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LIDOCAINE: A STUDY IN MICROSOMAL NITROGEN METABOLISM

by

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B.S., University of Washington, 1968

DISSERTATION

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ABSTRACT

LIDOCAINE: A STUDY IN MICROSOMAL NITROGEN METABOLISM

by

SIDNEY D. NELSON, JR.

UNDER THE SUPERVISION OF PROFESSOR WILLIAM F. TRAGER

Amines and amine derivatives (e.g., amides) provide the spice of life (or death, depending on your viewpoint) in the area of xenobiotic metabolism. No other functional group undergoes such a variety of biotransformations or utilizes such an array of enzymes in the biotransformation process. Among the pathways found for amine metabolism are deamination, N-dealkylation, and direct N-oxidation reactions which yield hydroxylamines, oximes, tertiary amine N-oxides, and N-hydroxyamides.

Most of these reactions are carried out by a suspected multiplicity of closely related enzymes which reside in the cellular endoplasmic reticulum and are called microsomal oxygenases. Currently, the mechanism proposed for the majority of microsomal oxidation reactions involves a prosthetic group called cytochrome P-450 which contains iron in the ferric state and binds with substrate. The enzyme-substrate complex is

reduced by NADPH to produce a ferrous complex which binds oxygen. One atom of the oxygen molecule is reduced to water and the other oxidizes the substrate.

The microsomal oxygenase system has proven to be extremely important in determining the biological fate of almost all foreign substances such as drugs, food additives, pollutants and other chemicals introduced into the human body. The purpose of the proposed research was to learn more about the chemical reactions involved in oxidative nitrogen metabolism by this enzyme system with particular emphasis on the biotransformation of the widely used local anesthetic and antiarrhythmic drug, lidocaine. The reasons for using lidocaine as the substrate were as follows:

1. Little was known at the inception of the thesis work concerning the metabolism of this agent in man or other animals.
2. By studying the metabolism of lidocaine a better understanding of its pharmacological mode of action, toxicities, and interactions with other drugs or chemicals might be gained, as well as learning about the metabolizing enzymes involved.
3. The chemical structure of lidocaine offers two distinct sites that contain nitrogen and which are susceptible to metabolic transformation: the tertiary amino group, which can yield further reactive secondary and primary amines, and an aromatic amide which upon hydrolysis can yield a further biotransformable aromatic amine.

Those aspects of the work that will be reported in this thesis can be divided into the following five areas:

1. Quantification of lidocaine metabolites in man.
2. Synthesis of and search for potential carcinogenic lidocaine metabolites.
3. The interaction of a metabolite of alcohol with a metabolite of lidocaine and its potential significance in the mechanism of N-dealkylation.
4. The microsomal metabolism of lidocaine and its deuterated analogs.
5. Molecular orbital studies concerning potential intermediates in microsomal amine oxidations.

Quantification of the suspected metabolites of lidocaine in man was carried out using stable isotope labeling and the relatively new technique of chemical ionization mass spectrometry (CIMS). This involved the synthesis of 15 suspected metabolites of lidocaine as their deuterated analogs to be used in a reverse isotope dilution of serial human plasma and urine samples following oral administration of a 250 mg dose of lidocaine hydrochloride monohydrate. Average results obtained by analyzing the 0-24 hour urine of three normal human male subjects indicated the presence of 7 of the possible 15 metabolites in the following amounts (% of administered dose based on free base): lidocaine (1.95%), MEGX (4.90%), GX (0.88%), m- and/or p-hydroxylidocaine (0.73%), m- and/or

p-hydroxyMEGX (0.56%), 2,6-dimethylaniline (0.97%), and 4-hydroxy-2,6-dimethylaniline (63.5%).

Two new potential metabolites, the N-hydroxyamides of lidocaine and MEGX, were synthesized, characterized, and are presently being tested for potential carcinogenic activity based on the carcinogenic propensity of other aromatic N-hydroxyamides. CIMS quantification using deuterated internal standards showed the presence of only minor quantities (0.5%) of the two N-hydroxyamides in 24-hour human urine samples. Quantification in a 24-hour urine sample collected under constant acidic (pH 5.0-5.3) urinary conditions also showed the absence of any major quantities of the two suspected metabolites.

Microsomal enzyme studies were carried out using both radiolabeled lidocaine (double-labeled ^{3}H- and ^{14}C) and various specifically deuterated analogs as substrates. Results from the studies with radiolabeled lidocaine show in the extractable phase, after a 15 minute incubation period with rat liver microsomes, the presence of lidocaine (78 ± 4%), MEGX (15 ± 2%), cyclic metabonate (2 ± 1%), and other (~5%). Lidocaine, labeled specifically with deuterium on both the methylene and methyl carbon atoms of the N-ethyl side chains, was used to probe the mechanism of the oxidative N-deethylation reaction. Deuterium substitution at the methylene carbon atoms, the presumed site of initial oxygen insertion, revealed a $k_H/k_D = 1.49 \pm .11$ and a $K_m^D/K_m^H = 1.23$. Deuterium substitution at the terminal methyl carbon atoms showed a $k_H/k_D = 1.52 \pm .10$ and a

$K_m^D/K_m^H = 0.92$. The results are explained in terms of both primary and secondary isotope effects on a possible rate-determining step in the N-dealkylation sequence.

Ab initio and CNDO/2 molecular orbital calculations were carried out on some potential intermediates in microsomal amine metabolism. The nature of the charge distribution in trimethylamine N-oxide allows us to conclude that if this N-oxide is an intermediate, its transformation to a carbinolamine probably goes through a radical mechanism. The electronic structure of some substituted imminium ions and oxaziridinium ions was also examined with reference to their potential intermediary roles in oxidative amine metabolism.

To my wife, Karen,

whose devotion and

understanding supported me

throughout the course of

this thesis;

and

To my parents,

whose initial guidance

made it possible.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. William F. Trager for his continuing interest, inspiration, and supervision during the course of the research.

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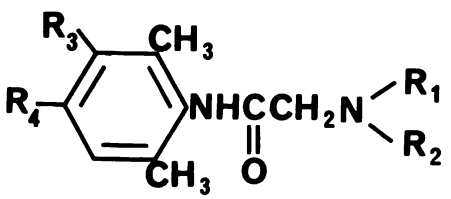
NOMENCLATURE

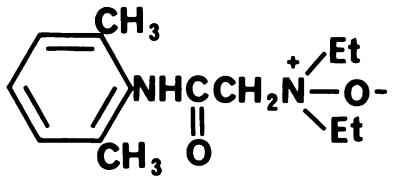
Throughout the text of this thesis reference is made to lidocaine and a number of compounds derived from it. In order to increase the ease with which the thesis can be read, the following trivial names and abbreviations are used.



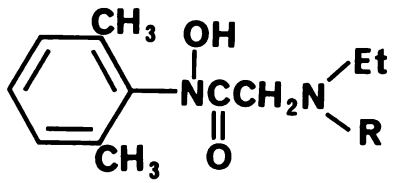
TRIVIAL NAME	CHEMICAL NAME	R_1	R_2	R_3	R_4
1. Lidocaine	ω-Diethylamino-2,6-dimethylacetanilide	Et	Et	H	H
2. MEGX	ω-Ethylamino-2,6-dimethylacetanilide or Monoethylglycine-xylidide	Et	H	H	H
3. GX	ω-Amino-2,6-dimethylacetanilide or Glycinexylidide	H	H	H	H
4. m-Hydroxylidocaine	ω-Diethylamino-3-hydroxy 2,6-dimethylacetanilide	Et	Et	OH	H
5. p-Hydroxylidocaine	ω-Diethylamino-4-hydroxy 2,6-dimethylacetanilide	Et	Et	H	OH
6. m-HydroxyMEGX	ω-Ethylamino-3-hydroxy-2,6-dimethylacetanilide	Et	H	OH	H

Other variants on the structure include the following:

1. Lidocaine N-oxide



2. N-Hydroxylidocaine

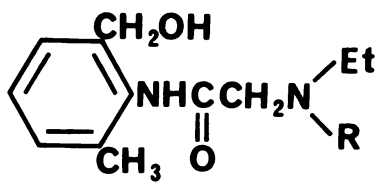


R = Et

3. N-HydroxyMEGX

R = H

4. Lidocaine benzyl alcohol

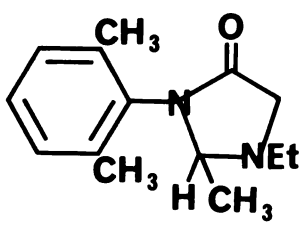


R = Et

5. MEGX benzyl alcohol

R = H

6. Cyclic metabonate



7. The term lidocaine will be used when referring to doses administered as lidocaine hydrochloride monohydrate. If a dose is administered as the free base, this will be specifically stated.

CHAPTER I

IN VIVO LIDOCAINE METABOLISM

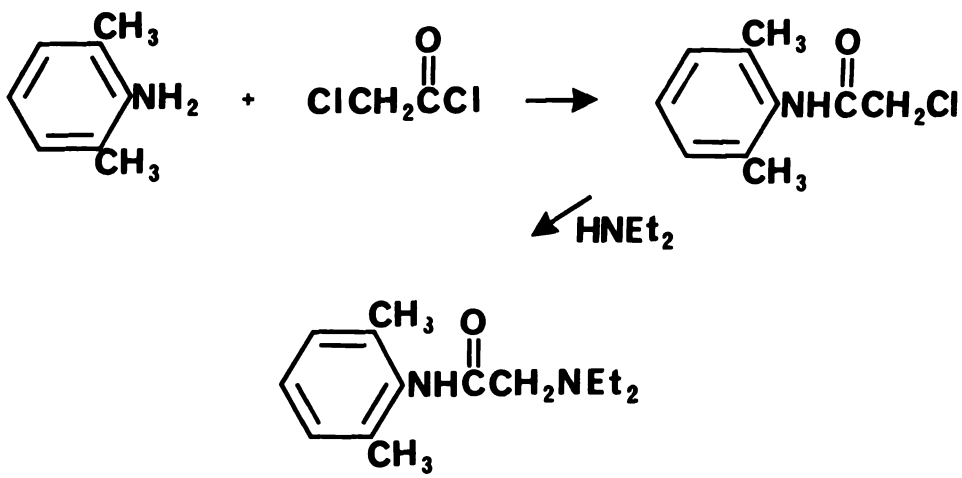
A. INTRODUCTION

Lidocaine is a widely used local anesthetic and antiarrhythmic agent. At present it is generally considered the most useful drug in suppressing ventricular arrhythmias which occur in approximately 80% of all myocardial infarction cases.[1-3] Because of its clinical importance and widespread use, a good understanding of the chemical properties, metabolism, and excretion of lidocaine and its metabolites is necessary to insure proper handling, dosage, and patient care when the drug is administered. By studying the in vivo metabolism of lidocaine, we might also gain a better understanding of its mode of action, toxicities, interactions with other drugs and chemicals, and possibly some insight into the biochemical reaction mechanisms involved in the biotransformation. Hopefully, this insight might be used to increase the efficacy and/or decrease the toxicity of the drug. This chapter will review those studies which have been undertaken in quest of this insight with inclusion of the available literature through October 1973.

