

Controlled potential electrolysis at an applied potential of 1.2 V was performed using solutions of nicotine (0.4 M) in 0.2 M sodium cyanide in methanol-water (1:1) to determine the nature of the products generated during the first oxidation wave. Oxygen was not excluded from these studies. Analysis of ether extracts demonstrated the formation of 2'-cyanonicotine (13% ± 4), the diastereomeric 5'-cyanonicotines (69 ± 5%) and N-cyanomethylnornicotine (19 ± 3%, Figure 62). Nornicotine was also observed. The reaction profile was similar when solutions of the bistartrate salt of 22 (0.5 and 1 mM) were employed. The percentages of oxidation at the 2'-, 5'- and N-methyl position, were 4 ± 2%, 84 ± 4% and 12 ± 2%, respectively, based on GC peak areas. No nornicotine was detected with the bistartrate salt since the methylene iminium ion 86 was more efficiently trapped as the corresponding N-methyl cyano adduct 73. An unknown compound eluted just prior to nornicotine. GC-EI mass spectral analysis indicated that this compound was nicotine. GC-EIMS analysis of the dichloromethane extracts demonstrated that 2'-cyanonicotine was more readily extracted by this solvent than ether as suggested by the increase in this cyano adduct relative to 44a and 44b. The product ratio for the 5'-cyanonicotines:2'-cyanonicotine:N-cyanomethylnornicotine in the dichloroethane extracts was 6:3:1. The preferential oxidation of the 5'- and 2'-positions is consistent with reports that the oxidation of cyclic amines tend to occur at ring carbon atoms.[216] The extent of oxidation at the 5'-position was 5% as determined by the GC tracings.

The labeled 5'-(R) and 5'-(S)-deuteronicotines were then used to determine if the electrochemical oxidation of 22 exhibited any diastereoselectivity in the proton loss at the 5'-position. The controlled potential electrolysis was done using a 1 mM solution of the labeled

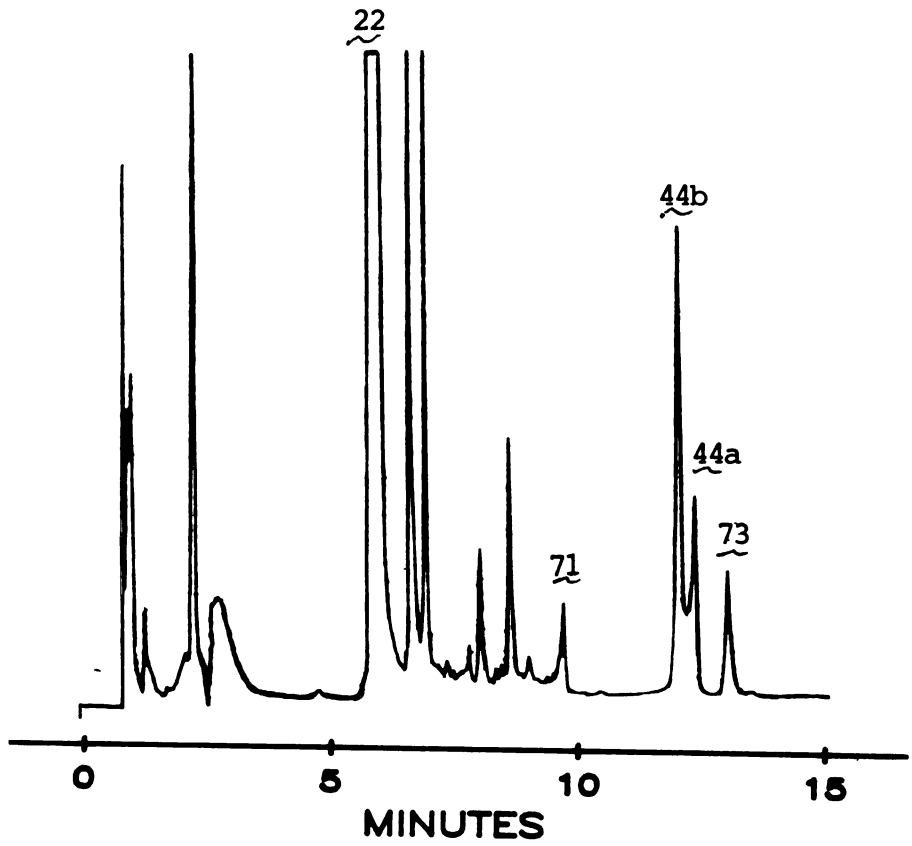
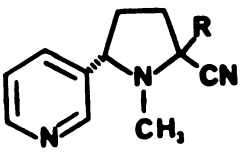
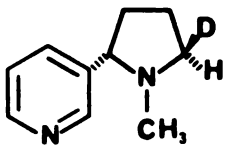
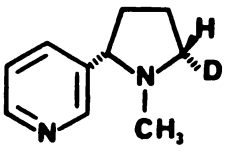


Figure 62. The capillary tracing of the dichloromethane extract obtained from the electrochemical oxidation of (S)-nicotine bis-l-tartrate in 0.2 M sodium cyanide in methanol-water (1:1). (S)-Nicotine, 22; 2'-cyanonicotine, 71; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

material at an applied potential of 1.2 V for 15 minutes at 0°-5° C. The dichloromethane extracts were analyzed by GC-EIMS in the selected ion mode to determine the deuterium content of the 5'-cyanonicotines generated during the electrolysis. The mass chromatographs of the extracts are displayed in Figure 63. The data (Table 28) show that 80% of the deuterons are retained during the oxidation of the 5'-position of 57 while only 37% are retained during the oxidation of 58. In both cases the pro-(R) proton is selectively removed. There is a slight preference for hydrogen abstraction when the 5'-pro-(R) position is labeled with deuterium. This observation suggests that a small kinetic isotope effect is influencing the choice between the pro-(R) and pro-(S) proton.

Table 28. The deuterium composition of the 5'-cyanonicotines formed in the electrochemical cyanation of nicotine-(E)-5'-d_1 and nicotine-(Z)-5'-d_1 (n=3).*



Substrate	% R=H	% R=D ± s.d.
	20	80 ± 0.5
	63	37 ± 1.0

*The data has been corrected for the stereochemical and isotopic impurities of 57 and 58 (see Section II.C for details).

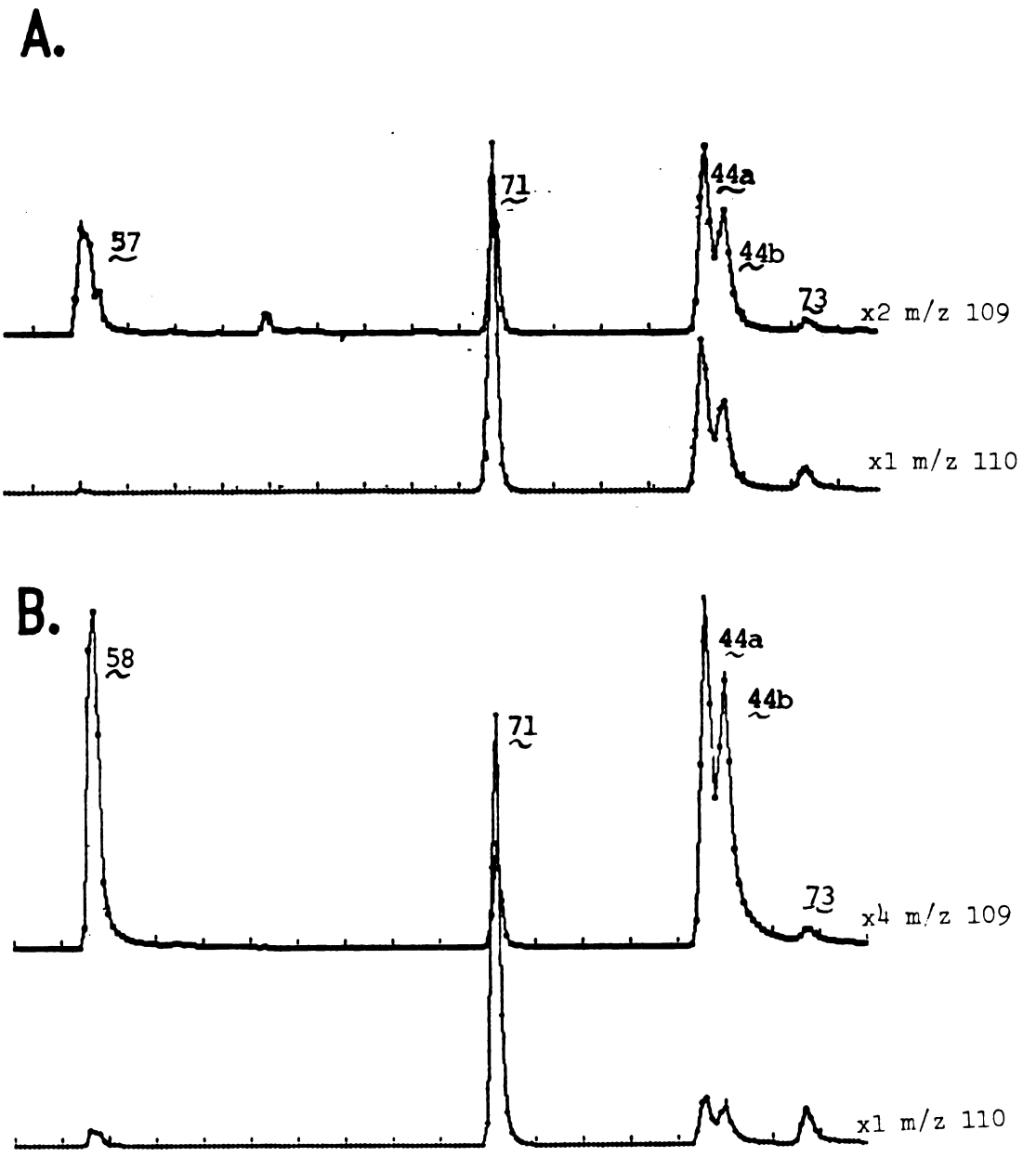


Figure 63. The mass chromatographs of the dichloromethane extracts obtained from the electrochemical oxidation of the monodeutero diastereomers, (S)-nicotine-(E)-5'-d_1 (A.) and (S)-nicotine-5'-(Z)-d_1 (B.) in 0.2 M sodium cyanide in methanol-water (1:1). (S)-Nicotine-(E)-5'-d_1, 57; (S)-nicotine-(Z)-5'-d_1, 58; 2'-cyanonicotine, 71; (E)- and (Z)-5'-cyanonicotines, 44a and 44b, respectively; and N-cyanomethylnornicotine, 73.

The role of isotope effects in the oxidation of the 5'-position was investigated further by measuring the deuterium content of the cyanoadducts generated during the competive oxidation of a 1:1 mixture of $\underset{\sim}{22}$-d_0 and $\underset{\sim}{22}$-5'-d_2. Following a 15 min electrolysis period, the reaction mixture was extracted with dichloromethane and the extract was analyzed by GC-EIMS monitoring the ion current at m/z 109, 110 and 111. The data (Table 29) indicate an isotope effect of 2.0 ± 0.2. The observation of an enrichment of deuterium in the other cyanoadducts suggests that the presence of deuterium in the 5'-position slightly shifts the oxidation to the 2'-position. Only a small amount of the reaction is shunted to the N-methyl group. This observation is consistent with the report of Chiba and Takata who found that tertiary pyrrolidine compounds are preferrably oxidized at an endocyclic carbon atom.[216]

Table 29. Deuterium kinetic isotope studies of the electrochemical oxidation of (S)-nicotine (n=3).

	unlabeled/labeled product
5'-cyanonicotines	2.0 ± 0.2
2'-cyanonicotine	0.67 ± 0.01
N-cyanomethylnornicotine	0.95 ± 0.01

The electrochemical oxidation of $\underset{\sim}{22}$ proceeded regioselectively at the 5'-position. A significant amount of oxidation also occurred at the 2'-position. the choice of the 2'- versus the 5'-position seems to be governed by the number of α-protons present at these positions. That is, the endocyclic regioselectivity is statistically determined. The preferential oxidation of the endocyclic positions is believed to be due

to steric effects.[216] Amines are believed to be absorbed onto the surface of the electrode prior to their oxidation.[216] The deprotonation then proceeds at positions closest to the anode surface.[216] Since the 5-membered ring is nearly planar for access, the ring α-carbon atoms can approach closer.[216]

The use of the monodeuterated diasteromers 57 and 58 demonstrated that the electrochemical oxidation of the 5'-position proceeds with the selective loss of the pro-(R) proton. This diastereoselectivity, as that observed in the photochemical oxidation of 22, is likely the result of stereoelectronic effects. The pro-(R) proton is selectively lost because, when this proton is removed, the forming p-orbital on the 5'-carbon has maximal overlap with the planar aminium radical (see Figure 59). One might conclude that the electrode does not govern which proton is lost in the oxidation process since the more hindered 5'-proton is selectively lost, that is, the proton cis to the pyridine ring. The N-methyl group may hinder the approach of the molecule to the electrode surface since it is trans to the pyridine on a time average basis in solution.[107] However, inversion at the nitrogen atom can occur to place the methyl moiety cis to the pyridine ring, allowing the lone pair of the nitrogen atom to approach the electrode surface. Consequently, the steric bulk of the pyridine moiety probably determines how the molecule approaches the electrode surface. Furthermore, the methyl group should have little influence on the stereochemical outcome of proton abstraction from the radical since the radical is planar. Thus, one would predict the pro-(S) hydrogen atom, which is trans to the pyridine group, would be selectively lost if the proton transfer step results in the loss of the atom closest to the electrode. Since we found that the pro-(R) proton, which is cis to the pyridine moiety, was selectively lost,

our result disagree with the conclusions of Chiba and Takata[216] that the positions closest to the electrode surface are preferentially lost.

This reaction is less regio- and stereoselective than the photochemical reaction. One possible reason is that deprotonation is more rapid in the electrochemical oxidation, so that stereoelectronic effects are not as important in this process as in the photosensitized reaction. Assuming that partial overlap of the p-orbital of the forming carbon radical with the p-orbital of the aminium radical is sufficient for deprotonation,[151] the 2'- and 5'-pro-(S) protons also can be lost. Also, it is possible that interactions between substrates and the dye in the photosensitized reaction play a significant role in directing the course of the reaction.

The stereochemical course of this oxidation is slightly altered by the presence of the label in the pro-(R) 5'-position, suggesting a small isotope effect for this reaction. Intermolecular isotope studies revealed an isotope effect of 2.0 ± 0.2. Since the oxidation was switched to the 2'-position to some degree when the 5'-position was labeled, the isotope effect for this oxidation may be greater than 2. Assuming that absorption onto the electrode surface is not rate determining, this value is consistent with a reaction mechanism involving aminium radical formation and not C-H bond breakage as the rate determining step.

G. Final Conclusions.

The stereochemical results from our studies on the cytochrome P-450 catalyzed oxidation of (S)-nicotine using the monodeuterated (S)-nicotine-5'-d_1 diastereomers, along with the stereochemical results from the model oxidizing systems are summarized in Table 30. The regioselectivity of the various oxidation reactions also are included. In addition, an apparent isotope effect for 5'-proton versus 5'-deuteron abstraction from the monodeuterated nicotine diastereomers 57 and 58 was determined by dividing the sum of the fraction of labeled 5'-cyanonicotines formed from 57 and 58 (proton abstraction) by the sum of the fraction of the unlabeled 5'-cyanonicotines formed from 57 and 58 (deuteron abstraction).

In our investigations of the stereochemical course of the human and rodent liver microsomal enzyme catalyzed oxidation of (S)-nicotine to the iminium metabolite 42, we found that the pro-(S) hydrogen atom is selectively lost in a process which appears to be relatively uninfluenced by deuterium isotope effects (see Table 30). In all cases, the cytochrome P-450 catalyzed α-carbon oxidation also was quite regioselective for the 5'-position. The preferential loss of the pro-(S) proton and the marked regioselectivity exhibited by this reaction have led us to propose that the nature of the binding of 22 at the enzyme's active site is a major determinant in the overall course of this reaction.

The results obtained from a series of model systems (Table 30) are consistent with this proposal. Substitution of iodosobenzene and cumene hydroperoxide for NADPH/O_2 in the cytochrome P-450-catalyzed oxidation had no effect on the regioselectivity of the enzymatic reaction. While there were slight oxidant-dependent variations in the

Table 30. A summary of the stereoselectivity and regioselectivity observed in the oxidation of (S)-nicotine by microsomal preparations and model systems.

Study	Stereoselectivity: Deuterium Content of 44a and 44b — Substrate	R=H	R=D ± s.d.	Regioselectivity: 5':N-CH_3:2'	"isotope effects": labeled 44a and 44b from 57 and 58 / unlabeled 44a and 44b from 57 and 58
Human liver Microsomes (n=20)	57	90	10 ± 10	9.9: <0.1:0	1.02
	58	11	89 ± 4		
New Zealand Rabbit Microsomes (phenobarbital-pretreated) (n=11)	57	92	8 ± 2	1: 1 :0	1.00
	58	8	92 ± 2		
Rat Microsomes (untreated) (n=2)	57	93	7 ± 1		.90
	58	12	88 ± 0		
Dutch Rabbit Microsomes NADPH/O_2 (n=38)	57	74	26 ± 5	9: 1 :0	1.20
	58	17	83 ± 2		
Dutch Rabbit Microsomes Cumene Hydroperoxide (n=8)	57	78	22 ± 3	9: 1 :0	1.22
	58	12	88 ± 2		
Dutch Rabbit Microsomes Iodosobenzene (n=8)	57	55	45 ± 6	9: 1 :0	1.53
	58	32	68 ± 2		
TPPFeIIICl/Iodosobenzene (n=3)	57	55	45 ± 1	1: 1 :trace	1.30
	58	32	68 ± 1		
Methemoglobin/H_2O_2 (n=3)	57	29	71 ± 1	9:trace :1	2.00
	58	38	62 ± 1		
Horseradish peroxidase/H_2O_2 (n=3)	57	22	78 ± 5	9: 4.5 :1	2.70
	58	32	68 ± 7		
Chloroperoxidase/EtOOH (n=2)	57	6	94 ± 0	4:trace :1	4.88
	58	28	72 ± 0		
Methylene Blue-sensitized Photochemical Oxidation (n=2)	57	2	98 ± 1	3: 1 :0	1.30
	58	85	15 ± 2		
Electrochemical Oxidation (n=3)	57	20	80 ± 1	6: 1 :3	1.41
	58	63	37 ± 1		

diastereoselectivity of the 5'-α-oxidation, the trans-5'-proton/deuteron of the monodeuterated substrate probes was abstracted preferentially in all cases. These observations suggest that the interactions of (S)-nicotine with the active site of the monooxygenase have a greater influence on the stereochemical course of the oxidation than the source of the active oxygen species.

The selective loss of the trans-5'-proton/deuteron in the iodosobenzene/$TPPFe^{III}Cl$ experiments imply that the interaction of (S)-nicotine with the planar porphyrin system plays a significant role in directing the diastereochemical course of the cytochrome P-450-mediated reaction. On the other hand, the absence of regioselectivity with respect to the oxidation of the 5'- versus the N-methyl group when this synthetic catalyst is employed demonstrates that the protein environment of cytochrome P-450's heme pocket directs the regiochemical course of the enzymatic reaction. The enhanced regioselectivity exhibited by the cytochrome P-450-mediated process relative to that observed in the electrochemical and methylene-blue-sensitized photochemical oxidation of 22 further supports the conclusion that enzyme-substrate interactions are highly structured and dictate specific sites of oxidative attack.

Although steric interactions between the planar porphyrin ring and substrate must contribute to the stereochemical restrictions governing this 2-electron oxidation, studies with the hemoprotein models revealed that other factors also are involved in determining the stereochemical course of the cytochrome P-450-mediated process. In the model reactions, loss of the 5'-α-hydrogen atom versus deuterium atom of the monodeuterated substrates was determined primarily by deuterium isotope effects and not by stereochemical factors as was the case with the cytochrome P-450 catalyzed oxidation. One possible interpretation of

these differences between the peroxidase- and cytochrome P450-catalyzed reactions is that the enzymes do not catalyze the oxidation of (S)-nicotine by the same mechanism. Methemoglobin and horseradish peroxidase catalyzed N-methylation reactions are believed to proceed through a reaction pathway different than the cytochrome P-450 catalyzed reaction.[20] Our results are consistent with this proposal. Another possibility is that, due to the restricted nature of the heme pocket of these two hemoproteins,[199,217,218] (S)-nicotine is unable to approach the heme iron in the same manner that it does with the active site of cytochrome P-450 and $TPPFe^{III}Cl$.

Chloroperoxidase, on the other hand, is proposed by others to have an active site very similar to that of cytochrome P-450[195] and to follow a similar mechanism for the N-dealkylation of N,N-dimethylaniline.[20] However, the dramatic difference in the diastereoselectivity of 5'-α-carbon oxidation of (S)-nicotine exhibited by cytochrome P-450 and chloroperoxidase may be due to differences in the way the substrate binds to the active sites of these two enzymes or may reflect differences in reaction pathways.

All of the model systems demonstrate that cytochrome P-450 strongly controls the regio- and stereochemical course of the oxidation. It is difficult, however, to extract mechanistic information about the cytochrome P-450-catalyzed reaction since none of the models truly mimics the enzymatic process. The chemical model studies demonstrate that the (Z)-5'-proton/deuteron is selectively lost. The two chemical reactions studied are thought to involve aminium radical intermediates. Since the cytochrome P-450 catalyzed reaction results in the selective loss of the thermodynamically disfavored (E)-5'-proton, one might conclude that the

enzymatic process does not involve initial aminium radical production. This conclusion, however, assumes that the molecule is unrestricted at the active site so as to allow the loss of the thermodynamically preferred proton. Differences in regio- and diastereoselectivity between the chemical model systems and cytochrome P-450 indicate that the enzyme does impose considerable constraint on the substrate. Consequently, the conformation of an enzymatically-formed aminium radical may be different from that generated in the chemical systems, and this conformation favors the loss of the pro-(S) proton. This may be the case in the oxidation of (S)-nicotine by cytochrome P-450 since the diastereoselectivity of both the chemical and the mono-oxygenase-mediated reactions are relatively uninfluenced by deuterium isotope effects.

One might also expect that the loss of the proton from the putative aminium radical intermediate would be assisted by a basic site provided by the protein. Thus, the proton closest to such a "hydrogen abstractor" would be preferentially removed. This hypothesis is supported by the enhanced regio- and stereoselectivity of the methylene blue-sensitized photochemical oxidation. It is resonable to postulate that π-π-interactions between the dye and the pyridine ring of (S)-nicotine may orient the aminium radical intermediate in such a way that the pro-(R) 5'-proton is closer to the dye than the pro-(S) 5'-proton or the 2'-proton. This would account for the regio- and diastereoselectivity which accompanies this oxidation. If (S)-nicotine approaches the porphyrin system with the bulky pyridine pointed away from the planar surface as shown in Figure 64, the pro-(S) 5'-hydrogen atom comes closest to the postulated active oxygen species. However, it should be noted that the direct abstraction of a hydrogen atom from (S)-nicotine itself also would require this type of approach. Nevertheless, a

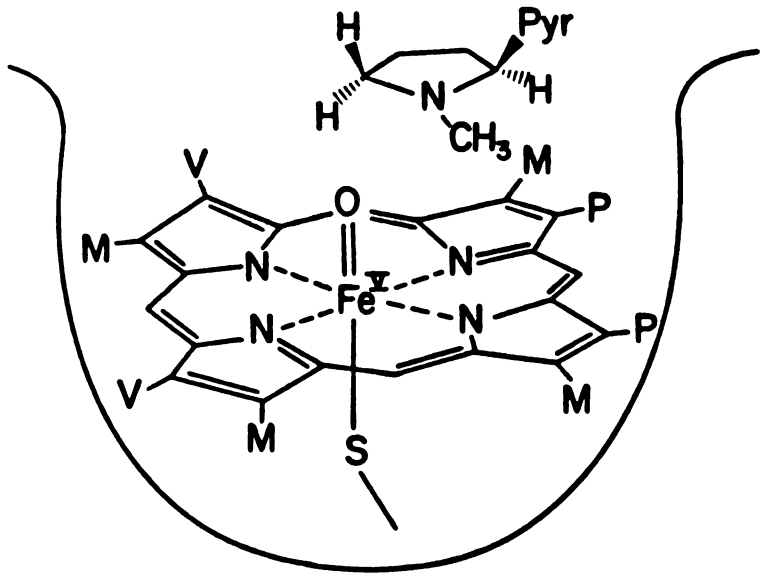


Figure 64. Proposed mode of binding of (S)-nicotine at the active site of cytochrome P-450.

hydrogen abstraction mechanism would be expected to result in a large isotope effect when the pro-(S) 5'-position is labeled with deuterium as has been observed for carbon hydroxylation reactions.[62,70,142] Such an isotope effect might alter the stereochemistry of 5'-hydrogen/deuterium atom abstraction. We found that the stereochemical course of the 5'-α-carbon oxidation in the microsomal studies was relatively unaffected by deuterium isotope effects. However, the diastereotopic relationship of the 5'-hydrogen atoms prevents us from observing true "intramolecular" isotope effects which would be useful in making mechanistic interpretations. Therefore, the consequence of an isotope effect might be suppressed since these hydrogen atoms will react through diastereomeric transition states. Furthermore, any isotope effect will be further suppressed if the choice of the 5'-protons is determined upon binding of the molecule to the enzyme's active site.

In conclusion, we have demonstrated that the cytochrome P-450-catalyzed α-carbon oxidation of (S)-nicotine is highly regio- and stereoselective and involve the loss in the removal of the pro-(S) 5'-hydrogen atom. The results obtained in the model studies revealed that the nature of the interaction of (S)-nicotine with the active site of the mono-oxygenase is important in controlling the regio- and stereochemical course of the reaction. While mechanistic interpretations are not possible from our studies, they indicate that the complex formed between (S)-nicotine and cytochrome P-450 is a highly constrained system.

Chapter IV

EXPERIMENTAL SECTION

General

(S)-Cotinine,[219] (R,S)-nornicotine,[113] N-cyanomethylnornicotine,[30] (S)-nicotine-5'-d_2,[86] iodosobenzene,[220] (S)-nicotine-N'-oxide,[132] and 5-methylnicotine[117] were prepared as described previously. 5-Methylcotinine, N-ethylnornicotine, and N-methyl-N-(3-pyridyl)methyl propanamide were gifts from Dr. P. Jacob, III, San Francisco General Hospital, San Francisco, Ca. All other chemicals were reagent grade or, in the case of dichloromethane used for GC-EIMS analysis, HPLC grade. Bovine methemoglobin, chloroperoxidase, horseradish peroxidase (Type IV), NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Sigma. Proton NMR spectra were recorded either on a Varian FT-80 (80 MHz) or a home-built 240 MHz NMR instrument linked to a Nicollet 1180 computer. Unless otherwise indicated, the spectra were recorded in $CDCl_3$. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard; s=singlet, d=doublet, t=triplet, m=multiplet, and br=broad. Melting points were measured on a Thomas Hoover melting point apparatus and are uncorrected. Elemental analyses were determined by the Microanalytical Laboratory of the University of California, Berkeley, California.

Analytical GC was performed on a Hewlett Packard 5880A series instrument with a nitrogen/phosphorous detector utilizing a 25 m x 0.2 mm i.d. silica SE 54 capillary column (Hewlett Packard) with helium as the carrier gas. Preparative HPLC was carried out on a Beckman model 100A pump with a Whatman M9 Partisil column linked to an Altex model 153

ultraviolet (UV) detector. Analytical HPLC employed an Altex 110A pump connected to a Lichrosorb SI-60 silica gel column (Altex Scientific Inc.) and a Hitachi 100-10 variable UV detector. GC-EIMS was performed on a J&W 30 m x 0.325 mm i.d. DB5 fused silica capillary column (1 μ) with a Varian 3700 instrument linked to a Kratos MS25 instrument.