B. GENERAL CHEMISTRY

Lidocaine was synthesized by Lofgren[4] in 1946 according to the reaction shown in Scheme 1. The protonated diethylamino group has a pK_a of 7.85.

SCHEME I-1



Soon after the synthesis of lidocaine, Goldbergh[5] and Gordh[6] demonstrated the long-lasting local anesthetic effect of the compound and its high chemical stability. Compared to the previously available benzoate or benzamide anesthetics, the 2,6-disubstituted anilide anesthetic was expected (based on steric factors) to be chemically more stable to hydrolysis. Lidocaine is stable to hydrolysis in plasma, unlike procaine, and because of this approximately 70% of the metabolism occurs in the liver in man.[7]

Based on the chemical make-up of lidocaine and a knowledge of the metabolizing enzymes known to be present in the liver, certain predictions

can be made as to where the molecule might be metabollically altered. Lidocaine has three reactive centers incorporated into its chemical structure: 1) a tertiary amino group 2) an amide linkage 3) an aromatic ring. The tertiary amino group is susceptible to N-oxidation and oxidative N-dealkylation by liver microsomes.[8] The amide linkage is susceptible to hydrolysis by microsomal amidases[9] and to oxidation by liver microsomes to an N-hydroxy amide.[8] The aromatic ring, including the aryl methyl groups, is susceptible to hydroxylation by liver microsomes.[8] Many of these reactions have been detected since the inception of the research presented in this thesis.

C. A REVIEW OF STUDIES ON LIDOCAINE METABOLISM

The importance of the metabolic disposition of lidocaine is immediately apparent when the question of oral administration is considered.[10-12] The effective oral dose required (~500 mg) leads to CNS toxicities, therefore, many studies concerning the metabolism of lidocaine have been undertaken in order to gain insight into the nature of its metabolites. Two preliminary studies were reported by McMahon and Woods[13,14] in 1951 indicating that only 10-20% of a sub-cutaneous dose of lidocaine in mongrel dogs was excreted unaltered in 24-hour urine samples. These authors suggested that the aromatic ring was oxidized to a phenolic compound which was further conjugated with sulfate.

In 1954 Sung and Truant[15] also used a non-specific assay to determine that in rats 2-6% of an injected dose (40 mg/Kg) of lidocaine

appeared in the 24-hour urine, while in a male monkey only 1% appeared at a dose of 15 mg/Kg. In two human subjects, 3-11% of an injected dose (3 mg/Kg) was found in 24-hour urine samples. These authors also reported that phenolic compounds, both free and conjugated, appeared in the urine compared to controls. Further studies indicated the liver to be the most active organ involved in lidocaine metabolism in rats.

Geddes and Douglas[16] in a communication in 1956 and Geddes[17] in later articles reported results with incubations of carbonyl-^{14}C-labeled lidocaine in various rat tissues. Liver slices alone appeared to yield major quantities of metabolites, one of which Geddes identified as N,N-diethylglycine from chromatographic data. This indicated hydrolysis of the amide linkage, a reaction difficult to achieve by chemical means with lidocaine.[18] The authors noted that the rate of hydrolysis was increased by flushing the system with oxygen.

Hollunger[19] in 1960 studied some of the liver enzymes responsible for the biotransformation of lidocaine in rabbits. He, too, found that the metabolism was oxygen dependent and that lidocaine was N-deethylated by the microsomal fraction to produce acetaldehyde plus MEGX. MEGX was then further metabolized by hydrolysis of the amide linkage to yield N-ethylglycine (monoethylglycine) and xylidine. Hollunger localized the amidase activity to the microsomes and purified a component of rabbit liver microsomes which hydrolyzed MEGX rapidly, but lidocaine only slowly.

Beckett, et al.,[20] studied the urinary excretion rates and plasma levels of lidocaine and MEGX in three human volunteers whose urine was maintained at an acidic pH by oral ammonium chloride treatment following an i.v. dose (50 mg) of lidocaine. An additional study was carried out with MEGX and, based on a computer analysis of the results, these researchers concluded that N-dealkylation of lidocaine represents approximately 40% of the dose. Further dealkylation to produce GX was not detected. The authors suggested that some of the CNS toxicity associated with lidocaine administration such as nausea, dizziness, and convulsions may be due to MEGX. More recently, Smith and Duce[21] studied the toxicology of MEGX in mice and dogs and found it to contribute to the antiarrhythmic and toxic effects in both species.

Breck and Trager[22] reported N^1-ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-imidazolidinone (= cyclic metabonate) as a metabolite of lidocaine in man. The authors suggested that this metabolite may arise during the process of oxidative N-dealkylation or from condensation of MEGX with acetaldehyde. This topic will be pursued in Chapter III of this thesis.

DiFazio and Brown[23] reported on lidocaine metabolism in normal and phenobarbital-treated dogs. These authors found both MEGX and GX in urine extracts and amounts of both metabolites increased with phenobarbital pretreatment which is a procedure known to increase microsomal oxygenase activity.

The most comprehensive study to date on lidocaine metabolism was reported in 1972 by Keenaghan and Boyes[24] who used radio-labeled lidocaine combined with a gas-chromatographic technique to study the tissue distribution and urinary excretion of lidocaine and its metabolites in rats, guinea pigs, dogs, and man (250 mg oral dose). The authors favor the sequence of metabolic pathways shown in Scheme 2 based on the results they obtained shown in Table 1.

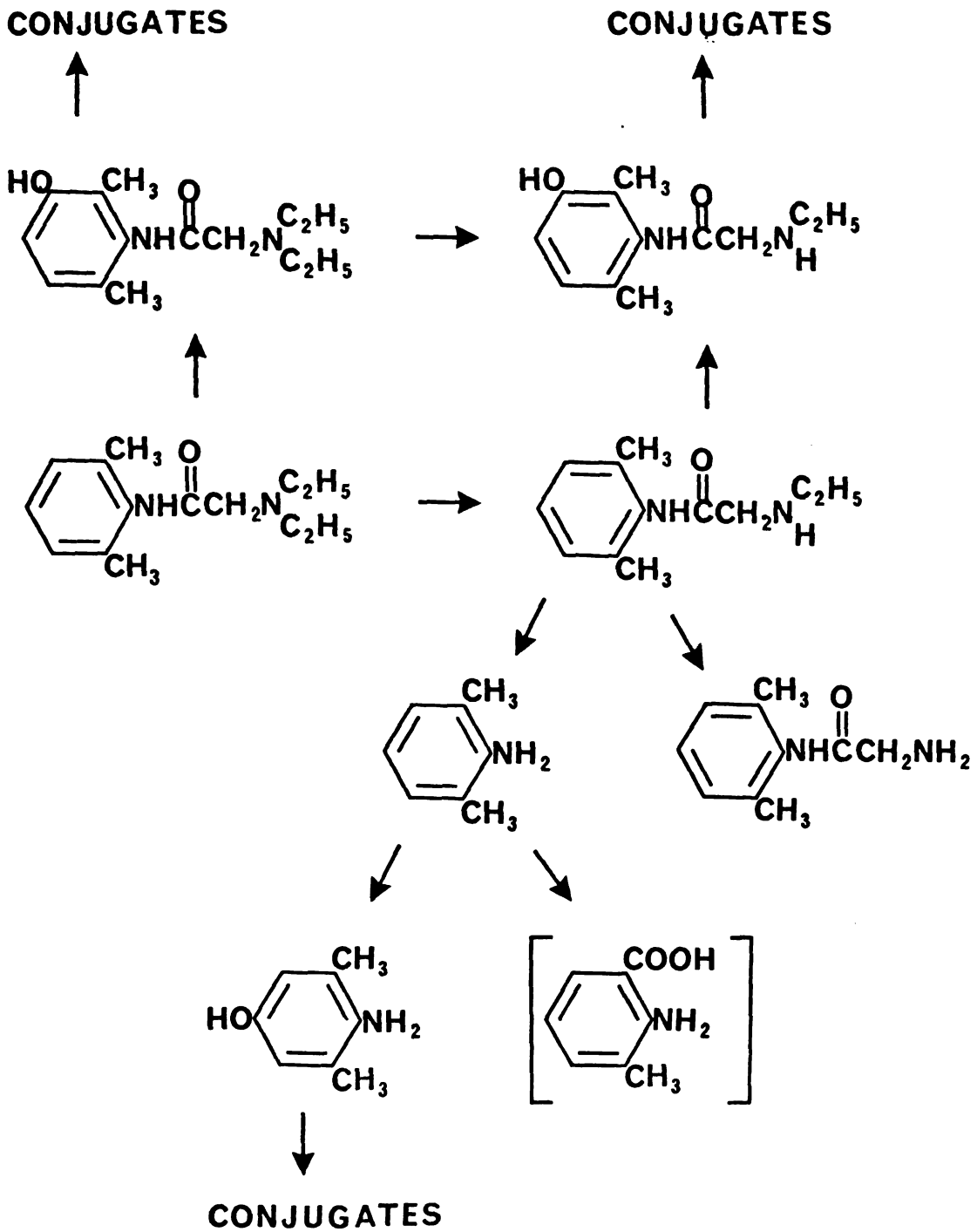
TABLE I-1

Urinary Metabolites of Lidocaine in Various Species

Compound	Percentage of Dose Recovered in Urine			
	Rat	Guinea Pig	Dog	Man
Lidocaine	0.2	0.5	2.0	2.8
MEGX	0.7	14.9	2.3	3.7
GX	2.1	3.3	12.6	2.3
3-hydroxylidocaine	31.2	0.5	6.7	1.1
3-hydroxy-MEGX	36.9	2.0	3.1	0.3
2,6-xylidine	1.5	16.2	1.6	1.0
4-hydroxy-2,6-xylidine	12.4	16.4	53.2	72.6
TOTAL	85.0	53.8	63.5	83.8

Species variation is evident from the table, the results in man being quite different from any of the other species. Especially noteworthy is that greater than 70% of the dose in man was recovered as the oxidized hydrolysis product, 4-hydroxy-2,6,dimethylaniline.

Scheme I-2. The metabolic transformations of lidocaine as proposed by Keenaghan and Boyes (1972).


CONJUGATES
CONJUGATES
HO
CH3
O
NHCCH2N
C2H5
C2H5
CH3
HO
CH3
O
NHCCH2N
C2H5
H
CH3
CH3
O
NHCCH2N
C2H5
C2H5
CH3
CH3
O
NHCCH2N
C2H5
H
CH3
CH3
NH2
CH3
CH3
O
NHCCH2NH2
CH3
CH3
HO
NH2
CH3
COOH
NH2
CH3
CONJUGATES

More recently, Thomas and Meffin,[25] using a gas-chromatographic method, detected only trace amounts (0.01-0.2%) of m-hydroxylidocaine after an oral dose of 115mg lidocaine, along with lesser amounts of p-hydroxylidocaine, both as conjugates. In a subsequent paper Mather and Thomas[26] presented preliminary evidence that in man, treated with ammonium chloride to maintain a constant urinary pH 4.5-5.5, approximately 10% of a 115mg oral dose of lidocaine is excreted as N-hydroxylidocaine, primarily as a conjugate. Another 6-7% was thought to be excreted as N-hydroxyMEGX. Both of these conjectured metabolites are potential carcinogens based on the carcinogenic propensity of other aromatic N-hydroxyamides. This subject will be considered further in Chapter II of this thesis.

Adjepon-Yamoah and Prescott[27] communicated in 1973 their findings that after intramuscular injection of 200 mg of lidocaine, the urinary excretion of lidocaine and MEGX plateaued rapidly compared to GX, which in one patient reached a value of 19% of the administered dose after 24 hours. This result is at variance with the results obtained by Keenaghan and Boyes[24] after a 250 mg oral dose of the same drug.

D. NEW APPROACHES TO THE QUANTIFICATION OF LIDOCAINE AND ITS METABOLITES

1. Introduction

Much of the early work in drug metabolism involved the use of non-specific colorimetric assays and chromatographic techniques. More

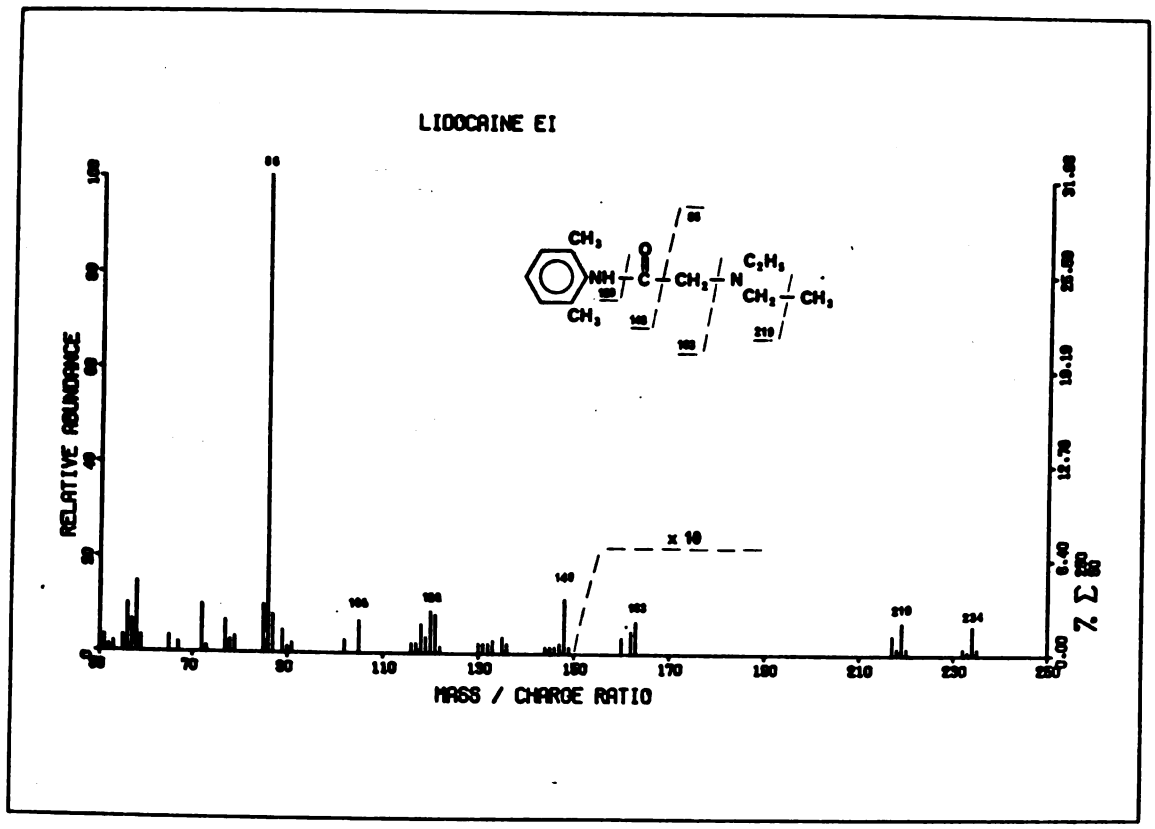
recently[24-27] the sensitive and more specific gas-chromatographic method has been used extensively to quantify drugs and their metabolites. In the last ten years mass spectrometry has become a key analytical technique for the qualitative determination of drugs and metabolites from biological fluids, its particular usefulness being in the more exact elucidation of metabolite structures.[28,29] Many studies are now combining mass spectrometry with gas-chromatography (GC-MS) to identify and quantify compounds in biological fluids.[30,31] In these techniques the mass spectrometer serves as a sophisticated detector to either monitor a single ion (mass fragmentography) or several ions (multiple ion detection) in the gas chromatographic eluent.[32,33]

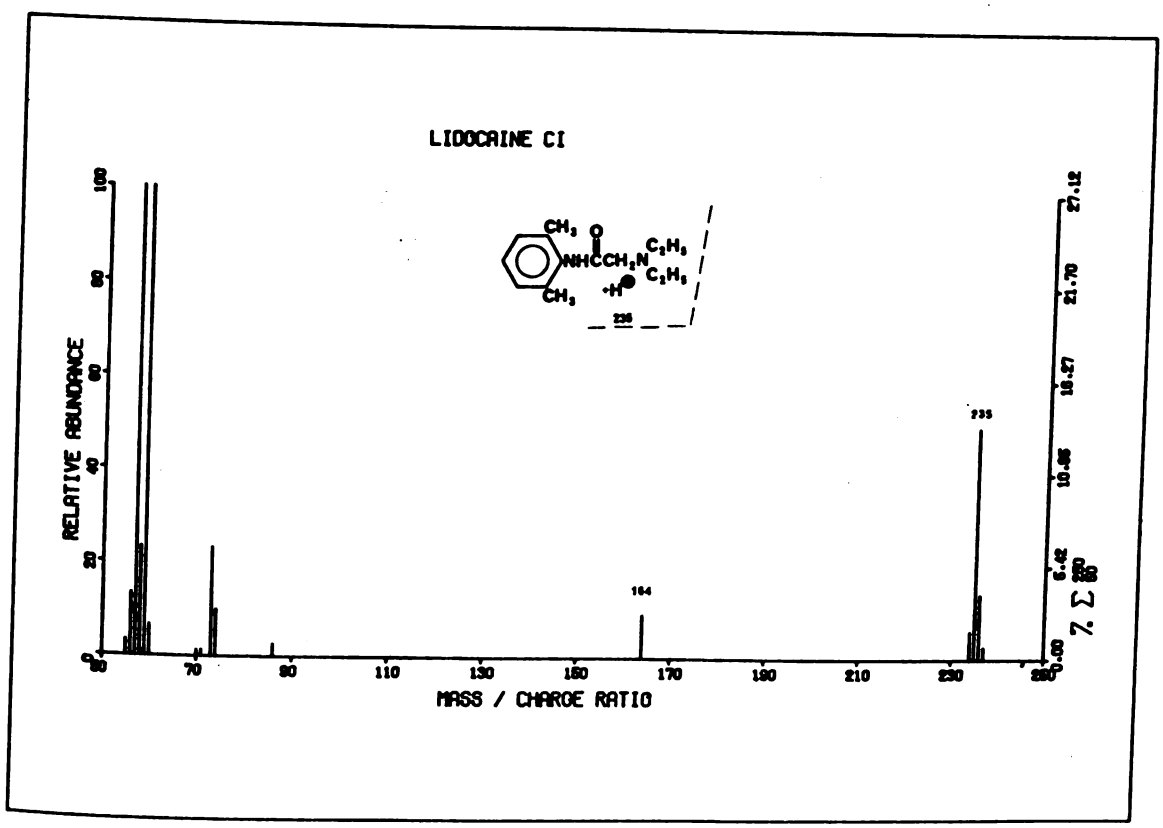
Strong and Atkinson[34,35] reported in 1972 the use of GC-MS to detect and quantify lidocaine and its two N-deethylated metabolites, MEGX and GX, in the plasma and urine of patients receiving i.v. infusions of the drug for the treatment of cardiac arrhythmias. Trimecaine, ω-diethylamino-2,4,6-trimethylacetanilide, was used as an internal standard. Under the conditions of i.v. infusion, lidocaine levels ranged from 1.2-15.0 μg/ml, MEGX from 0.2-2.4 μg/ml, and GX from <1.0-2.7 μg/ml with GX levels <1.0 μg/ml not measurable. The authors noted that a rapid, specific analytical technique for monitoring these metabolites might be useful since one or more of these biotransformation products may be responsible for some of the CNS toxicity associated with lidocaine therapy.

A more direct quantification of lidocaine and its metabolites in human plasma and urine has been employed in the course of this research due primarily to the inventive work of Dr. William A. Garland. The technique utilizes a combination of stable isotope labeling and chemical ionization mass spectrometry (CIMS), and offers an alternate approach to the GC-MS method with certain advantages. The analysis is carried out without recourse to either derivatization or chromatography. As described by Fales and Milne,[36] the biological fluid is extracted with an organic solvent and the residue remaining after solvent evaporation is placed directly into the CIMS ion source via a direct insertion probe. A successful analysis relies on both the fact that a drug or drug metabolite is typically present in greater concentration in the extract than endogenous biochemicals, and on the simple mass spectra (predominantly MH^+ ions) obtained using isobutane chemical ionization.

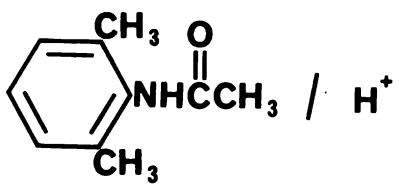
An example of the difference between the EI (electron impact) and CI mass spectra of lidocaine is shown in Figure 1. Note that under electron impact lidocaine yields a small parent ion and a number of more intense fragment ions. The isobutane CI mass spectrum, on the other hand, shows primarily the MH^+ ion at m/e = 235 for protonated lidocaine with only a small peak at m/e = 164 from mass 100 to 235. The exact origin of the peak at m/e = 164 is not known although it corresponds to the structure shown on page 12. The origin of this ion is now under investigation and appears to be a function of the geometry of the ion source. This ion disappears when an exit slit rather than a hole is used. Other major peaks

Figure I-1. Comparative electron impact (EI) and chemical ionization (CI) mass spectra of lidocaine.


LIDOCAINE EI
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
x 10
86
120
140
165
210
234


LIDOCAINE CI
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
235
164

in the spectrum at m/e = 57 and m/e = 73 correspond to peaks arising from the isobutane reagent gas.