The photochemical experiments were carried out with a Hannovia medium pressure mercury lamp (450 Watt) contained in a quartz immersion well with a pyrex insert filter. The electrochemical experiments were conducted with a BAS voltammograph connected to a Soltec VP-64145 x-y recorder.

(E)- and (Z)-5'-Cyanonicotine (44a and 44b).[57,58]

A 70% solution of sodium dihydrobis(2-methoxyethoxy)aluminate (4.3 g., 14.4 mmol) in dry ether (50 mL) was added dropwise to an ice-cooled, stirred solution of cotinine (3 g., 17 mmol) in ether (60 mL) in a flame dried flask under nitrogen over 30 minutes. The reaction mixture was allowed to stir in the ice bath for 3 hours. An aqueous solution (50 mL) of KCN (6 g., 91.2 mmol) was added slowly. The pH was adjusted to 5-7 with acetic acid. The reaction mixture was allowed to warm to room temperature and was made basic with a saturated K_2CO_3 solution and extracted three times with ether (50 mL). The extracts were combined and dried over K_2CO_3 (anhyd.). After filtration and removal of solvent under reduced pressure, a yellow oil was obtained. The oil was purified on a silica gel column using an ether:ammonia (2000:1) system as the eluent. The product was isolated as a pale yellow oil in 62% yield: b.p. (0.8 mmHg) 125° C. The 80 MHz ^{1}H-NMR spectrum (Figure 4) was identical to that reported by Hubert-Brierre et al.[91]: δ 1.5-2.5 (complex m, 4H, C3' and C4'), 2.3 and 2.32 (2s, 3H, N-CH_3 of the (E)- and

(Z)-isomers), 3.5 (complex m, 1.4H, C2' and 5'H of the (E)-isomer), 4.1 (d of d, 0.6H, 5'-H of the (Z)-isomer), 7.28 (m, 1H, C5), 7.6 (m, 1H, C4), 8.55 (m, 2H, C2 and C6). Separation of the diastereomers was acheived by analytical HPLC on a normal phase column using ether-ammonia as the eluent (flow: 1 mL/min): (E)-5'-cyanonicotine: ^{1}H-NMR: δ 1.5-2.5 (complex m, 4H, C3' and C4'), 2.32 (s, 3H, NCH_3), 3.5 (t, 1H, C2'), 4.1 (br d, 1H, C5'), 7.28 (m, 1H, C5), 7.6 (m, 1H, C4), 8.55 (m, 2H, C2 and C6); MS, 187 (M^+), 109 (100%); (Z)-5'-cyanonicotine: ^{1}H-NMR: δ 1.5-2.5 (complex m, 4H, C3' and C4'), 2.30 (s, 3H, NCH_3), 3.5 (complex m, 2H, C2' and C5'), 7.28 (m, 1H, C5), 7.7 (m, 1H, C4), 8.55 (m, 2H, C2 and C6); MS: 187 (M^+), 109 (100%). The NMR spectra of the (E)- and (Z)-5'-cyanonicotines are shown in Figures 8 and 9, respectively. The mass spectra are shown in Figure 6.

Nicotine Δ-$^{1'(5')}$-Iminium Diperchlorate (42).[58]

A 70% perchloric acid solution (2.6 mL, 30.2 mmol) was diluted with ethanol (10 mL) and added slowly to an ice-cooled ethanolic solution (20 mL) of 5'-cyanonicotine (2.6 g., 13.8 mmol). The white crystals which precipitated out were filtered, washed with acidic methanol, and recrystallized from acidic methanol to yield 2.3 g. (41.7% yield) of product: m.p. 237-241° C with decomposition (lit.[58] 230°-232° C). The 80 MHz ^{1}H-NMR spectrum in 10% DCl in D_2O (see Figure 10) agreed with that reported previously:[58] δ 2.0-2.35 (complex multiplets, 4H, C3' and C4'), 3.28 (br s, 3H, N-CH_3), 7.5-9.0 (m, 5H, 4 aromatic pyridine H and C5').[58]

Anal. Cal'd for $C_{10}H_{15}N_2Cl_2O_6$: C, 33.24; H, 3.91; N, 7.75. Found: C, 33.12; H, 3.90; N, 7.69.

(S)-Nicotine-5'-(E)-d_1 (57).

Platinium oxide (700 mg) was suspended in a 2.5% DCl in D_2O solution (70 mL) with stirring under one atmosphere of D_2 for one half hour. The substrate, 42 bisperchlorate (7.1 g, 17.3 mmol) was added and more D_2 was introduced. The reaction mixture was allowed to stir at room temperature for 3.5 hours and then was filtered through a celite pad. The filtrate was made basic with a saturated K_2CO_3 solution, extracted with dichloromethane (5x70 mL), dried (K_2CO_3), and filtered. The solvent was removed at atmospheric pressure and a yellow oil (4.34 g) was obtained: 240 MHz ^{1}H-NMR: δ 1.5-2.1 (complex m, 4 H, C3' and C4'), 2.2 (s 3H, N-CH_3), 3.1 (t, 1H, C2'), 3.3 (br d, 0.86H, $H_{5'Z}$), 7.3 (m, 1 H, C5), 7.6 (d of t, 1H, C4), 8.3-8.5 (m, 2H, C2 and C6); MS: m/z 163 (M^+, 40%), 134 (46%), 85 (100%). An expansion of the ^{1}H-NMR spectrum is shown in Figure 13 and the mass spectrum is shown in Figure 14.
The di-(l)-tartrate of 57 (5.16 g, 56.8% overall yield) was prepared as reported by Aceto et al.[105]: m.p. 138.5-139.5° C (lit.[105] 138-139.5° C); ^{2}H-NMR (D_2O): δ 3.4 ppm. The ^{2}H-NMR spectrum is shown in Figure 15.
Anal. Calc'd for $C_{18}H_{25}DN_2O_{12}$: C, 46.65; H, 5.87; N, 6.04. Found: C, 46.44; H, 5.85; N, 5.79.

Mercuric Acetate Oxidation of Nicotine.[91]

A stirred solution of (S)-nicotine (2.0 g, 12.4 mmol) and mercuric acetate (15 g, 47 mmol) in 50 mL 5% acetic acid solution was heated at 80° C for 2 hours. After cooling, the reaction mixture was filtered. Hydrogen sulfide was bubbled through the ice-cooled filtrate and a black precipitate formed. The precipitate was removed by filtration through a celite pad in the presence of carbon black. This process was repeated until no more precipitate formed. The solution made basic with sat-

urated K_2CO_3 solution and stirred for 1 hr following the addition of KCN (1.7 g, 26 mmol). The resulting mixture was extracted with ether (3x50 mL) and the combined organic extracts were dried ($MgSO_4$) and concentrated to give 1.04 g of crude product. This mixture was separated by column chromatography (silica gel, 30 g) with ammonia saturated ether-hexane (2:3) to give 22.8 mg of 2'-cyanonicotine (1% yield): ^{1}H-NMR ($CDCl_3$): δ 1.9-2.4 (m, 3H), 2.24 (s, 3H, N-CH_3), 2.4-2.9 (m, 2H), 3.1-3.5 (m, 1H), 7.25 (q, 1H, C5), 7.9 (d of t, 1H, C4), 8.6 (d, 1H, C6), 8.85 (m, 1H, C2); MS: m/z 187 (M^+), 160, 109 (100%); and 0.421 g of the (E)- and (Z)-5'-cyanonicotines: ^{1}H-NMR ($CDCl_3$): δ 1.5-2.5 (complex m, 4H, C3' and C4'), 2.30 and 2.32 (2s, 3H, N-CH_3 of the (E)- and (Z)-isomers), 3.5 (complex m, 1.4H, H2' and 5'-H of the (E)-isomer), 4.1 (d of d, 0.6H, 5'-H of the (Z)-isomer), 7.28 (m, 1H, C5), 7.6 (m, 1H, C4), 8.55 (m, 2H, C2 and C6). The ^{1}H-NMR spectrum of 2'-cyanonicotine is shown in Figure 16 and the EI-mass spectrum is shown in Figure 17.

Preparation of Sodium Aluminum Hydride.[108]

Under an argon atmosphere in a flame-dried 3-necked round bottom flask (25 mL) fitted with a reflux condenser, NaD (1.35 g, 61.14% dispersed in oil, 33 mmol) was rinsed twice with freshly distilled THF. The cleaned NaD was resuspended in anhydrous THF (8 mL) and $LiAlD_4$ (1.17 g, 27.9 mmol) was added. The suspension was heated to 60° C for 5 minutes and stirring was continued at room temperature for five hours. The slurry was poured into a dry centrifuge tube and centrifuged to pellet the solids. The liquid was removed and stored in dry screw top test tubes under argon. The solution (13 mL) was found to be 1.7 M $NaAlD_4$ as determined by the volume of D_2 gas produced when a known amount of the solution was added to n-butanol in a closed system.

5'-Cyanonicotine-5'-d_1 (44a- and 44b-5'-d_1).[57]

A solution of freshly prepared $NaAlD_4$ (3 mL, 20 mmol hydride) in 50 mL THF was added dropwise to an ice-cooled, stirred solution of (S)-cotinine (3g, 17 mmol) in 50 mL dry THF under nitrogen. The reaction mixture was allowed to warm slowly to room temperature. After 3.5 hours, the mixture was cooled and another aliquot of $NaAlD_4$ (1 mL) was added slowly. The system was allowed to warm to room temperature and stirring was continued for 45 minutes. A saturated NH_4Cl solution (20 mL) was added and the THF layer was removed. The aqueous phase was extracted with ether (4x50 mL). The organic layers were combined and dried (K_2CO_3). Removal of the solvent under reduced pressure afforded 2.04 g of crude product. The product was purified by preparative HPLC (silica column; mobile phase: ether:NH_4 2000:1; flow rate: 5 mL/min) to yield 1.6 g of a pale yellow oil (50% yield). The 80 MHz ^{1}H-NMR spectrum of the mixture of (E)- and (Z)-5'-cyanonicotine-5'-d_1 (Figure 18) is identical to that of a mixture of unlabeled 5'-cyanonicotines except for the absence of the 5'-proton signals at 3.5 and 4.1 ppm: δ 1.9-2.6 (complex m, 4H, C3' and C4'), 2.30 and 2.32 (2s, 3H, N-CH_3 of the diastereomers), 3.5 (m, 1H, C2'), 7.3 (m, 1H, C5), 7.6 (m, 1H, C4), 8.55 (m, 2H, C2 and C6); EI-MS (Figure 21): m/z 188 (M^+), 110 (100%).

Nicotine $\Delta^{1'(5')}$-Iminium Diperchlorate-5'-d_1 (42-5'-d_1).[57]

A 70% perchloric acid solution (2.1 mL, 25 mmol) was diluted with ethanol (10 mL) and added slowly to an ice-cooled ethanolic solution (15 mL) of 5'-cyanonicotine-5'-d_1 (0.93g, 5 mmol). Crystals formed over several hours. The crystals were filtered and washed with acidic methanol to yield 1.15 g of product. The crude product was recrystal-

lized from hot methanol containing a trace of perchloric acid to yielded 0.80 g of white crystals (44% yield): m.p. 240°-243° C with decomposition (lit.[58] 230°-232° C). The 80 MHz ^{1}H-NMR spectrum in 10% DCl in D_2O (Figure 20) is similar to the unlabeled (S)-nicotine Δ-1'(5')-iminium bisperchlorate except for the absence of the 5'-proton signal at 8.9 ppm: δ 2.0-3.0 (complex m, 4H, C3' and C4'), 3.37 (br s, 3H, N-CH_3), 5.6 (t, 1H, C2'), 8.0 (m, 1H, C5), 8.25-8.9 (m, 3H, C4, C6, and C2).

Nicotine-Z-5'-d_1 (58).

Platinum oxide (80 mg), in 2.5% HCl in H_2O (8 mL), was prereduced for 30 minutes in the presence of one atmosphere of H_2 gas. Crystalline 42-d_1 diperchlorate (0.79 g., 2.12 mmol) was added and the mixture was allowed to stir at room temperature under one atmosphere of H_2 gas for 4 hours. The catalyst was removed by filtration through a celite pad. The filtrate was made basic with a saturated K_2CO_3 solution and extracted with methylene chloride (5x10 mL). The extracts were combined, dried (K_2CO_3), filtered, and concentrated at atmospheric pressure. The residue was purified on a silica gel column using ether-ammonia (3:1) as the eluent to yield 0.30 g of product (83.6% yield): ^{1}H-NMR: δ 1.5-2.1 (complex m, 4H, C3' and C4'), 2.17 (s, 3H, N-CH_3), 2.3 (m, 1H, $H_{5'E}$), 3.1 (t, 1H, C2'), 3.3 (br d, 0.13H, $H_{5'Z}$), 7.3 (m, 1H, C5), 7.6 (d of t, 1H, C4), 8.3-8.5 (m, C2 and C4); MS: m/z 163 (M^+), 134, 85 (100%). An expansion of the 240 MHz spectrum is shown in Figure 22 and the EI-mass spectrum is displayed in Figure 21.

The di-(l)-tartrate salt of 58 was prepared according to the procedure reported by Aceto et al.:[105] m.p. 138.5°-139.5° C (lit.[105] 138°-139.5° C); ^{2}H-NMR (D_2O) (Figure 23): 3.8 ppm.

Anal. Calc'd for $C_{18}H_{25}DN_2O_{12}$: C, 46.65; H, 5.87; N, 6.04. Found: C, 45.76; H, 5.61; N, 6.02.