MH^+ = m/e 164

Quantification using the CIMS technique is accomplished by adding a known amount of a stable isotope analog of the drug or metabolite into the biological fluid prior to extraction, preferably as soon after the sample is collected as possible. This serves to convert the mass spectral peak height information obtained into absolute concentrations by comparing the MH^+ ions generated in the mass spectrum from the compound to be quantified and its stable isotope analog. The same procedure is standard analytical practice in many isotopic dilution assays except the internal standard used is radioactive and the detection is achieved by a scintillation counter. The use of stable isotope analogs in mass spectral assays has recently become quite popular.[31, 37-40]

The CIMS-stable isotope technique combined with some of the other more classical techniques has confirmed the presence of many suspected

lidocaine metabolites and shown the absence of others, at least under the in vivo conditions of dosage and analysis used in this study. In order to carry out this analysis the 17 suspected metabolites of lidocaine and the deuterated analogues shown in Figure 2 were synthesized according to procedures described in the experimental section. The results will be described in two general parts following an outline of preliminary procedures used in the in vivo experimentation: 1) quantification in plasma 2) quantification in urine.

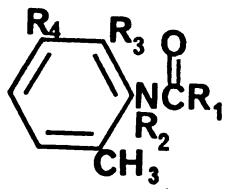
2. Preliminary Procedures

Prior to the in vivo studies the following control studies were carried out with results as indicated:

a. Mass Spectral Studies on Synthetic Metabolites

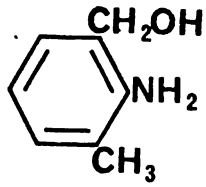
CIMS was performed on all suspected lidocaine metabolites and their deuterated analogs. One important finding was that all of the benzyl alcohols (6, 7, 10 - Figure 2) produced major (MH^+ - 18) ions as a result of a loss of water from the MH^+ ion. This fascile water loss probably results from the mechanism outlined on page 16. Because of this characteristic water loss, any benzyl alcohol metabolites should be easily differentiated from the isomeric phenolic metabolites (4, 5, 11 - Figure 2). Figure 3 shows comparative CI mass spectra of lidocaine benzyl alcohol and m-hydroxylidocaine.

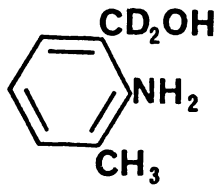
Figure I-2. Synthetic lidocaine metabolites and deuterated analogs.

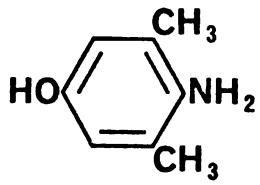


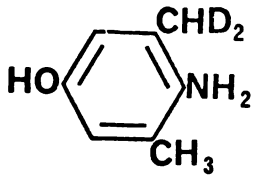
(• = no change)

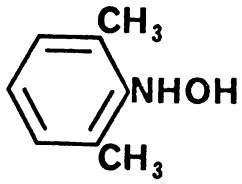
Undeuterated				Deuterated			
R_1	R_2	R_3	R_4	R_1	R_2	R_3	R_4
1. $CH_2N(CH_2CH_3)_2$	H	CH_3	H	$CH_2N(CD_2CH_3)_2$	•	•	•
2. $CH_2N(H)CH_2CH_3$	H	CH_3	H	$CH_2N(H)CH_2CD_3$	•	•	•
3. CH_2NH_2	H	CH_3	H	CD_2NH_2	•	•	•
4. $CH_2N(CH_2CH_3)_2$	H	CH_3	OH	$CH_2N(CD_2CH_3)_2$	•	•	•
5. $CH_2N(H)CH_2CH_3$	H	CH_3	OH	$CH_2N(H)CH_2CD_3$	•	•	•
6. $CH_2N(C_2H_5)_2$	H	CH_2OH	H				
7.				$CH_2N(H)C_2H_5$	H	CD_2OH	H
8. $CH_2N(CH_2CH_3)_2$	OH	CH_3	H	$CH_2N(CD_2CH_3)_2$	•	•	•
9. $CH_2N(H)CH_2CH_3$	OH	CH_3	H	$CH_2N(H)CH_2CD_3$	•	•	•

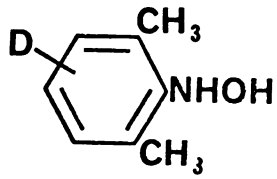
10. 

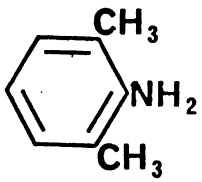


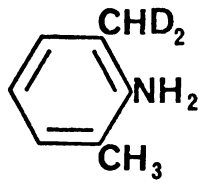
11. 

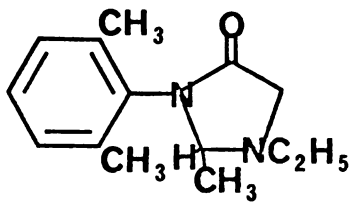


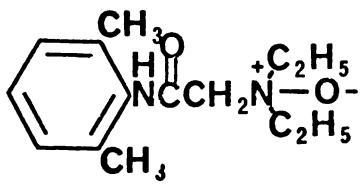
12. 

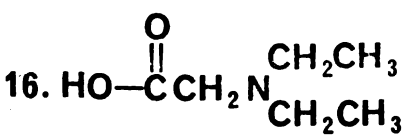


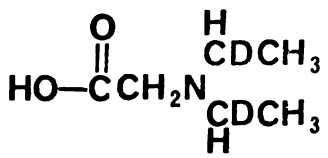
13. 

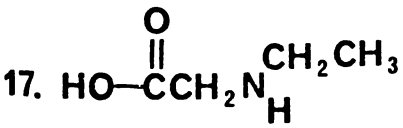


14. 

15. 

16. 



17. 

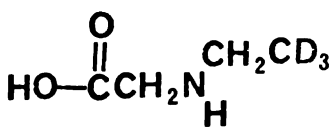
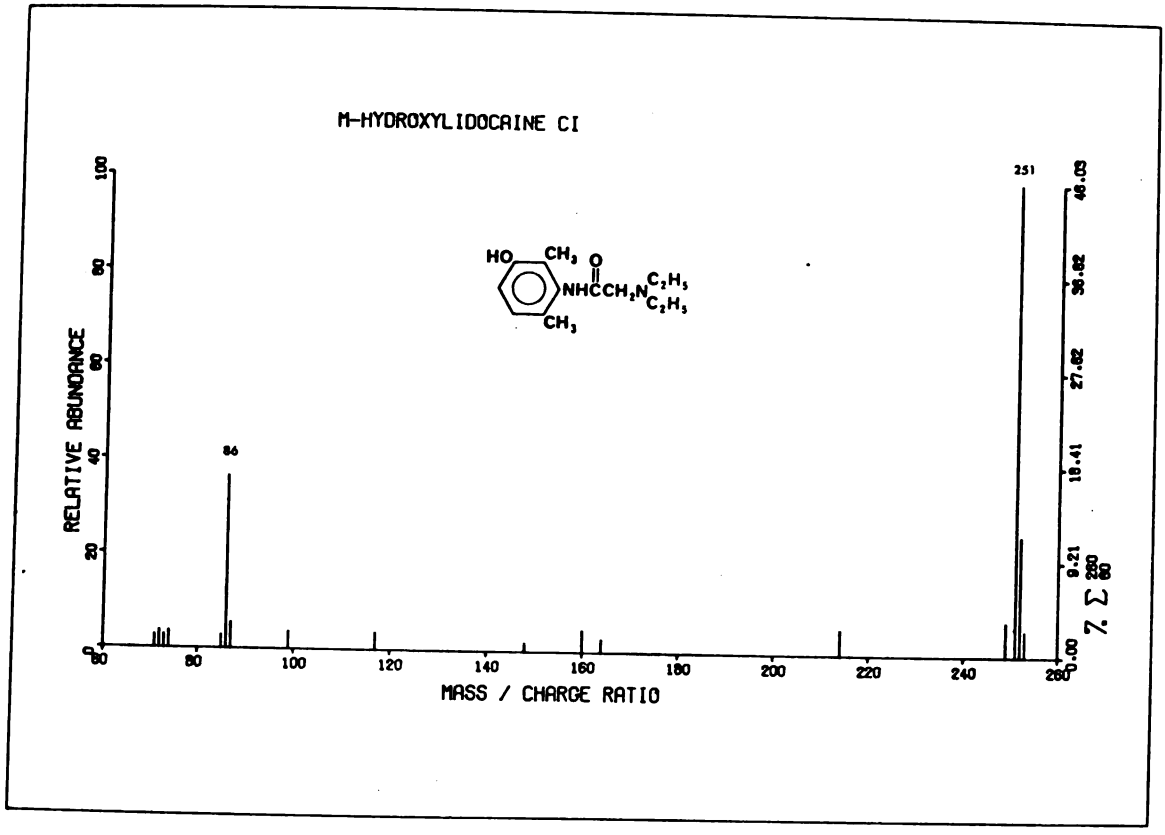
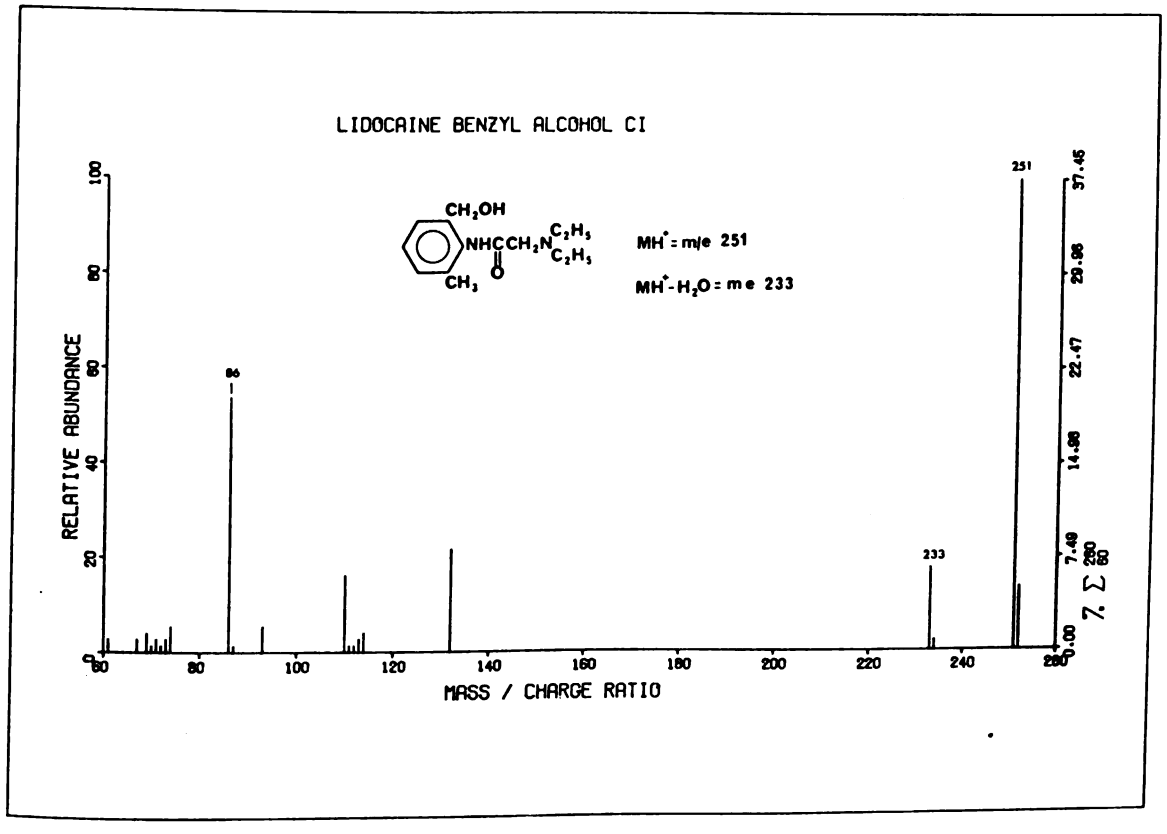
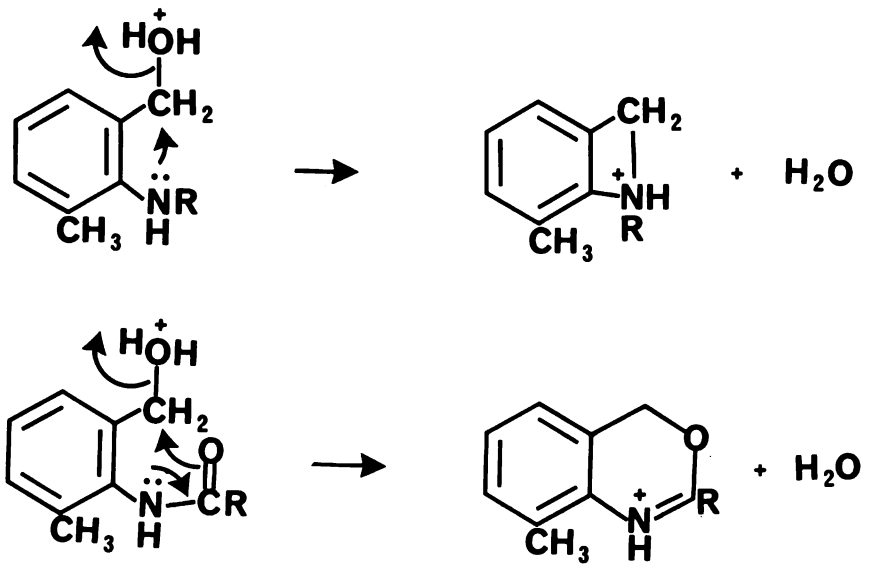


Figure I-3. Comparative CI mass spectra of lidocaine benzyl alcohol and m-hydroxylidocaine.


M-HYDROXYLIDOCAINE CI
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
86
251
HO
CH3
O
NHCCH2N
C2H5
C2H5
CH3


LIDOCAINE BENZYL ALCOHOL CI
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
86
233
251
CH2OH
NHCCH2N
C2H5
C2H5
CH3
O
MH+ = m/e 251
MH+-H2O = me 233



Unfortunately, the N-hydroxyamides (8, 9 - Figure 2), 2, 6-dimethylphenylhydroxylamine (12), and lidocaine N-oxide (15) lose oxygen under all temperature and CIMS conditions employed to give (MH^+ -16) ions which interfere with quantification of lidocaine, MEGX, and 2, 6-dimethylaniline. These possible oxygenated metabolites were therefore quantified by other methods which will be described in Chapter II. This fascile loss of oxygen is probably the result of a thermal homolytic cleavage of the weak nitrogen-oxygen bond and has been observed by others.[41, 42]

b. Preliminary In Vivo Experiment

To determine the spectrum of lidocaine metabolites detectable by CIMS in plasma and urine samples, a healthy 75 Kg male subject received orally an encapsulated dose of 202 mg of lidocaine as the free base. Prior to receiving the dose a 24-hour urine control sample was collected and stored in a freezer. Immediately prior to dosing a 15 ml "0-time" blood sample was collected in a heparinized tube and plasma separated by spinning the sample at 800xg for 15 minutes. Similar samples were collected at 30, 60, 90 and 180 minutes after dosing and stored in a freezer at -15° until further work-up. Serial urine samples were collected from 0-2, 2-4, 4-8, and 8-24 hours with storage of urine in glass bottles in a freezer.

Plasma and urine samples were extracted at pH 8.5 with two volumes of methylene chloride and the residue after evaporation of solvent was subjected to CIMS. Compared to the "0-time" plasma sample, only MH^+ ions corresponding to lidocaine, MEGX, and possibly GX could be seen in the plasma extracts. In the urine extracts MH^+ ions corresponding to 2,6-dimethylaniline, 4-hydroxy-2,6-dimethylaniline, GX, MEGX, hydroxylated MEGX, lidocaine, and hydroxylated lidocaine could readily be detected. No major peaks corresponding to the $(MH^+ - 18)$ loss of water from possible benzyl alcohol metabolites could be found even though these compounds were readily extractable from urine and plasma.

c. Standardization Experiments

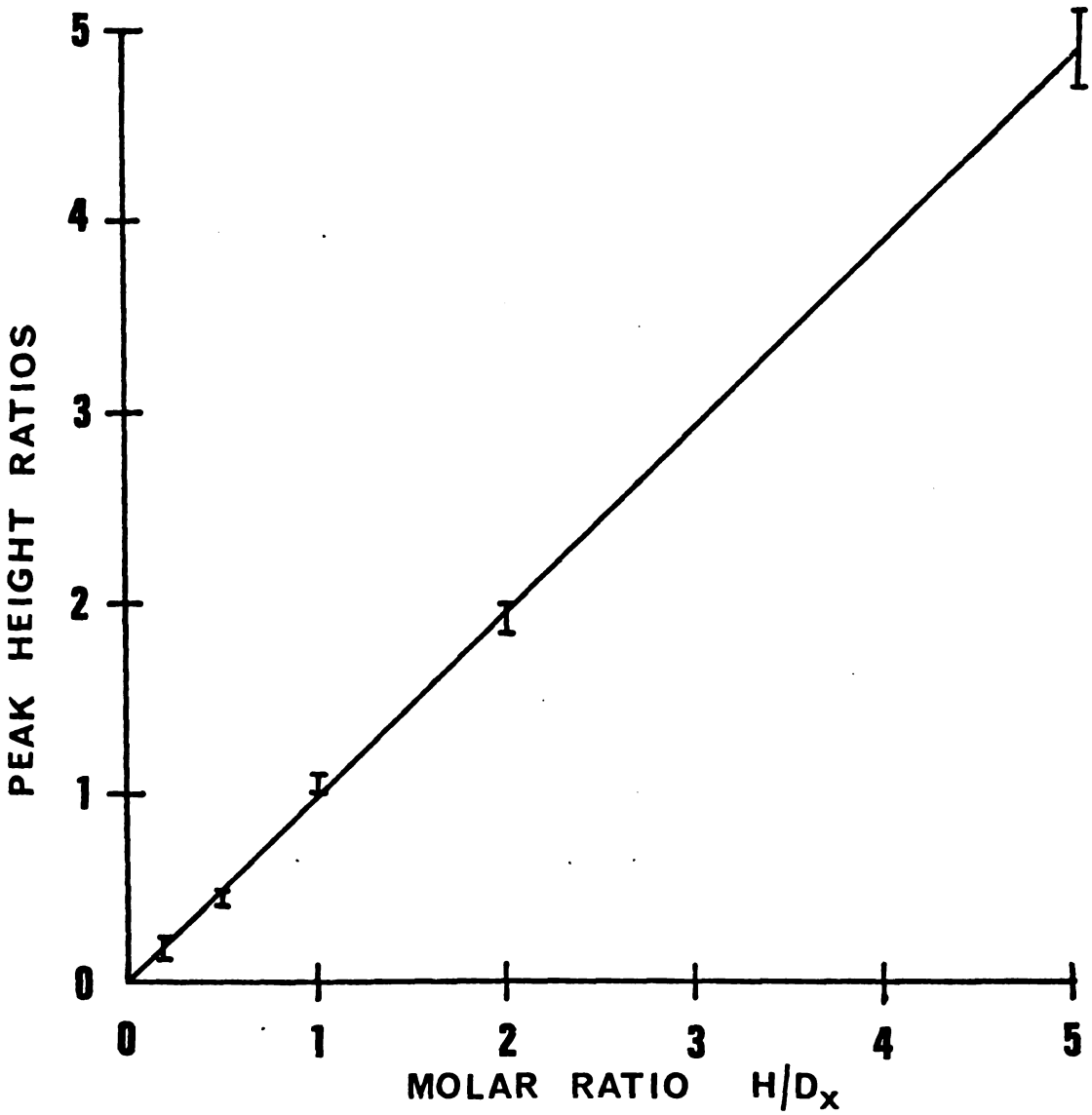
Previous work has shown that extraction and mass spectral response to a compound and a deuterated analog of that compound are equivalent.[39,40] This was tested under the CIMS conditions used, both with blank plasma and urine samples as described in the experimental section. CIMS analysis of the peak-height ratios of metabolite vs. deuterated standards showed good agreement between known and calculated concentrations with variances ranging from 1-5% for the compounds analyzed. Figure 4 shows a standard curve for the quantification of lidocaine and six of its metabolites found in urine.

3. Results of In Vivo Experiments

a. Plasma

Using deuterated internal standards and the CIMS technique described, lidocaine and MEGX were quantified in serial plasma samples from two male subjects (A, B) after a single oral dose of 250 mg lidocaine, and of one male subject (C) after a single oral dose of 202 mg of lidocaine as the free base. The range of detection was from 5 ng - 4 μg for lidocaine, and 0.1-1.0 μg for MEGX illustrating the great dynamic range of the direct CIMS technique. An attempt was also made to analyze for GX. Although the MH^+ at m/e = 181 for the GX-d_2 internal standard was readily seen in all spectra at the 0.5 μg level, no corresponding peaks above background could be found for the MH^+ ion of GX at m/e = 179.

Figure I-4. Standard curve of lidocaine and six of its metabolites vs. their deuterated analogs from CI mass spectral analysis of human urine extracts.



Standard curve of lidocaine and six of its metabolies plus their deuterated standards extracted from urine. The bars (I) represent the range of peak height ratios observed in CI mass spectral analysis for various amounts of lidocaine and the six metabolites added to 10 ml blank urine samples containing the following amounts of deuterated standards: 100 μg lidocaine-d_4, 150 μg MEGX-d_3, .100 μg GX-d_2, 50 μg m-hydroxylidocaine-d_4, 50 μg m-hydroxyMEGX-d_3, 50 μg 2, 6-dimethylaniline-d_2, and 200 μg 4-hydroxy-2, 6-dimethylaniline-d_2. The variances observed for all compounds and their deuterated analogs were from 1-5% except for samples of GX vs. GX-d_2 where variances as much as plus or minus 10% were found. This was due to interfering ions observed in the CI mass spectra of control urine at m/e 179, 180 and 181.