N-(1-Cyanoethyl)nornicotine (75).[30]

Nornicotine (200 mg, 1.36 mmol) and NaCN (200 mg, 4.1 mmol) were dissolved in water (45 mL) containing a trace amount of concentrated H_2SO_4. An aqueous solution (20 mL) of acetaldehyde (1 mL, 17.8 mmol) was added slowly and the mixture was allowed to stir 30 minutes. A 1 N NaOH solution was used to basify the solution (pH<9) which then was extracted with ether (5x5 mL). The organic layers were dried ($MgSO_4$) and concentrated under reduced pressure to yield crude product; ^{1}H-NMR: δ 1-3.5 (complex, 7H, pyrrolidine protons); 1.35 and 1.60 (2d, 3H, N-CH_3); 3.55 and 4.6 (2q, J=7 Hz, 1H, C<u>H</u>CNCH$_3$), 7.3 (q, 1H, C5); 7.7 (d of t, 1H, C4); 8.5 (complex m, 2H, C2 and C6); EI-MS: m/z M^+ 174, 96 (100%). The NMR spectrum of the N-(1-cyanoethyl)nornicotines is shown in Figure 27 and the EI-mass spectra was shown in Figure 29.

5'-Cyano-5-methylnicotine (76).[91]

A stirred solution of 5-methylnicotine (0.2324 g, 1.32 mmol) and mercuric acetate (1.6 g., 5.0 mmol) in 5% acetic acid (5 mL) was heated to 80° C for 2.5 hrs, then cooled. Hydrogen sulfide gas was bubbled through the ice-cooled solution. A black precipitate formed and was removed by filtration through a celite pad. This step was repeated until no more precipitate was formed. Potassium cyanide (0.5g, 7.6 mmol) was added and the solution made basic with K_2CO_3. After stirring

for 1.5 hrs, the mixture was extracted with ether (5x25 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to yield crude product (0.344 g). Column chromatographic purification on basic alumina using a mobile phase consisting of ether-ammonia (2000:1) yielded 0.221 g of product (83.3%): The ^{1}H-NMR spectrum in $CDCl_3$ (Figure 32) was identical to that of a mixture of (E)- and (Z)-5'-cyanonicotine except for the presence of the 5-methyl proton signal at 2.34 ppm: δ 1.9-2.6 (complex m, 4H, C3' and C4'), 2.30 and 2.32 (2s, 3H, N-CH_3 of the diastereomers), 2.34 (s, 3H, 5-CH_3), 3.5 (m, 1H, C2'), 7.2 (m, 1H, C5), 7.6 (m, 1H, C4), 8.55 (m, 2H, C2 and C6).

5'-Cyanonicotine-N-d_3 (77).[91,221]

Nornicotine (0.31 g, 2.1 mmol) was placed in deuterium oxide (12 mL) and an acidic deuterium oxide solution of paraformaldehyde (93 mg, 3.1 mmol) was added. The pH was adjusted to 6 with acetic acid-d_1. Sodium cyanoborodeuteride (0.235g, 3.6 mmol) was added and the reaction was stirred at room temperature under an N_2 atmosphere. After 3 hours, the solution was made basic and extracted with ether (3x10 mL). The extracts were combined, dried (K_2CO_3), and concentrated at atmospheric pressure. The crude nicotine-N-d_3 (0.306 g) was passed through a short column of basic alumina using ammonia-saturated ether as the mobile phase and was then used to prepare 5'-cyanonicotine according to the procedure of Hubert-Brierre.[91] A 5% acetic acid solution (5 mL) of nicotine-N-d_3 (200 mg, 1.2 mmol) and mercuric acetate (1.5g, 4.7 mmol) was stirred at 80-90° C for 2 hrs. The reaction mixture was cooled and filtered. Hydrogen sulfide was bubbled through the ice-cooled filtrate. The mixture was filtered through a celite pad in the presence of carbon black. Sodium cyanide (210 mg, 4.3 mmol) was added to the filtrate and

the pH was adjusted to 6. After 1/2 hr, the solution was made basic and extracted with ether (4x10 mL). The extracts were combined, dried over $NaSO_4$ and concentrated under reduced pressure. The crude product (180 mg) was purified by column chromatography using basic alumina and eluting with ether. A pale yellow oil (138 mg, 60% yield) was obtained after concentration of the eluent at reduced pressure. The 80 MHz ^1H-NMR in $CDCl_3$ (Figure 35) was identical to that obtained for a mixture of unlabeled (E)- and (Z)-5'-cyanonicotines except for the absence of the $N-CH_3$ signals at 2.30 and 2.32 ppm: δ 1.5-2.5 (complex m, 4H, C3' and C4'), 3.5 (t, 1H, C2'), 4.1 (br d, 1H, C5'), 7.28 (m, 1H, C5) 7.6 (m, 1H, C4) 8.55 (m, 2H, C2 and C6); EI-MS (Figure 36): 190 (M^+), 112 (100%). GC-EIMS analysis demonstrated that the product was contaminated with a small amount of N-cyanomethylnornicotine-d_2.

Metabolic Studies.

a. Dutch Rabbit Liver Microsomes.

Six month old male dutch rabbits were stunned by a blow to the neck and decapitated. The liver was perfused with ice cold 1.15% KCl (250 mL) and removed. The liver was homogenized in KCl solution (2 mL/g liver), doing 7 passes. Another aliquot of KCl (1 mL/g liver) was added and one more pass was made. The homogenate was centrifuged for 20 minutes at 10,000xg (0°-4° C). The supernatant was removed and centrifuged at 100,000xg for 75 minutes (0°-4° C). The supernatant from this spin was discarded, the pellet was resuspended in KCl solution (2x1 mL), and centrifuged a second time at 100,000xg. The pellet was resuspended in pH 7.4 phosphate buffer (0.2 M, 2x1 mL). Protein concentrations were

determined by the method described by Lowry et al.[221] At this point, the microsomal preparation was used immediately or frozen at -78° C under N_2 for future experiments.

b. Other Rodent Microsomal Preparations.

Phenobarbital-pretreated New Zealand rabbit liver microsomal preparations were obtained from Mr. M. Shigenaga and Ms. K. Hoag. A liver microsomal preparation made from the livers of seven rats was obtained from Dr. B. Ferraiolo.

c. Human Liver Microsomes.

Human liver was obtained from J. Trudell (Stanford University, Palo Alto, Ca.) and from L. Waskell (Veteran's Administration Hospital, San Francisco, Ca.). Microsomes were prepared as described above for the Dutch rabbit liver microsomes, however, the initial homogenization was done with 4 mL KCl solution/g liver. A third human microsomal preparation was obtained from F. Kadlubar (National Center for Toxicological Research, Jefferson, AR).

d. Incubations.

The various nicotine compounds (0.5 mM) were incubated with microsomal preparations (3 mg protein/mL) in 0.2 M potassium phosphate buffer, pH 7.4 (final volume 2.5-5 mL) containing magnesium chloride (1.5 mM), sodium cyanide (1 mM) and NADPH (1 mg/mL), a NADPH-regenerating system ($NADP^+$ (0.5 mM), glucose-6-phosphate dehydrogenase, and glucose-6-phosphate), iodosobenzene (1 mM), or cumene hydroperoxide (1 mM). Following an incubation period of 5, 10, 15 or 30 min at 37° C, the reactions were terminated by freezing at -78° C or by placing on ice. They were extracted with an equal volume of $FeSO_4$ washed ether. The aqueous phase was frozen on dry ice and the ether decanted into a

prewashed vial. The solvent was removed under a N_2 stream and the residue was resuspended in methylene chloride (50 µL) for GC-EIMS analysis (injection port: 220° C, ion source: 150° C, inlet line: 200° C). The sample was injected onto the column at room temperature. After the solvent had eluted (approx. 2 min.), the column was rapidly heated to 180° C and held there. The ion current at m/z 109, 110 and 111 was obtained by computer-assisted selected ion monitoring techniques. In some incubation mixtures, 5'-cyanonicotine-N-d_3 (5 µg) was added as an aqueous solution to the incubation mixtures for the quantitation of the metabolically generated 5'-cyanonicotines. In these studies, the ion current was monitored at m/z 109, 110, 111, and 112. Quantitation of the diastereomeric 5'-cyanonicotines was achieved by comparing the ratio of the sum of the ion currents at m/z 109, 110, and 111 to the ion current at m/z 112 with values obtained from a standard curve. Standard curves were generated using known amounts of the synthetic unlabeled and monodeutero-5'-cyanonicotine diastereomers.

Separate incubations were done to quantitate the amount of nicotine. Analysis was done using the HPLC assay of Shigenaga.[222] The metabolic incubations (1 mL) were terminated with 1 M K_2CO_3 (2 ml) and the internal standard, N-methyl-N-(3-pyridyl)methyl propanamide was added. The mixture was extracted with an equal volume of methylene chloride by vortexing for 30 seconds followed by centrifugation for 2-3 minutes at 1000 x g. An aliquot (100 µL) was subjected to HPLC analysis. Quantitation of nicotine and its metabolites was achieved by comparing the peak height ratios of compound/internal standard to values obtained from standard curves generated with synthetic cotinine,

nicotine, (E)- and (Z)-5'-cyanonicotines, and nicotine Δ-1'(5')-iminium ion. The HPLC analysis was performed on a silica column with a mobile phase consisting of acetonitrile-1% propylamine (v/v; flow rate: 2.0 mL/min).

Cotinine analyses were done by Neal Benowitz's laboratory at San Francisco General Hospital with 1 mL aliquots of the aqueous phase of the metabolic mixture following ether extraction employing the method of Jacob et al.[113]

e. Metabolic Studies with Synthetic (E)- and (Z)-5'-Cyanonicotine.

The incubations were done as described above using 50 μM 5'-cyanonicotine in place of nicotine. Incubations were done using a 50:50 mixture of 5'-cyanonicotine-5'-d_0 and -5'-d_1 or the individual materials in the presence or absence of NADPH. When the 50:50 mixture was used, the deuterium content of the remaining 5'-cyanonicotine was determined by GC-EIMS analysis of the ether extracts as described in the nicotine metabolic studies. Cotinine analysis was performed as described by Jacob et al.[113]

Tetraphenylporphinatoiron(III) Chloride-Iodosobenzene Catalyzed Oxidation of (S)-Nicotine.[177]

The tartrate salts of the monodeuterated nicotine diastereomers 57 and 58 or a 1:1 mixture of (S)-nicotine-d_0 and (S)-nicotine-5'-d_2 were converted to the free base form by dissolution in 5 M NaOH. These solutions were extracted with ether and concentrated under N_2. The residue was then used in the following experiment. Triplicate mixtures of nicotine (7.5 mg, 46 nmol), iodosobenzene (0.5 mg, 2.5 nmol) and $TTPFe^{III}Cl$ (0.22 mg, 0.31 nmol) in CH_2Cl_2-MeOH (4:1, 0.25 mL) were stirred under argon for 1 hr at room temperature. A pH 6 0.2 M sodium

phosphate solution of sodium cyanide (100 ul of 200 mg/mL) was added. After 15 min, the aqueous phase was made basic with a saturated aqueous potassium carbonate solution. The CH_2Cl_2 phase was removed and the aqueous phase was extracted with ether (2 mL). The organic phases were combined and analyzed by the capillary GC-EIMS selected ion monitoring assay described eariler. Controls were performed with (S)-nicotine-d_0 in the absence of catalyst or in the absence of iodosobenzene. Similar proportions of reactants were used in initial studies done with (S)-nicotine in CH_2Cl_2 or dry ethanol and nicotine-N'-oxide in ethanol.

Hydrogen Peroxide-Methemoglobin Catalyzed Oxidation of (S)-Nicotine.[197]

Triplicate incubations (2.5 mL) were performed with the labeled nicotine bis-l-tartrates (10 mM) in the presence of bovine methemoglobin (1.8 mg), and hydrogen peroxide in pH 7.4 phosphate buffer at 30° C for 30 minutes. The mixtures were cooled on ice prior to the addition of sodium cyanide (30 mg). They were then extracted with CH_2Cl_2 (5 mL) The CH_2Cl_2 were concentrated to 200 µL and analyzed by the previously described GC-EIMS selected ion monitoring assay (see the liver microsomal experiments. Controls were performed in the absence of either hydrogen peroxide or hemoprotein.

Hydrogen Peroxide-Horseradish Peroxidase Catalyzed Oxidation of (S)-Nicotine.[189]

a. The Determination of the Enzyme Intermediates in the Hydrogen Peroxide-Horseradish Peroxidase Catalyzed Oxidation of (S)-Nicotine.

The study was conducted in 3 mL aliquots of pH 6.8 0.1 M phosphate buffer in quartz cuvettes. The absorption spectra of solutions containing native enzyme (4.7 μM horseradish peroxidase, Type IV), enzyme (4.7 μM) and hydrogen peroxide (4.7 μM), and enzyme (4.7 μM), hydrogen peroxide (4.7 μM) and (S)-nicotine (4 mM) were measured with a DW2 Aminco UV-visible spectrophotometer between 340 and 660 nm.

b. Stereochemical Studies.

The monodeuterated nicotine diastereomers 57 and 58 or a 1:1 mixture of (S)-nicotine and (S)-nicotine-5',5'-d_2 (0.85 mM) were incubated with horseradish peroxidase (Type IV, 0.1 mg protein/mL) and hydrogen peroxide (0.136 mM) in pH 6.8 0.1 M phosphate buffer (total volume: 5 mL) at 30° C or 37° C. Controls were done in the absence of the peroxidase or hydrogen peroxide. After 30 minutes the incubation mixtures were cooled on ice and sodium cyanide (30 mg) was added. The mixtures were extracted with ether or CH_2Cl_2 (5 mL). Following concentration under a N_2 stream, the extracts were redissolved in CH_2Cl_2 (50-100 μL) for GC-EIMS selected ion monitoring assay as described in the liver microsomal experiments.

Ethyl Peroxide-Chloroperoxidase Catalyzed Oxidation of (S)-Nicotine.[20]

The monodeuterated nicotine diastereomers 57 or 58 as their bis-l-tartrate salts (0.85 mM) were incubated in duplicate with chloroperoxidase (66 μg protein/mL) and ethyl peroxide (1.7 mM) in pH 6.8 0.1 M phosphate buffer (total volume: 2.5 mL) at 25° C for 30 minutes. Sodium cyanide (30 mg) was added to the incubation mixtures after they were cooled on ice. After 5-10 minutes CH_2Cl_2 (5 mL) extractions were performed. The residue obtained after concentration under a N_2 stream was redissolved in CH_2Cl_2 (200 μL) for analysis by the previous described GC-EIMS selected ion monitoring assay.