The results of the quantification are presented in Table 2. It is noteworthy that in all samples with measurable MEGX levels small but measurable amounts of cyclic metabonate could be detected. Based on the peak height ratio of the MH^+ of cyclic metabonate at m/e = 233 vs. the MH^+ ion of lidocaine at m/e = 235, the concentration of cyclic metabonate in plasma was found to follow approximately the same time course as MEGX.* The peak concentration appears at 60 minutes and reaches a concentration level of approximately one-fifth that of the MEGX level in subjects A and B as shown in Table 2. These concentrations of cyclic metabonate must be considered as rather crude estimates. However, the presence of this metabolite is confirmed since there was no concomitant increase in m/e 236 compared to control values, which would be expected if the metabonate was arising from the condensation of exogenous acetaldehyde with MEGX. The increase would result from condensation of acetaldehyde with added MEGX-d_3 standard. The problem of the cyclic metabonate will be considered in detail in Chapter III.

Figure 5 depicts the results of the study in graphic form and indicates peak plasma levels of lidocaine 30 min. after administration to subjects A and B, whereas the peak level in subject C, who received lidocaine as the free base, probably occurred between the 90 and 180

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*Although the MH^+ ion for cyclic metabonate was found in all plasma samples, the deuterium standard of the cyclic metabonate was not added to the plasma because of its fascile hydrolysis to MEGX. Due to the almost identical extraction characteristics of cyclic metabonate and lidocaine, the deuterated lidocaine was used as the reference standard.

TABLE I-2

Serial Plasma Levels of Lidocaine, MEGX, and Cyclic Metabonate

Time[a]	Subject	Lidocaine m/e 235[b] / m/e 239	Lidocaine µg/ml	MEGX m/e 207[b] / m/e 210	MEGX µg/ml	Cyclic Metabonate m/e 233 / m/e 235	Cyclic Metabonate µg/ml
30 min	A	84.2/29.6	2.03	22.3/90.0	0.13	.049	0.10
	B	93.5/82.3	0.81	10.2/38.7	0.13	.043	0.03
	C	18.1/90.6[c]	0.002	not measurable		not measurable	
60 min	A	90.6/44.0	1.47	45.2/41.0	0.55	.075	0.11
	B	60.3/64.8	0.67	17.4/34.5	0.25	.079	0.05
	C	5.3/64.2	0.05	5.8/50.3	0.06	.093	0.04
90 min	A	60.5/34.9	1.22	11.9/26.6	0.23	.048	0.06
	B	50.7/77.8	0.47	21.6/34.5	0.21	.054	0.02
	C	40.7/68.8	0.42	7.3/57.7	0.06	.076	0.04
180 min	A	49.0/37.0	0.95	8.7/19.7	0.22	.066	0.06
	B	38.7/121	0.23	10.1/26.6	0.16	.063	0.01
	C	58.7/102	0.41	7.9/60.8	0.06	.112	0.04

[a]Refers to the time the blood sample was taken after subject received an oral dose of lidocaine hydrochloride monohydrate (A, B) or lidocaine as the free base (C).

[b]The peak heights as measured from the CI mass spectrum after correcting for C-13 abundances and deuterium incorporation.

[c]Based on C-13 satellite peak of lidocaine-d_4 internal standard.

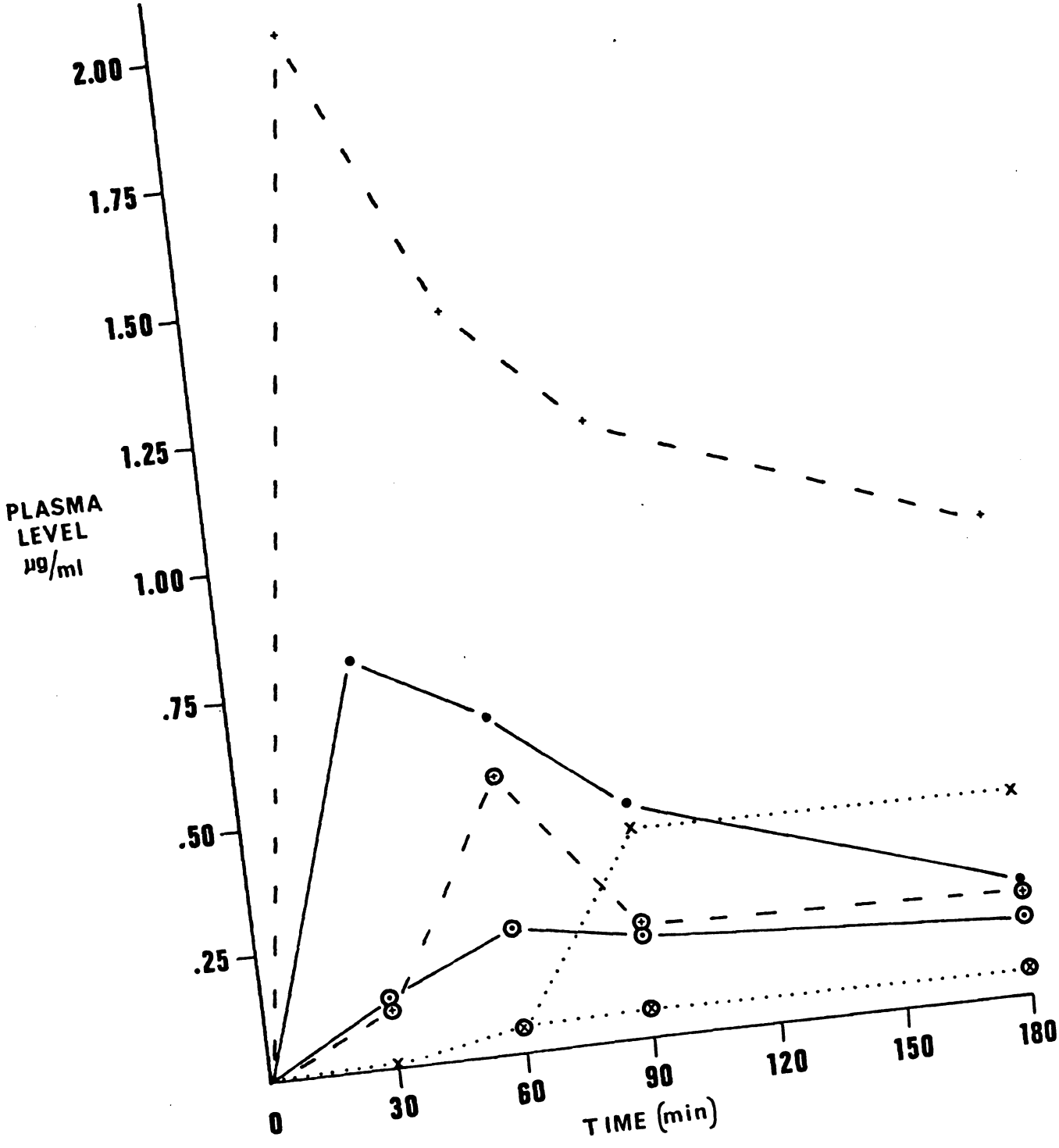
minute sampling period. This slower absorption of the free base would be expected considering its much lower water solubility compared to the soluble lidocaine hydrochloride monohydrate salt. Peak plasma levels of MEGX occur approximately 30 min. after peak plasma lidocaine levels.

Figure I-5. Plasma levels of lidocaine and MEGX in two male human subjects (A, B) after oral administration of 250 mg of lidocaine hydrochloride monohydrate, and subject C after 202 mg of lidocaine as the free base.

+----+ subject A, lidocaine; ⊕----⊕ subject A, MEGX

●——● subject B, lidocaine; ⊙——⊙ subject B, MEGX

x....x subject C, lidocaine; ⊗....⊗ subject C, MEGX


2.00
1.75
1.50
1.25
1.00
.75
.50
.25
PLASMA LEVEL µg/ml
0
30
60
90
120
150
180
TIME (min)

Although subjects A and B were of similar weight and physical make-up, there was a great difference in peak plasma levels of lidocaine and MEGX. From the work of Boyes, et al.,[12] and Adjepon-Yamoah and Prescott,[27] subject A has higher plasma levels than are normally found after oral administration. An added "subjective" note is that A was the only volunteer to feel dizzy during the first 60 minutes of the experiment. The peak plasma levels and time course of both lidocaine and MEGX in subject B are similar to those encountered in the studies cited above, and the normalized CI mass spectrum of the 60 minutes plasma sample of this subject is presented in Figure 6.

b. Urine

1) Basic Metabolites

Lidocaine and six of its metabolites were quantified simultaneously using the CIMS—stable isotope dilution procedure. Basically, this involved collection of serial urine samples with immediate addition of a stock solution containing known amounts of the deuterated standards to urine aliquots. The urine was then stored in a freezer (-15°) until further work-up. Using this procedure, any structural change occurring in these metabolites was reflected in identical changes in the added standards. Quantification was carried out before and after β-glucuronidase-sulfatase treatment. The results of the quantification are presented in Table 3, and cumulative excretion curves of the various metabolites for subjects A, B, and C are displayed in Figures 7-10. Comparative CI mass spectra of

Figure I-6. Galvanometer (x3) trace of isobutane chemical ionization mass spectrum of subject B 60 minute plasma sample (benzene extract) after oral administration of 250 mg lidocaine hydrochloride monohydrate. Ion at m/e = 239 represents lidocaine-d_4 internal standard, m/e = 235 represents lidocaine in plasma; m/e = 210 represents MEGX-d_3 internal standard, m/e = 207 represents MEGX in plasma; m/e = 181 represents GX internal standard. The large peak at m/e = 195 is from caffeine and was the largest ion found in the 0-time blood sample as well.