The Methylene Blue-sensitized Photochemical Oxidation of (S)-Nicotine.[91]

The monodeuterated nicotine bis-l-tartrates (27.1 mg, 58.5 nmol), methylene blue (0.8 mg, 2.1 nmol), potassium cyanide (3.8 mg, 78 nmol), and sodium pyruvate (6.5 mg, 59 nmol) were dissolved in an aqueous solution of potassium phosphate solution (0.2 M, 1.5 mL). The pH was adjusted to pH 9 with 5 N NaOH. Each sample was irradiated with a Hannovia lamp filtered through pyrex for 30 or 60 minutes with oxygen bubbling through the solution. The mixture was extracted with CH_2Cl_2 (2x2 mL). The organic phases were combined, dried over $MgSO_4$ (anhyd.), filtered, and analyzed directly by the previously described GC-MS selected ion monitoring assay (for a description of the assay, see the liver microsomal experiments). Duplicate experiments were done for 57, 58 and mixtures of (S)-nicotine and (S)-nicotine-5'-d_2.

The Electrochemical Cyanation of (S)-Nicotine.[216]

a. Cyclic Voltametry: (S)-nicotine (0.65 g, 4 mmol) was dissolved in a methanol-water (1:1) solution of sodium cyanide (0.2 M). A platinum wire electrode was used to cycle from 0 V to 2 V at a rate of 200 mV/sec against a standard calomel electrode.

b. Controlled Potential Electrolysis: A 25 mL H-type electrolysis cell (The Electrosynthesis Company, Inc.) with porous glass frits was employed for these experiments. The labeled nicotine bis-l-tartrates (0.725 mg, 3 μmoles) were dissolved in 0.2 M sodium cyanide methanol-water (1:1) solution (15 mL). The reaction was carried out between 0°-5° C at 1.2 V versus a saturated calomel electrode with platinum wire electrodes. After 15 minutes, the solution was extracted with CH_2Cl_2 (5 mL). The extract was analyzed by the previously described capillary GC-EIMS selected ion monitoring assay. The experiment was done in triplicate for each of the labeled compounds.

V. REFERENCES

1. S.D. Nelson, "Metabolic Activation and Drug Toxicity," J. Med. Chem., 25, 753-765 (1982).

2. J.R. Gillette, J.R. Mitchell, and B.B. Brodie, "Biochemical mechanisms of drug toxicity," Ann. Rev. Pharmacol., 14, 271-288 (1974).

3. D.H. Swenson, "Metabolic activation and detoxication of aflatoxins," in Reviews in Biochemical Toxicology Vol. 3 (E. Hodgson, J.R. Bend, and R.M. Philpot, eds.), Elsevier, New York, 1981, pp. 155-192.

4. M.R. Boyd and L.T. Burka, "In vivo studies on the relationship between target organ alkylation and the pulmonary toxicity of a chemically reactive metabolite of 4-ipomeanol," J. Pharmacol. Exp. Ther., 207, 687-697 (1978).

5. D.R. Thakker, H. Yagi, W. Levin, A.W. Wood, A.H. Conney, and D.M. Jerina, "Polycyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens," in Bioactivation of Foreign Compounds (M.W. Anders, eds.), Academic Press, New York, 1985, 177-242.

6. D.J. Jollow, J.R. Mitchell, W.Z. Potter, D.C. Davis, J.R. Gillette and B.B. Brodie, "Acetominophen-induced hepatic necrosis. II. Role of covalent binding in vivo," J. Pharmacol. Exp. Ther., 187, 195-202 (1980).

7. J.L. Holtzman, "Role of reactive oxygen and metabolite binding in drug toxicity," Life Science, 30, 1-9 (1982).

8. L.L. Smith, M.S. Rose, and I. Wyatt, "The pathology and biochemistry of paraquat," Ciba Found. Symp., 65, 321-341 (1979).

9. G.M. Rosen, E.J. Rauckman, S.P. Ellington, D.C. Dahlin, J.L. Christie, and S.D. Nelson, "Reduction and glutathione conjugation reactions of N-acetyl-p-benzoquinoneimine and two methylated analogs," Mol. Pharmacol., 25, 151-157 (1984).

10. G.M. Cohen, "Metabolic basis of target organ toxicity," in Biological Basis of Detoxification, (J. Caldwell and W.B. Jakoby, eds.), Academic Press, New York, 1983, pp. 325-347.

11. F.P. Guengerich and D.C. Liebler, "Enzymatic activation of chemicals to toxic metabolites," CRC Crit. Rev. Tox., 14, 259-307 (1985).

12. J. Rose and N. Castagnoli, Jr., "The metabolism of tertiary amines," Med. Res. Rev., 3, 73-88 (1983).

13. K. Chiba, A. Trevor, and N. Castagnoli, Jr., "Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase," Biochem. Biophys. Res. Commun., 120, 574-578 (1984).

14. R.E. Heikkila, L. Manzino, F.S. Cabbat, and R.C. Duvoisin, "Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine by monoamine oxidase inhibitors," Nature (London), 311, 467-469 (1984).

15. P.S. Caldera, J.I. Salach, N. Castagnoli, Jr., A.J. Trevor, and T.P. Singer, "Studies on the chemical and biochemical behavior of $MPDP^+$," in MPTP: A Neurotoxin Producing a Parkinsonian Syndrome," (N. Castagnoli, Jr., S. Markey, A. Trevor, and I.J. Kopin, eds.), Academic Press, New York, in press.

16. J.W. Langston, I. Irwin, and E.G. Langston, "Pargyline prevents MPTP-induced parkinsonism in primates," Science, 225, 1480-1482 (1984).

17. R.E. White and M.E. Coon, "Oxygen activation by cytochrome P-450," Ann. Rev. Biochem., 49, 315-356 (1980).

18. M.S. Fish, N.M. Johnson, E.C. Horning, "t-Amine oxide rearrangments. N,N-Dimethyltryptamine oxide," J. Am. Chem. Soc., 78, 3668-3671 (1956).

19. T. Shono, T. Toda, and N. Oshino, "Electron transfer from nitrogen in microsomal oxidation of amine and amide. Simulation of microsomal oxidation by anodic oxidation," J. Am. Chem. Soc., 104, 2639-2641 (1982).

20. G.T. Miwa, J.S. Walsh, G.L. Kedderis, and P.F. Hollenberg, "The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P-450- and peroxidase-catalyzed N-demethylation reactions," J. Biol. Chem., 258, 14445-14449 (1983).

21. R.P. Hanzlik and R.H. Tullman, "Suicidal inactivation of cytochrome P-450 by cyclopropylamines. Evidence for cation-radical intermediates," J. Am. Chem. Soc., 104, 2048-2050 (1982).

22. P.L. Ashley and B.W. Griffin, "Involvement of radical species in the oxidation of aminoantipyrine by cumene hydroperoxide in rat liver microsomes," Mol. Pharmacol., 19, 146-152 (1981).

23. D. Beké, "Heterocyclic pseudo bases," Adv. Heterocyclic Chem., 1, 167-188 (1963).

24. M. Overton, J.A. Hickman, M.D. Threadgill, K. Vaughan, and A. Gescher, "The generation of potentially toxic reactive iminium ions from the oxidative metabolism of xenobiotic N-alkyl compounds," Biochem. Pharmacol., 34, 2055-2061 (1985).

25. S.D. Nelson, G.D. Breck, and W.F. Trager, "In vivo metabolite condensations. Formation of N'-ethyl-2-methyl-N'-(2,6-dimethylphenyl)-4-imidazolidinone from the reaction of a metabolite of alcohol with a metabolite of lidocaine," J. Med. Chem., 16, 1106-1112 (1973).

26. R. Ziegler, B. Ho, and N. Castagnoli, Jr., "Trapping of metabolically generated electrophilic species with cyanide ion: metabolism of methapyrilene," J. Med. Chem., 24, 1133-1138 (1981).

27. B. Ho and N. Castagnoli, Jr., "Trapping of metabolically generated electrophilic species with cyanide ion: the metabolism of 1-benzylpyrrolidine," J. Med. Chem., 23, 133-139 (1980).

28. D.P. Ward, A.J. Trevor, A. Kalir, J.D. Adams, T.A. Baillie, and N. Castagnoli, Jr., "Metabolism of phencyclidine: the role of iminium ion formation in covalent binding to rabbit microsomal protein," Drug Met. Disp., 10, 690-695 (1982).

29. P.J. Murphy, "Enzymatic oxidation of nicotine to nicotine $\Delta^{1'(5')}$-iminium ion. A newly discovered intermediate in the metabolism of nicotine," J. Biol. Chem., 248, 2796-2800 (1975).

30. T.L. Nguyen, L.D. Gruenke, and N. Castagnoli, Jr., "Metabolic N-demethylation of nicotine. Trapping of a reactive iminium ion species with cyanide ion," J. Med. Chem., 19, 1168-1169 (1976).

31. T.-L. Nguyen, L.D. Gruenke, and N. Castagnoli, Jr., "Metabolic oxidation of nicotine to chemically reactive intermediates," J. Med. Chem., 22, 259-263 (1979).

32. J. Weissman, A. Trevor, K. Chiba, L.A. Peterson, P. Caldera, N. Castagnoli, Jr., and T. Baillie, "Metabolism of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by liver homogenate fractions," J. Med. Chem., 28, 997-1001 (1985).

33. B. Ketterer, S.K.S. Srai, B. Waynforth, D.L. Tullis, F.E. Evans, and F.F. Kadlubar, "Formation of N-(glutathion-S-methylene)-4-aminoazobenzene following metabolic oxidation of the N-methyl group of the carcinogen, N-methyl-4-aminoazobenzene," Chem.-Biol. Interact., 38, 287-302 (1982).

34. C.J. Logan, F.H. Coffee, and J.A. Page, "The formation of a novel mercapturic acid during the metabolism of an N-methyl aromatic amine, 4-cyano-N,N-dimethylaniline," Biochem. Pharmacol., 33, 2345-2346 (1984).

35. A.R.. Mattocks, "Mechanisms of pyrrolizidine alkaloid toxicity," Proc. 5th Int. Conf. Pharmacol., 2, 114-123 (1973).

36. J.N. Lown, K.C. Majunder, A.J. Meyers, and Altech, "Studies related to antitumor antibiotics X. Reactions of maytansine and its analogs with DNA in vitro," Bioorganic Chem., 6, 453-463 (1977).

37. M.K.P. Hoag, A.J. Trevor, Y. Asscher, J. Weissman, and N. Castagnoli, Jr., "Metabolism-dependent inactivation of liver microsomal enzymes by phencyclidine," Drug Met. Disp., 12, 371-375 (1984).

38. M.M. Ames, M.E. Saunders, and W.S. Tiede, "Role of N-methylolpentamethylmelamine in the metabolic activation of hexamethylmelamine," Cancer Res., 43, 500-504 (1983).

39. K. Chiba, L.A. Peterson, K.P. Castagnoli, A.J. Trevor, and N. Castagnoli, Jr., "Studies on the molecular mechanism of bioactivation of the selective nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine," Drug Met. Disp., 13, 342-347 (1985).

40. L.A. Peterson, P.S. Caldera, A. Trevor, K. Chiba, and N. Castagnoli, Jr., "Studies on the 1-methyl-4-phenyl-2,3-dihydropyridinium species 2,3-MPDP$^+$, the monoamine oxidase catalyzed oxidation product of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)," J. Med. Chem., 28, 1432-1436 (1985).

41. United States Department of Health and Human Services, "The health consequences of smoking," in Cancer USPHS Publication No. 82-50179, Government Printing Office, Washington, D.C., p. 322 (1982).

42. Surgeon General, "Smoking and Health," US Department of Health and Welfare, Department of Health, Education and Welfare Publication No. (PHS) 79-50066, Washington, D.C. (1979).

43. E.L. Wynder and D. Hoffman, "Certain constituents of tobacco smoke," in Tobacco and Tobacco Smoke, Academic Press, New York, 1967, pp. 330-407.

44. S.S. Armed, C.B. Mosehos, M.M. Lyons, H.A. Oldewurtel, R.J. Coumbis, and T.J. Regan, "Cardiovascular effects of long term smoking and nicotine administration," Am. J. Cardiol., 37, 33-40 (1976).

45. E. Boyland, "The possible carcinogenic actions of alkaloids of tobacco and betelnut," Planta, 16 suppl., 13-23 (1968).

46. E.C. Hammond, L. Garfinkel, H. Seidman, and E.A. Lew, "Some recent findings concerning cigarette smoking," in Origins of Cancer. Book A: Incidence of Cnacer in Humans (H.H. Hiatt, J.D. Watson, and J.A. Winsten, eds.), Cold Spring Harbor Laboratory, New York, 1977, pp. 101-112.

47. P. Hill and E.L. Wynder, "Smoking and cardiovascular disease," Am. Heart J., 87, 491-496 (1974).

48. D.W. Irving and T. Yamamoto, "Cigarette smoking and cardiac output," Br. Heart J., 25, 126-132 (1963).

49. D. Hoffman and S.S. Hecht, "Perspectives in cancer research: nicotine derived N-nitrosoamines and tobacco related cancer: current status and future directions," Cancer Res., 45, 935-944 (1985).

50. J.W. Gorrod and P. Jenner, "The metabolism of tobacco alkaloids," Essays Toxicol., 6, 35-78 (1975).

51. A. Pilotti, "Biosynthesis and mammalian metabolism of nicotine," Acta Physiol. Scand. Suppl., 479, 13-17 (1980).

52. B. Hucker, J.R. Gillette, and B.B. Brodie, "Enzymic pathway for the formation of cotinine, a major metabolite of nicotine in rabbit liver," J. Pharmacol. Exp. Ther., 129, 94-100 (1960).

53. J.W. Gorrod and A.R. Hibberd, "The metabolism of nicotine-$\Delta^{1'(5')}$-iminium ion, in vivo and in vitro," Eur. J. Drug Met. Pharmacokinetics, 7, 293-298 (1982).

54. A.R. Hibberd and J.W. Gorrod, "Enzymology of the metabolic pathway from nicotine to cotinine in vitro," Eur. J. Drug Met. Pharmacokinetics, 8, 151-162 (1983).