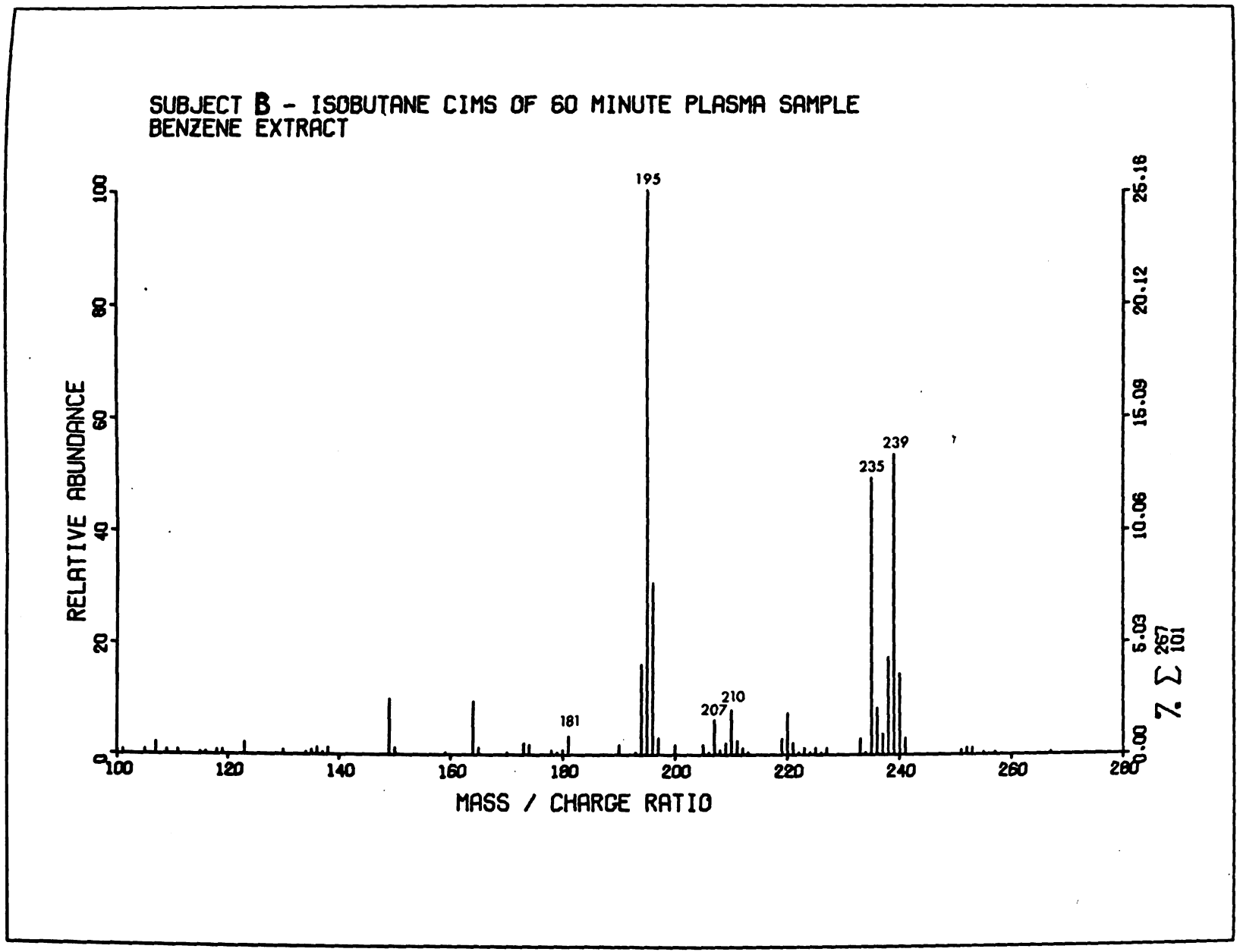

SUBJECT B - ISOBUTANE CIMS OF 60 MINUTE PLASMA SAMPLE
BENZENE EXTRACT
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
195
239
235
210
207
181
% Σ 267 101
0
20
40
60
80
100
100
120
140
160
180
200
220
240
260
280
0.00
5.03
10.06
15.09
20.12
25.18

TABLE I-3

Quantification of Lidocaine and its Metabolites in Human Urine

Compound	Percentage of Administered Dose Recovered in 0-24 Hour Urine[a]					
	Subject A		Subject B		Subject C	
	Before β-gluc. %	After[b] β-gluc. %	Before β-gluc. %	After[b] β-gluc. %	Before β-gluc. %	After β-gluc. %
Lidocaine	1.78	1.51	3.33	2.90	0.74	0.74
MEGX	4.17	4.02	8.80	8.36	1.74	1.73
GX	0.23	--[c]	1.32	--[c]	1.08	--[c]
m- and/or p-Hydroxylidocaine	0.06	0.80	0.10	0.43	0.06	0.97
m- and/or p-HydroxyMEGX	0.	0.62	0.20	0.80	0.03	0.25
2,6-Dimethylaniline	1.41	--[c]	0.27	--[c]	1.22	--[c]
4-Hydroxy-2,6-dimethylaniline	1.37	68.2	0.70	61.7	0.	60.5
TOTAL	9.02	75.2	14.7	74.2	4.87	64.2

[a] Quantified as molar concentrations of free bases in subjects A and B after an oral dose of 250 mg lidocaine hydrochloride monohydrate, and in subject C after an oral dose of lidocaine as the free base.

[b] After treatment with β-glucuronidase-sulfatase for 24 hours some of the metabolite levels were found to inexplicably decrease somewhat.

[c] The β-glucuronidase-sulfatase treatment somehow affected both the metabolite and internal standard so that they were inextractable.

Figure I-7. Cumulative urinary excretion curves for lidocaine (———) and GX (-----) in subject A (▲), subject B (●) and subject C (o).

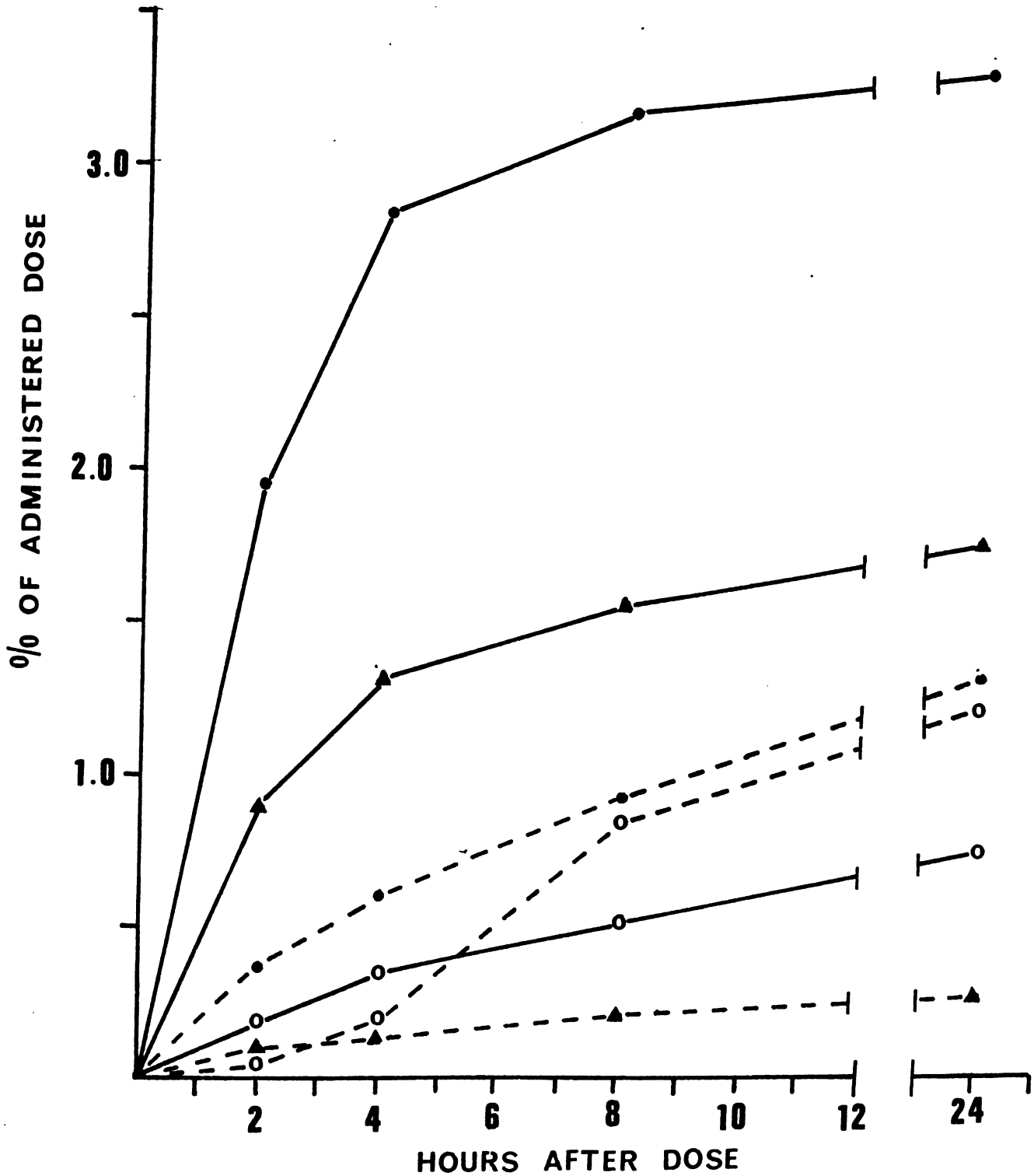

% OF ADMINISTERED DOSE
3.0
2.0
1.0
2
4
6
8
10
12
24
HOURS AFTER DOSE

Figure I-8. Cumulative urinary excretion curves for MEGX in subject A ▲——▲, subject B ●——●, and subject C o——o.