55. S. Brandange and L. Lindblom, "The enzyme 'aldehyde oxidase' is an iminium oxidase. Reaction with nicotine Δ-1'(5')iminium ion," Biochem. Biophys. Res. Commun., 91, 991-996 (1979).

56. B.C. Barass, J.W. Blackburn, R.W. Brimblecombe, and P. Rich, "Modification of nicotine toxicity by pretreatment with different drugs," Biochem. Pharmacol., 18, 2145-2152 (1969).

57. E.B. Saunders, J.F. DeBardeleben, and T.S. Osdene, "Nicotine chemistry. 5'-Cyanonicotine," J. Org. Chem., 40, 2848-2849 (1975).

58. L. Lindblom and S. Brandange, "Synthesis, structure and stability of nicotine Δ1'(5')iminium ion, -an intermediary metabolite of nicotine," Acta Chem. Scand., 33B, 187-191 (1979).

59. P.F. Hollenberg, G.T. Miwa, J.S. Walsh, L.A. Dwyer, D.E. Rickert, and G.L. Kedderis, "Mechanisms of N-demethylation reactins catalyzed by cytochrome P-450 and peroxidase," Drug Met. Disp., 13, 272-275 (1985).

60. M.M. Abdel-Monem, "Isotope effects in enzymatic N-demethylation of tertiary amines," J. Med. Chem., 18, 427-530 (1975).

61. g.T. Miwa, W.A. Garland, B.J. Hodshon, A.Y.H. Lu, and D.B. Northrup, "Kinetic isotope effects in cytochrome P-450-catalyzed oxidation reactions. Intermolecular and intramolecular deuterium isotope effects during the N-demethylation of N,N-dimethylphentermine," J. Biol. Chem., 255, 6049-6054 (1980).

62. J.T. Groves, G.A. McCluskey, R.E. White, and M.J. Coon, "Aliphatic hydroxylation by highly purified liver microsomal cytochrome P-450. Evidence for a carbon radical intermediate," Biochem. Biophys. Res. Commun., 81, 154-160 (1978).

63. S. Bergstrom, S. Lindstredt, B. Samuelson, E.J. Corey, and G.A. Gregoriou, "The stereochemistry of 7α-hydroxylation in the biosynthesis of cholic acid from cholesterol," J. Am. Chem. Soc., 80, 2337-2338 (1958).

64. M. Hayano, M. Gut, R.I. Dorfman, O.K. Sebek, and D.H. Peterson, "Steric considerations in the enzymatic course of the hydroxylation of steriods," J. Am. Chem. Soc., 80, 2336-2337 (1958).

65. E.J. Corey, G.A. Gregoriou, and D.H. Peterson, "The stereochemistry of 11α-hydroxylation of steroids," J. Am. Chem. Soc., 80, 2338 (1958).

66. M.H. Gelb, D.C. Heimbrook, P. Mälkönen, and S.G. Sligar, "Stereochemistry and deuterium isotope effects in camphor hydroxylation by the cytochrome P-450$_{cam}$ monoxygenase system," Biochemistry, 21, 370-377 (1982).

67. A.B. Foster, M. Jarman, J.D. Stevens, P. Thomas, and J.H. Westwook, "Isotope effects in O- and N-demethylations mediated by rat liver microsomes: an application of direct insertion electron impact mass spectrometry," Chem.-Biol. Interact., 9, 327-340 (1974).

68. J.P. Shea, S.D. Nelson, and G.P. Ford, "MNDO calculations of kinetic isotope effects in model cytochrome P-450 oxidations," J. Am. Chem. Soc., 105, 5451-5454 (1983).

69. J.T. Groves, S. Krishnan, G.E. Avaria, and T.E. Nemo, "Studies of the mechanism of oxygen activation and transfer catalyzed by cytochrome P-450," in Biomimetic Chemistry (D. Dolphin, C. McKenna, Y. Marakami, and I. Tabushi, eds.), American Chemical Society, Washington, D.C., 1980, pp. 277-289.

70. L.M. Hjelmeland, L. Aronow, and J.R. Trudell, "Intramolecular determination of primary kinetic isotope effects in hydroxylations catalyzed by cytochrome P-450," Biochem. Biophys. Res. Commun., 76, 541-549 (1977).

71. A.Y.H. Lu, N. Harada, and G.T. Miwa, "Rate-limiting steps in cytochrome P-450-catalysed reactions: studies on isotope effects in the O-de-ethylation of 7-ethoxycoumarin," Xenobiotica, 14, 19-26 (1984).

72. G.T. Miwa, J.S. Walsh, and A.Y.H. Lu, "Kinetic isotope effects on cytochrome P-450-catalyzed oxidation reactions: the oxidative O-dealkylation of 7-ethyoxycoumarin," J. Biol. Chem., 259, 3000-3004 (1984).

73. S.D. Nelson, L.R. Pohl, and W.F. Trager, "Primary and β-secondary deuterium isotope effects in N-deethylation reactions," J. Med. Chem., 18, 1062-1065 (1975).

74. R.E. McMahon, H.W. Culp, J.C. Craig, and N. Ekwuribe, "Mechanism of the dealkylation of tertiary amines by hepatic oxygenases. Stable isotope studies with 1-benzyl-4-cyano-4-phenylpiperidine," J. Med. Chem., 22, 1100-1103 (1979).

75. G.T. Miwa, J.A. Walsh, and A.Y.H. Lu, "The application of stable isotopes to drug metabolism studies," in Advances in Pharmacology and Therapeutics, vol. II (H. Yoshida, Y. Hagihara, and S. Ebashi, eds.), Pergamon Press, New York, 1982, pp 207-213.

76. D.B. Northrup, "The expression of isotope effects on enzyme-catalyzed reactions," Ann. Rev. Biochem., 50, 103-131 (1981).

77. L. Melander and W.H. Saunders, Reaction Rates of Isotopic Molecules, Wiley Interscience, New York, 1980, pp. 91-128.

78. C. Elison, H.W. Elliot, M. Look, and H. Rappoport, "Some aspects of the fate and relationship of the N-methyl group of morphine to its pharmacological activity," J. Med. Chem., 6, 237-246 (1963).

79. T.-L. Nguyen, "α-Carbon hydroxylation in the metabolism of tobacco alkaloids," Dissertation, University of California, San Francisco, Ca., 1977.

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

81. J.W. Gorrod, P. Jenner, G. Keysell, and A.H. Beckett, "Selective inhibition of alternative oxidative pathways of nicotine metabolism in vitro," Chem.-Biol. Interact., 3, 269-270 (1971).

82. B. Ho, "Cyanide trapping of metabolically generated electrophilic intermediates derived from tertiary amines," Dissertation, University of California, San Francisco, Ca., 1981.

83. S. Probst, private communication.

84. C.G. Chavdarian, E.B. Saunders, and R.C. Bassfield, "Synthesis of optically active nicotinoids," J. Org. Chem., 47, 1069-1073 (1982).

85. M. Nakane and C.R. Hutchinson, "Biosynthetic studies of secondary plant metabolites with $^{13}CO_2$. Nicotiniana alkaloids. 2. New syntheses of nornicotine and nicotine. Quantitative carbon-13 NMR spectroscopic analysis of [2',3',N-CH_3-$^{13}C_3$]Nicotine," J. Org. Chem., 43, 3922-3931 (1978).

86. T.-L. Nguyen and N. Castagnoli, Jr., "The synthesis of deuterium labelled tobacco alkaloids: nicotine, nornicotine and cotinine," J. Labelled Cmpd. and Radiopharm., 14, 919-934 (1979).

87. F. Korte and H.-J. Schulze-Steinen, "Acyl-lacton-umlagerung XXII. Umlagerung von α-aroyl-pyrrolidonen in konz. Salzsäure zu pyrrollinderivaten," Chem. Ber., 95, 2444-2452 (1962).

88. M. Freifelder, Catalytic Hydrogenation in Organic Synthesis: Procedures and Commentary, Wiley and Sons, Inc., New York, 1978, Chapt. 2 and 8.

89. K. Harada and D. Yoshida, "Syntheses of optically active α-amino acids from ester of α-keto acids by hydrogenolytic assymmetric transamination, a solvent effect," Bull. Chem. Soc. Japan, 43, 921-925 (1970).

90. K. Harada and K. Matsumoto, "Sterically controlled syntheses of optically active organic compounds XIV. Syntheses of dipeptides from N-(α-ketoacyl)-α-amino acid esters," Bull. Chem. Soc. Japan, 44, 1068-1071 (1971).

91. Y.D. Hubert-Brierre, D. Herlem, and F. Khuong-Huu, "Oxydation photochimique d'amines tertiaires et d'alcaloides-VI. Oxydation photosensibilisee d'alcaloides comportant un heterocycle N-methyle (nicotine, N-methyl anabasine, ajmaline)," Tetrahedron, 31, 3049-3054 (1975).

92. J. Ohashi, T. Morishima, and T. Yonezawa, "Application of proton nmr shift reagents to the stereochemical analysis of nicotine," Bull. Chem. Soc. Japan, 44, 576-577 (1971).

93. J.F. Whidby and J.I. Seeman, "The configuration of nicotine. A nuclear magnetic resonance study," J. Org. Chem., 41, 1585-1590 (1973).

94. J.F. Whidby, W.B. Edwards, III., and T.P. Pitner, "Isomeric nicotines. Their solution conformation and proton, deuterium, carbon 13 and nitrogen 15 nuclear magnetic resonance," J. Org. Chem., 44, 794-798 (1979).

95. J.B. Lambert and W.L. Oliver, "Nitrogen inversion without retarding factor," J. Am. Chem. Soc., 91, 7774-7775 (1969).

96. E. Breuer and D. Melumad, "Nuclear magnetic resonace spectra of cyclic amines. Shielding of α protons trans to a lone pair and cis to an N-methyl group in pyrrolidines," J. Org. Chem., 38, 1601-1602 (1973).

97. S. Ohki and M. Yoshino, "Synthesis of pyrrolidine derivatives with anticholinergic properties. III. Syntheses and stereochemistry of 1,2,5-trimethylpyrrolidine and 3-(diphenylmethylene)-1,2,5-trimethylpyrrolidine," Chem. Pharm. Bull., 16, 269-274 (1968).

98. M. Uskokovic, H. Bruderer, C. von Planta, T. Williams, and A. Brossi, "The nuclear magnetic resonance spectra of the angular proton in benzo[a]- and indolo[a]quinolizidines," J. Am. Chem. Soc., 86, 3364-3367 (1964).

99. M. Cushman and N. Castagnoli, Jr., "The synthesis of trans-3'-methylnicotine," J. Org. Chem., 37, 1268-1271 (1972).

100. J.B. Lambert and R.G. Keske, "Factors contributing to the chemical shift of protons adjacent to nitrogen in piperidines," Tet. Let., 2023-2026 (1969).

101. R. Merenyi, "Structure determination of iminium salts by physical methods," Adv. Org. Chem., 9, 23-105 (1976).-

102. A.M. Duffield, H. Budzikiewicz, and C. Djerassi, "Mass spectrometry in structural and stereochemical problems. LXXII. A study of the fragmentation processes of some tobacco alkaloids," J. Am. Chem. Soc., 87, 2926-2932 (1965).

103. L. Lindblom, "Studies on some nicotine metabolites," Chem. Commun. Univ. Stockholm, 5-40 (1979).

104. A. Ozadi, Isotopic Studies of Hetereogeneous Catalysis, Academic Press, New York, 1977, p. 11.

105. A.D. Aceto, B.R. Martin, I.M. Uwaydah, E.L. May, L.S. Harris, C. Izazola-Condy, W.L. Dewey, T.J. Bradshaw, and W.C. Bincek, "Opically pure (+)-nicotine from (±)-nicotine and biological comparisons with (-)-nicotine," J. Med. Chem., 22 174-177 (1979).

106. M.L. Martin, G.L. Martin, J.-J. Depuech, Practical NMR Spectroscopy," Heyden, London, 1980, p. 362.

107. T.P. Pitner, W.B. Edwards, III, R.L. Bassfield, and J.F. Whidby, "The solution conformation of nicotine. A ^{1}H and ^{2}H nuclear magnetic resonance investigation," J. Am. Chem. Soc., 100, 246-251 (1978).

108. L.I. Zarharkin and V.V. Gavrilenko, "Mutual conversions in the alumohydrides of lithium, sodium, and potassium," Otdelenie Khimicheskikh Nauk, 1146-1149 (1962).

109. W.G. Brown, "Reductions by lithium aluminium hydride," Organic Reactions, 6, 469-507 (1951).

110. V.M. Mićović and M.W. Mihailović, "The reduction of acid amides with lithium aluminium hydride," J. Org. Chem., 18, 1190-1200 (1953).

111. G. Stork, A.A. Ozorio, and A.Y.W. Leong, "N,N-Diethylaminoacetonitrile: a generally useful latent acyl carbanion," Tet. Let., 5175-5178 (1978).

112. J.D. Albright, F.J. McEvoy, and D.B. Moran, "The use of α-(aryl)-4-morpholineacetonitriles (masked acyl anion equivalents) in 1,4-additions to α,β-unsaturated esters and nitriles. A versatile synthetic route to 6-aryl-3(2H)-pyridazinones," J. Heterocyclic Chem., 15, 881-892 (1978).

113. P. Jacob, M. Wilson, and N.L. Benowitz, "Improved gas chromatographic method for determination of nicotine and cotinine in biologic fluids," J. Chromatogr., 222, 61-70 (1981).

114. P. Jacob, private communication.

115. S. Brandänge and L. Lindblom, "N-vinyl as N-H protecting group. A convenient synthesis of myosmine," Acta Chem. Scand. B, 30, 93 (1976).

116. P. Jacob, III, "Resolution of (±)-5-bromonornicotine. Synthesis of (R)- and (S)-nornicotine of high enantiomeric purity," J. Org. Chem., 47, 4165-4167 (1982).

117. P. Jacob, III, N.L. Benowitz, L. Yu, and A.T. Shulgin, "Determination of nicotine-N-oxide by gas chromatography following thermal conversion to 2-methyl-6-(3-pyridyl)-tetrahydro-1,2-oxazine," Anal. Chem., in press.

118. A.Y.H. Lu, "Multiplicity of liver drug metabolizing enzymes," Drug Met. Rev., 10, 187-208 (1979).

119. P. Jenner, J.W. Gorrod, and A.H. Beckett, "Factors affecting the in vitro metabolism of R-(+)- and S-(-)-nicotine by guinea-pig liver preparations," Xenobiotica, 3, 563-572 (1973).

120. J.B. Shenkman, H. Remmer, and R.W. Estabrook, "Spectral studies of drug interaction with hepatic microsomal cytochrome," Mol. Pharmacol., 3, 113-123 (1967).

121. W. Levin, P.E. Thomas, L.M. Reik, D.E. Ryan, S. Bandiera, M. Haniu, and J.E. Shively, "Immunochemical and structural characterization of rat hepatic cytochrome P-450," in Microsomes and Drug Oxidations (A.R. Boobis, J. Caldwell, F. DeMatteis, and C.R. Elcombe, eds.), Taylor and Francis, London, 1985, pp. 13-22.

122. M.J. Coon, S.D. Black, V.S. Fujita, D.R. Koop, and G.E. Tarr, "Structural comparison of multiple forms of cytochrome P-450," in Microsomes and Drug Oxidations (A.R. Boobis, J. Caldwell, F. DeMatteis, and C.R. Elcombe, eds.), Taylor and Francis, London, 1985, pp. 42-51.

123. E.F. Johnson and G.E. Schwab, "Constitutive forms of rabbit liver microsomal cytochrome P-450: enzymatic diversity, polymorphism and allosteric regulation," Xenobiotica, 4, 3-18 (1984).

124. A.R. Boobis and D.S. Davies, "Human cytochrome P-450," Xenobiotica, 14, 151-185 (1984).

125. L.M. Distlerath, P.E.B. Reilly, M.V. Martin, G.G. Davis, G.R. Wilkinson, and F.P. Guengerich, "Purification and characterization of the human liver cytochrome P-450 involved in debrisoquine 4-hydroxylation and phenacetin O-deethylation, two prototypes for genetic polymorphism in oxidative drug metabolism," J. Biol. Chem., 260, 9057-9067 (1985).

126. A.W. Wood, D.E. Ryan, P.E. Thomas, and W. Levin, "Regio- and stereoselective metabolism of two C_{19} steroids by five highly purified and reconstituted rat hepatic cytochrome P-450 isozymes," J. Biol. Chem., 258, 8839-8847 (1983).

127. W.R. Porter, C. Wheeler, and W.F. Trager, "Changes in the metabolic profiles of R- and S-warfarin and R- and S-phenprocoumon as a probe to categorize the effect of inducing agents on microsomal hydroxylases," Biochem. Pharmacol., 30, 3099-3104 (1981).

128. H. Nakayama, T. Nakashima, and Y. Kuroguchi, "Heterogeneity of hepatic nicotine oxidase," Biochim. Biophys. Acta, 715, 254-257 (1982).

129. N.M. Papadopoulos, "Formation of nornicotine and other metabolites from nicotine in vitro and in vivo, Can. J. Biochem., 42, 435-442 (1964).

130. P. Jenner, J.W. Gorrod, and A.H. Beckett, "Species variation in metabolism of R-(+)- and S-(-)-nicotine by α-C and N-oxidation in vitro," Xenobiotica, 3, 573-580 (1973).

131. D.M. Ziegler and F.H. Pettit, "Formation of an intermediate N-oxide in the oxidative demethylation of N,N-dimethylaniline catalysed by liver microsomes," Biochem. Biophys. Res. Commun., 15, 188-193 (1964).

132. J.C. Craig and K.K. Purushothaman, "An improved preparation of tertiary amine N-oxides," J. Org. Chem., 35, 1721 (1970).

133. R.M. Dajani, J.W. Gorrod, and A.H. Beckett, "In vitro hepatic and extra-hepatic reduction of (-)-nicotine-1'-N-oxide in rats," Biochem. Pharmacol., 24, 109-117 (1975).

134. A.H. Conney, "Pharmacological implications of microsomal enzyme induction," Pharmacol. Rev., 19, 317-366 (1967).

135. M.J. Coon, J.L. Vermilion, K.P. Vatsis, J.S. French, W.L. Dean, and D.A. Haugen, "Biochemical studies on drug metabolism: isolation of multiple forms of liver microsomal cytochrome P-450," in Drug Metabolism Concepts (D.M. Jerina, ed.), American Chemical Society, Washington, D.C., 1977, pp 46-71.

136. P.P. Wang, P. Beaune, L.S. Kaminsky, G.A. Dannan, F.F. Kadlubar, D. Larrey, and F.P. Guengerich, "Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes," Biochemistry, 22, 5375-5383 (1983).

137. A.Y.H. Lu, N. Harada, J.S.A. Walsh, and G.T. Miwa, "Isotope effect and metabolic switching in the cytochrome P-450-catalyzed O-deethylation of 7-ethoxycoumarin," in Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications (E. Hietanen, M. Laitinen, and O. Hanninen, eds.), Elsevier Biomedical Press, Amsterdam, 1982, pp 405-412.

138. J. March, Advanced Organic Chemistry. Reaction, Mechanisms, and Structure (2nd ed.), McGraw Hill, New York, 1977, pp. 205-206.

139. D.B. Northrup, "Determining the absolute magnitude of hydrogen isotope effects," in Isotope Effects on Enzyme-Catalyzed Reactions (W.W. Cleland, M.H. O'Leary, and D.B. Northrup, eds.), University Park Press, Baltimore, 1977, pp. 122-152.

140. D.M. Jerina, D.P. Michaud, R.J. Feldman, K.P. Vyas, D.R. Thakker, H. Yagi, P.E. Thomas, D.E. Ryan, and W. Levin, "Stereochemical modeling of the catalytic site of cytochrome P-450c," in Microsomes, Drug Oxidations, and Drug Toxicity (R. Sato and R. Kato, eds.), Wiley Interscience, New York, 1982, pp. 195-201.

141. T. Poulos, private communication.

142. S. Shapiro, J.U. Piper, and E. Caspi, "Steric course of hydroxylation at primary carbon atoms. Biosynthesis of 1-octanol from (1R)- and (1S)-[1-^{2}H, ^{2}H, ^{1}H; 1-^{14}C]octane by rat liver microsomes," J. Am. Chem. Soc., 104, 2301-2305 (1982).

143. J.P. Shea, G.L. Valentine, and S.D. Nelson, "Source of oxygen in cytochrome P-450-catalyzed carbinolamine formation," Biochem. Biophys. Res. Commun., 109, 231-2335 (1982).

144. G.L. Kedderis, L.A. Dwyer, R.E. Rickert, and P.F. Hollenberg, "Source of the oxygen atom in the product of cytochrome P-450-catalyzed N-demethylation," Mol. Pharmacol., 23, 758-760 (1983).

145. H. Sayo and M. Masui, "Anodic oxidation of amines. Part IV. Cyclic voltametry and controlled potential electrolysis of 4-dimethylamino-antipyrine in acetonitrile," J. Chem. Soc. Perkin Trans. II, 1640-1645 (1973).

146. Y.L. Chow, W.C. Danen, S.F. Nelson, and D.H. Rosenblatt, "Nonaromatic aminium radicals," Chem. Rev., 73, 243-274 (1978).

147. O. Augusto, H.S. Beilan, and P.R. Ortiz de Montellano, "The catalytic mechanism of cytochrome P-450 spin trapping. Evidence for one-electron substrate oxidation," J. Biol. Chem., 257, 11288-11295 (1982).

148. R.P. Hanzlik, V. Kishore, and R. Tullman, "Cyclopropylamines as suicide substrates for cytochromes P-450," J. Med. Chem., 22, 759-761 (1979).

149. T.L. Macdonald, K. Zirvi, L.T. Burka, P. Peyman, and F.P. Guengerich, "Mechanism of cytochrome P-450 inhibition by cyclopropylamines," J. Am. Chem. Soc., 104, 2050-2052 (1982).

150. F.P. Guengerich, R.J. Willard, J.P. Shea, L.E. Richard, and T.L. Macdonald, "Mechanism-based inactivation of cytochrome P-450 by heteroatom-substituted cyclopropanes and formation of ring-opened products," J. Am. Chem. Soc., 106, 6446-6447 (1984).

151. F.D. Lewis and T.L. Ho, "On the selectivity of tertiary amines oxidations," J. Am. Chem. Soc., 102, 1751-1752 (1980).

152. D. Döppe and J. Heufer, "N-Demethylation of N,N-dimethylanilines by photoexcited 3-nitrochlorobenzene," Tet. Let., 23, 1553-1556 (1982).

153. T. Shono, T. Toda, and N. Oshino, "Preparation of N-dealkylated drug metabolites by electrochemical simulation of biotransformation," Drug Met. Disp., 9, 481-482 (1981).

154. F. Pettit and D.M. Ziegler, "The catalytic demethylation of N,N-dimethylaniline-N-oxide by liver microsomes," Biochem. Biophys. Res. Commun., 13, 193-197 (1963).

155. S. Hamill and D.Y. Cooper, "The role of cytochrome P-450 in dual pathway of N-demethylation of N,N'-dimethylaniline by hepatic microsomes," Xenobiotica, 14 139-149 (1984).

156. J.M. Machinist, W.H. Orme-Johnson, and D.M. Ziegler, "Microsomal oxidase. II. Properties of a pork liver microsomal N-oxide dealkylase," Biochemistry, 5, 2939-2943 (1966).

157. P. Willi and M.H. Bickel, "Liver metabolic reactions: tertiary amine N-dealkylation, tertiary amine N-oxidation, N-oxide reduction, and N-oxide-dealkylation. II. N,N'-Dimethylaniline," Arch. Biochem. Biophys., 156, 772-779 (1973).

158. P. Hlavica, "Biological oxidation of nitrogen in organic compounds and disposition of N-oxidized products," CRC Crit. Rev. Biochem., 11, 39-101 (1982).

159. R.E. McMahon and H.R. Sullivan, "Reduction oflevopropoxyphene N-oxide to propoxyphene by dogs in vivo and rat liver microsomal fraction in vitro," Xenobiotica, 7, 377-382 (1977).

160. A.L. Misra, R.B. Pontani, and N.L. Vadlamani, "Metabolism of norcocaine, N-hydroxynorcocaine, and cocaine-N-oxide in the rat," Xenobiotica, 9, 189-199 (1979).

161. F.P. Guengerich, "Oxidation of sparteines by cytochrome P-450: evidence against the formation of N-oxides," J. Med. Chem., 27, 1101-1103 (1984).

162. M.H. Bickel, "Liver metabolic reactions: tertiary amine N-dealkyation, tertiary amine N-oxidation, N-oxide reduction, and N-oxide N-dealkylation," Arch. Biochem. Biophys., 148, 54-62 (1971).

163. A.H. Beckett, M. Mietchard, and A.A. Shihab, "The influence of methyl substitution on the N-demethylation and N-oxidation of normethadone in animal species," J. Pharm. Pharmacol., 23, 941-946 (1971).

164. J.W. Gorrod and D.J. Temple, "The formation of an N-hydroxymethyl intermediate in the N-demethylation of N-methylcarbazole in vivo and in vitro," Xenobiotica, 6, 265-274 (1976).

165. S.G. Sligar, M.H. Gelb, and D.C. Heimbrook, "Bio-organic chemistry and cytochrome P-450-dependent catalyst," Xenobiotica, 14, 63-86 (1984).

166. A.D. Rahimtula and P.J. O'Brien, "Hydroperoxide-catalyzed liver microsomal aromatic hydroxylation reactions involving cytochrome P-450," Biochem. Biophys. Res. Commun., 60, 440-447 (1974).

167. J.T. Groves and D.V. Subramanian, "Hydroxylation by cytochrome P-450 and metalloporphyrin models. Evidence of allylic rearrangements," J. Am. Chem. Soc., 106, 2177-2181 (1984).

168. F.F. Kadlubar, K.C. Morton, and D.M. Ziegler, "Microsomal-catalyzed hydroperoxide-dependent C-oxidation of amines," Biochem. Biophys. Res. Commun., 54, 1255-1261 (1973).

169. A.D. Rahimtula, A.D. O'Brien, H.E. Seifried, and D.M. Jerina, "The mechanism of action of cytochrome P-450. Occurrence of the 'NIH' shift during hydroperoxide-dependent aromatic hydroxylations," Eur. J. Biochem., 89, 133-141 (1978).

170. J.-Å. Gustafsson, L. Rondahl, and J. Bergman, "Iodosylbenzene derivatives as oxygen donors in cytochrome P-450-catalyzed steroid hydroxylations," Biochemistry, 18, 865-870 (1979).

171. V. Ullrich, "The mechanism of cytochrome P-450 action," in Microsomes and Drug Oxidations (V. Ullrich, I. Roots, A. Hildebrandt, R.W. Estabrook, and A.H. Conney, eds.), Pergamon Press, Oxford, 1977, pp. 192-201.

172. J. Capdevila, R.W. Estabrook, and R.A. Prough, "Differences in the mechanism of NADPH- and cumene hydroperoxide-supported reactions of cytochrome P-450," Arch. Biochem. Biophys., 200, 186-195 (1980).

173. R. Rennenberg, J. Capdevila, N. Chacos, R.W. Estabrook, and R.A. Prough, "Hydrogen peroxide-supported oxidation of benzo(a)pyrene by rat liver microsomal fractions," Biochem. Pharmacol., 30, 843-848 (1981).

174. M.J. Coon, R.E. White, R.C. Blake, III, "Mechanistic studies with purified liver microsomal cytochrome P-450: comparison of O_2- and peroxide-supported hydroxylation reactions," in Oxidases and Related Redox Systems (T.E. King, H.S. Mason, and M. Morrison, eds.), Univ. Park Press, Baltimore, 1979, pp. 857-885.

175. R.W. Estabrook, C. Martin-Wixtrom, Y. Saeki, R. Renneberg, A. Hildebrant, and J. Werringloer, "The peroxidatic function of liver microsomal cytochrome P-450: comparison of hydrogen peroxide and NADPH catalysed N-demethylation reactions," Xenobiotica, 14, 87-104 (1984).

176. D. Mansuy, "The use of model systems in biochemical toxicology," in Reviews in Biochemical Toxicology Vol. 3 (E. Hodgson, J.R. Bend, and R.M. Philpot, eds.), Elsevier, New York, 1981, pp. 283-320.

177. P. Shannon and T.C. Bruice, "A novel P-450 model system for the N-dealkylation reaction," J. Am. Chem. Soc., 103, 4582-4583 (1981).

178. M.W. Nee and T.C. Bruice, "Use of the N-oxide of p-cyano-N,N-dimethylaniline as an 'oxygen'-donor in a cytochrome P-450 model system," J. Am. Chem. Soc., 104, 6123-6125 (1982).

179. J.T. Groves and T.E. Nemo, "Aliphatic hydroxylation catalyzed by iron porphyrin complexes," J. Am. Chem. Soc., 105, 6243-6248 (1983).

180. J.R.L. Smith and P.R. Sleath, "Model systems for cytochrome P-450 dependent mono-oxygenases. Part 1. Oxidation of alkenes and aromatic compounds by tetraphenylporphinatoiron(III) chloride and iodosobenzene," J. Chem. Soc. Perkins Trans. II, 1009-1015 (1982).

181. J.T. Groves, T.E. Nemo, and R.S. Meyers, "Hydroxylation and epoxidation catalyzed by iron-porphine complexes. Oxygen transfer from iodosobenzene," J. Am. Chem. Soc., 101, 1032-1033 (1979).

182. J.T. Groves and T.E. Nemo, "Epoxidation reactions catalyzed by iron porphyrins. Oxygen transfer from iodosobenzene," J. Am. Chem. Soc., 105, 5786-5791 (1983).

183. M.F. Powell, E.F. Pai, and T.C. Bruice, "Study of (tetraphenylporphinato)maganese(III)-catalyzed epoxidation and demethylation using p-cyano-N,N-dimethylaniline N-oxide as oxygen donor in a homogeneous system. Kinetics, radiochemical ligation studies, and reaction mechanism for a model cytochrome P-450," J. Amer. Chem. Soc., 106, 3277-3285 (1984).

184. G.L. Kedderis, D.R. Koop, and P.F. Hollenberg, "N-Demethylation reactions catalyzed by chloroperoxidase," J. Biol. Chem., 255, 10174-10182 (1980).

185. G.L. Kedderis and P.F. Hollenberg, "Characterization of the N-demethylation reactions catalyzed by horseradish peroxidase," J. Biol. Chem., 258, 8129-8138 (1983).

186. B.W. Griffin and P.L. Ting, "Mechanism of N-demethylation of aminopyrine by hydrogen peroxide catalyzed by horseradish peroxidase, metmyoglobin, and protohemin," Biochemistry, 17, 2206-2211 (1978).

187. R.N. Pandey, G.L. Kedderis, D.E. Rickert, and P.F. Hollenberg, "Oxygen-18 studies of the peroxidase-catalyzed oxidation of N-methylcarbazole," Fed. Proc., 44, 1779 (1985).

188. B.W. Griffin, D.K. Davis, and G.V. Bruno, "Electron paramagnetic resonance study of the oxidation of N-methyl-substituted aromatic amines catalyzed by hemeproteins," Bioorg. Chem., 10, 342-355 (1981).

189. F.S. Sariaslani, M.W. Duffel, and J.P. Rosazza, "One-electron oxidation of vindoline and 16-O-acetylvindoline catalyzed by peroxidase," J. Med. Chem., 28, 629-633 (1985).

190. S. Zaida, D. Gilhar, and J. Silman-Greenspan, "Replacing the mixed function oxidase N-demethylation by hydrogen peroxide and either hemoglobin or ferrous sulfate," Pharmaceutical Research, 277-279 (1984).

191. D.W. Starke, K.S. Blisard, and J.J. Mieyal, "Substrate specificity of the monooxygenase activity of hemoglobin," Mol. Pharmacol., 25, 467-475 (1984).

192. H.B. Dunford, "Peroxidases," Adv. in Inorg. Biochem., 4, 41-68

(1982).

193. P. George and D.H. Irvine, "The reaction between metmyoglobin and hydrogen peroxide," Biochem. J., 52, 511-517 (1952).

194. P. George and D.H. Irvine, "A kinetic study of the reaction between ferrimyoglobin and hydrogen peroxide," J. Colloid Sci., 11, 327-339 (1956).

195. S.P. Cramer, J.H. Dawson, K.O. Hodgson, and L.P. Hager, "Studies on the ferric forms of cytochrome P-450 and chloroperoxidase by extended x-ray absorption fine structure. Characterization of the Fe-N and Fe-S distances," J. Am. Chem. Soc., 100, 7282-7290 (1978).

196. R.E. Dickerson and I. Geis, Hemoglobin: Structure, Function, Evolution, and Pathology, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, Ca., 1983, p.27.

197. P.R. Ortiz de Montellano and C.E. Catalano, "Epoxidation of styrene by hemoglobin and myoglobin. Transfer of oxidizing equivalents to the protein surface," J. Biol. Chem., 260, 9265-9271 (1985).

198. K.L. Kunze and P.R. Ortiz de Montellano, "Formation of a σ-bonded aryliron complex in the reaction of arylhydrazines with hemoglobin and myoglobin," J. Am. Chem. Soc., 105, 1380-1381 (1983).

199. P.R. Ortiz de Montellano and D.E. kerr, "Inactivation of myoglobin by ortho-substituted arylhydrazines. Formation of prosthetic heme aryliron but not N-aryl adducts," Biochemistry, 24, 1147-1152 (1985).

200. J.F. Gibson, D.J.E. Ingram, and P. Nicholls, "Free radical produced in the reaction of metmyoglobin with hydrogen peroxide," Nature, 181, 1398-1399 (1958).

201. N.K. King, F.D. Looney, and M.E. Winfield, "Amino acid free radicals in oxidsed metmyoglobin," Biochim. Biophys. Acta, 133, 65-82 (1967).

202. S.A. Adidiran and H.B. Dunford, "Structure of horseradish peroxidase compound I. Kinetic evidence for the incorporation of the oxygen atom from the oxidizing substrate into the enzyme," Eur. J. Biochem., 132, 147-150 (1983).

203. T.H. Moss, A. Ehrenberg, and A.J. Bearden, "Mössbauer spectroscopic evidence for the electronic configuration of iron in horseradish peroxidase and its peroxide derivatives," Biochemistry, 8, 4159-4162 (1969).

204. D. Herlem, Y. Hubert-Brierre, and F. Khuong-Huu, "N-Demethyaltion photochimique d'amines tertiares. IV. Mise en evidence de la formation d'ions immoniums intermediaten preparation photochimique d'α-aminonitriles," Tet. Let., 4173-4176 (1973).

205. D. Herlem, Y. Hubert-Brierre, F. Khuong-Huu, and R. Goutarel, "Reactions photochimiques d'amines tertiaires et d'alcaloides-III" Tetrahedron, 29, 2195-2202 (1973).

206. R.H. Kayser and R.H. Young, "The photoreduction of methylene blue by amines-I. A. Flash photolysis study of the reaction between triplet methylene blue and amines," Photochem. and Photobiol., 24, 395-401 (1976).

207. R.F. Bartholomew and R.S. Davidson, "The photosensitised oxidation of amines," Chem. Commun., 1174-1175 (1970).

208. R.F. Bartholomew and R.S. Davidson, "The photosensitsed oxidation of amines. Part II. The use of dyes as photosensitisers: evidence that singlet oxygen is not involved," J. Chem. Soc. (C), 2347-2351 (1971).

209. F. Khuong-Huu, D. Herlem, and Y. Hubert-Brierre, "Reactions photochimiques d'amines tertiares et d'alcaloides V. Oxydation photosensibilisee d'amines tertiaires. Action du pyruvate de sodium sur la formation des composes oxygenes," Tet. Let., 359-362 (1975).

210. M. Masui, H. Sayo, and Y. Tsuda, "Anodic oxidati on of amines. Part I. Cyclic voltammetry of aliphatic amines at a stationary glassy-carbon electron," J. Chem. Soc. B., 973-976 (1968).

211. L.C. Portis, J.T. Klug, and C.K. Mann, "Electrochemical oxidation of some phenethylamines," J. Org. Chem., 39, 3488-3494 (1974).

212. J.R.L. Smith and D. Masheder, "Amine oxidation. Part IX. The electrochemical oxidation of some tertiary amines: the effect of structure on reactivity," Perkins Trans. II, 47-51 (1976).

213. C.K. Mann, "Cyclic stationary electrode. Voltammetry of some aliphatic amines," Anal. Chem., 36, 2424-2426 (1964).

214. M. Masui and H. Sayo, "Anodic oxidation of amines. Part II. Electrochemical dealkylation of aliphatic tertiary amines," J. Chem. Soc. B, 1593-1596 (1971).

215. P.J. Smith and C.K. Mann, "Electrochemical dealkylation of aliphatic amines," J. Org. Chem., 34, 1821-1826 (1969).

216. T. Chiba and Y. Takata, "Anodic cyanation of tertiary aliphatic and heterocyclic amines," J. Org. Chem., 42 2973-2977 (1975?).

217. A. Schejter, A. Lanir, and N. Epstein, "Binding of hydrogen donors to horseradish peroxidase: a spectroscopic study," Arch. Biochem. Biophys., 174, 36-44 (1976).

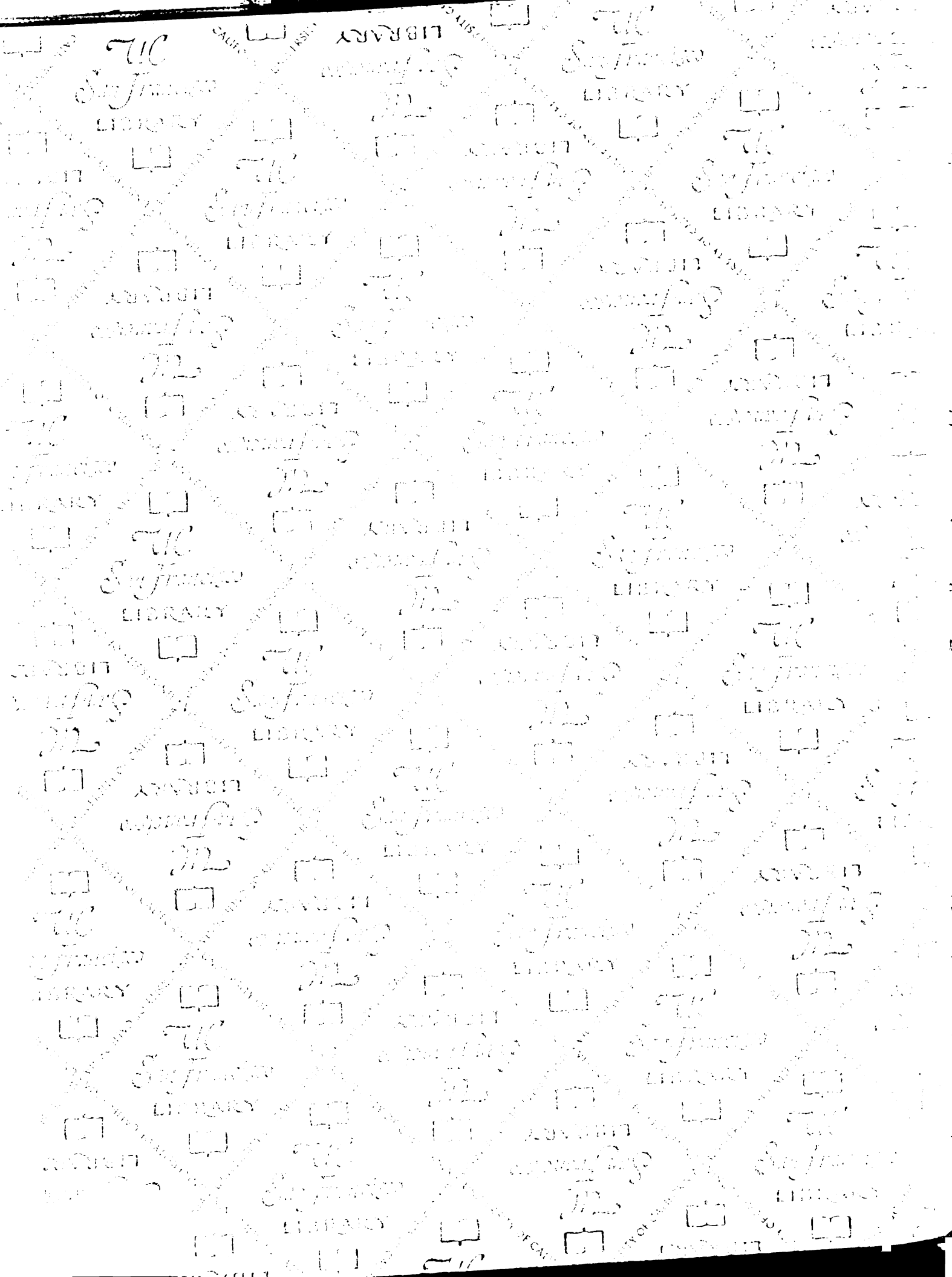
218. I. Morishima and S. Ogawa, "Nuclear magnetic resonance studies of hemoproteins. Binding of aromatic donor molecules to horseradish peroxidase," J. Biol. Chem., 254, 2814-2820 (1979).

219. E.R. Bowman and H. McKennis, Jr., "(-)-Cotinine," Biochem. Prep., 10, 36-39 (1963).

220. H. Saltzman and J.G. Sharefkin, "Iodosobenzene," Org. Syn., 43, 60-61 (1963).

221. O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.J. Randall, "Protein measurement with the Folen phenol reagent," J. Biol. Chem., 193, 265-275 (1951).

222. M. Shigenaga, private communication.


LIBRARY