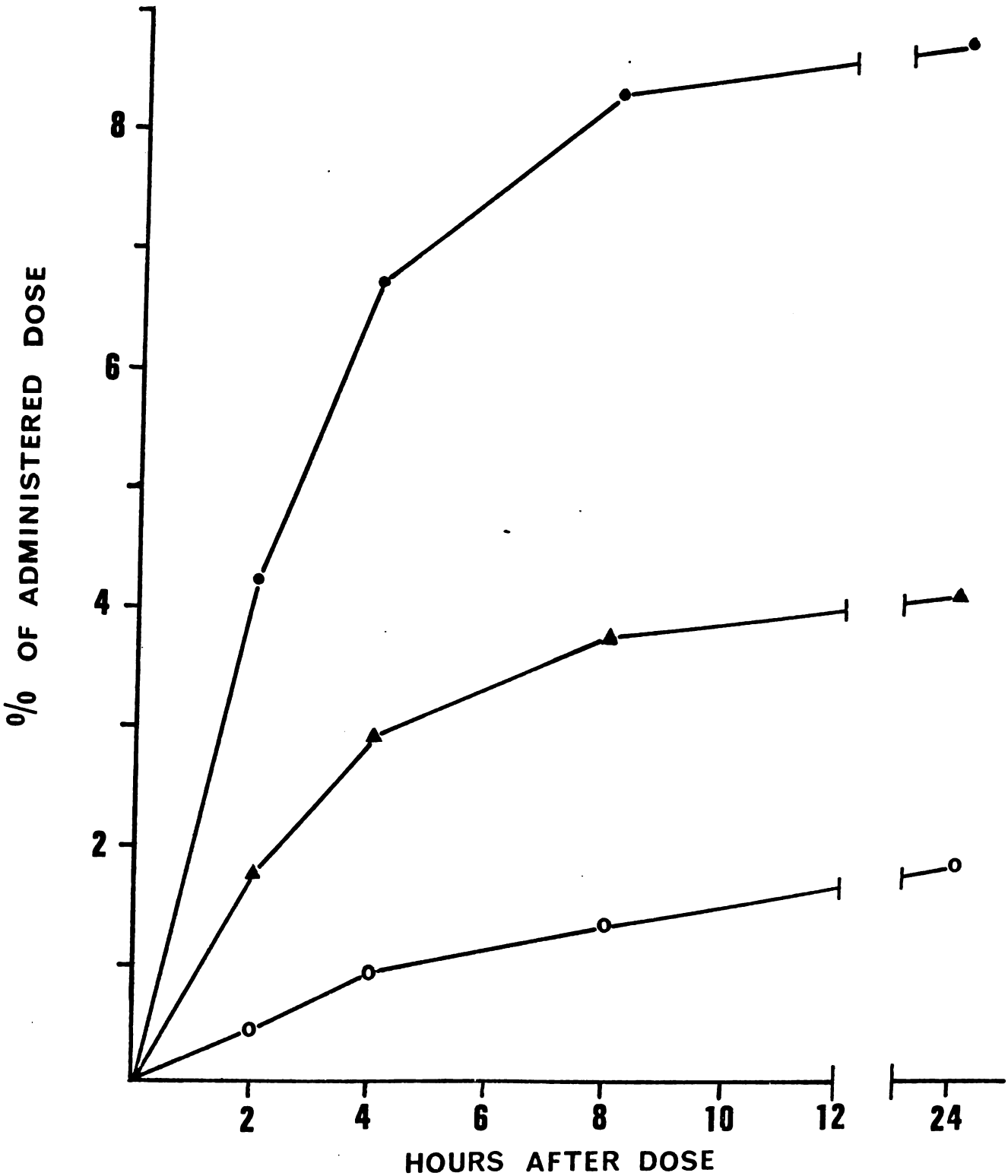

% OF ADMINISTERED DOSE
8
6
4
2
2
4
6
8
10
12
24
HOURS AFTER DOSE

Figure I-9. Cumulative urinary excretion curves for m- and/or para-hydroxylidocaine (———) and for m- and/or para-hydroxyMEGX (------) in subject A (▲), subject B (●) and subject C (o).

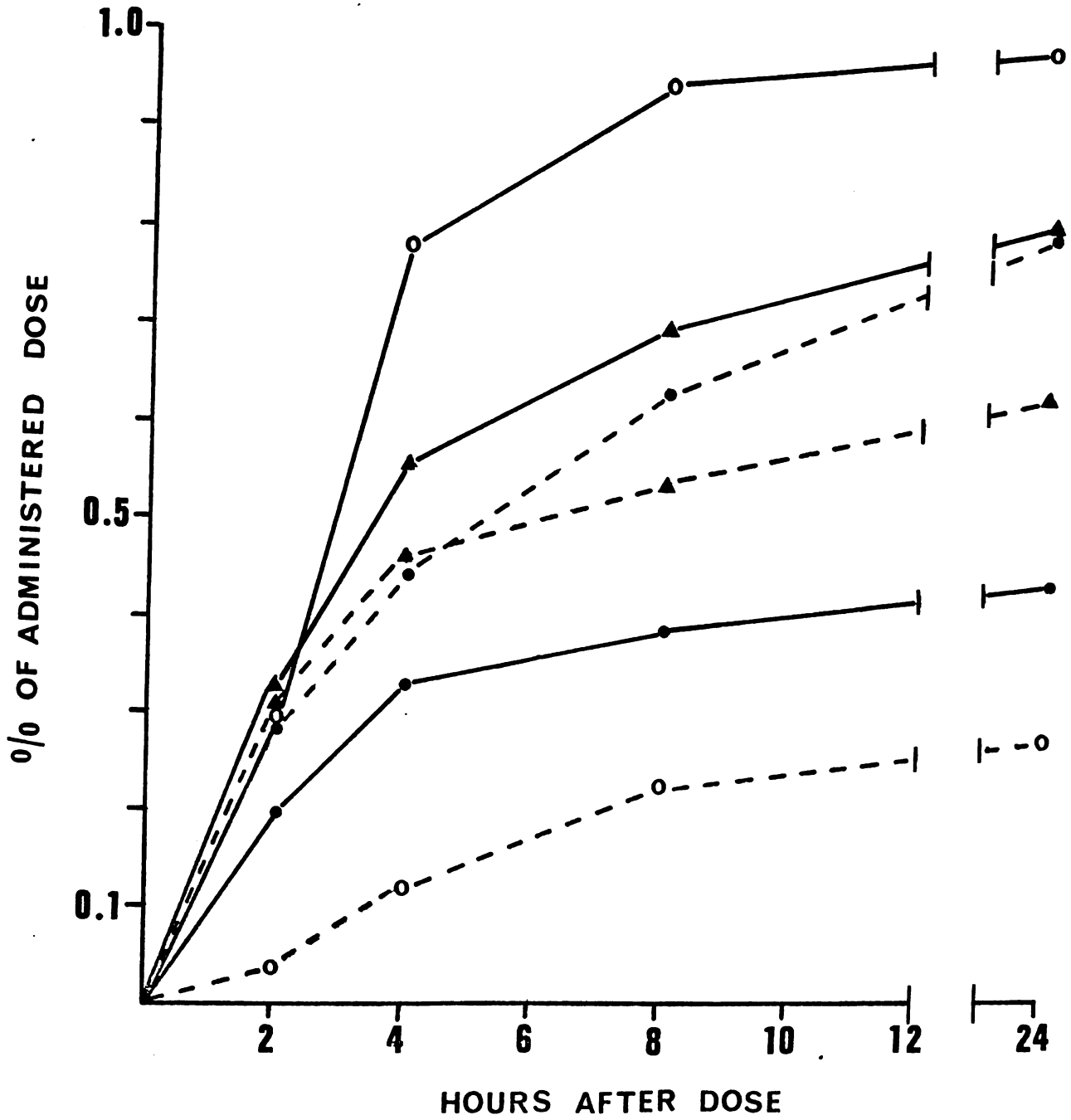
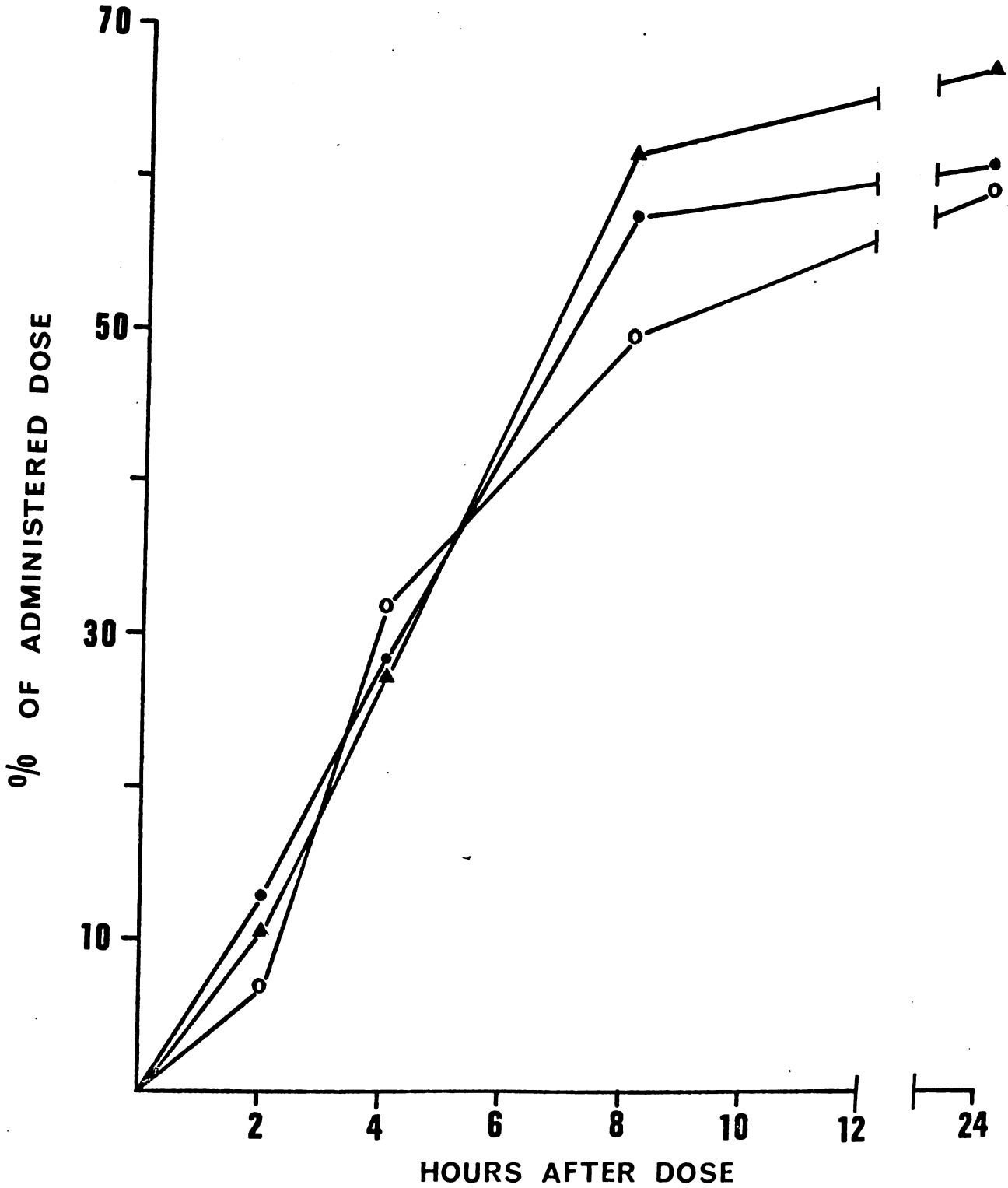

1.0
0.5
0.1
% OF ADMINISTERED DOSE
2
4
6
8
10
12
24
HOURS AFTER DOSE

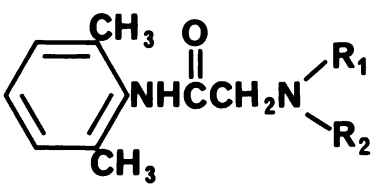
Figure I-10. Cumulative urinary excretion curve for 4-hydroxy-2,6-dimethylaniline in subject A (▲———▲), subject B (●———●) and subject C (o———o).

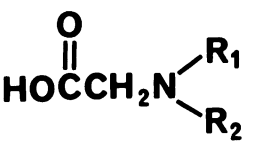

70
50
30
10
% OF ADMINISTERED DOSE
2
4
6
8
10
12
24
HOURS AFTER DOSE

the 0-2 and 8-24 hour urine extracts of subject B are shown in Figure 11, and comparative spectra of the 4-8 hour urine extracts of subject C before and after β-glucuronidase-sulfatase treatment in Figure 12.

2) Amino Acids

Three possible amino acids can result from hydrolysis of the amide linkage of lidocaine and its two N-deethylated metabolites MEGX and GX as shown.



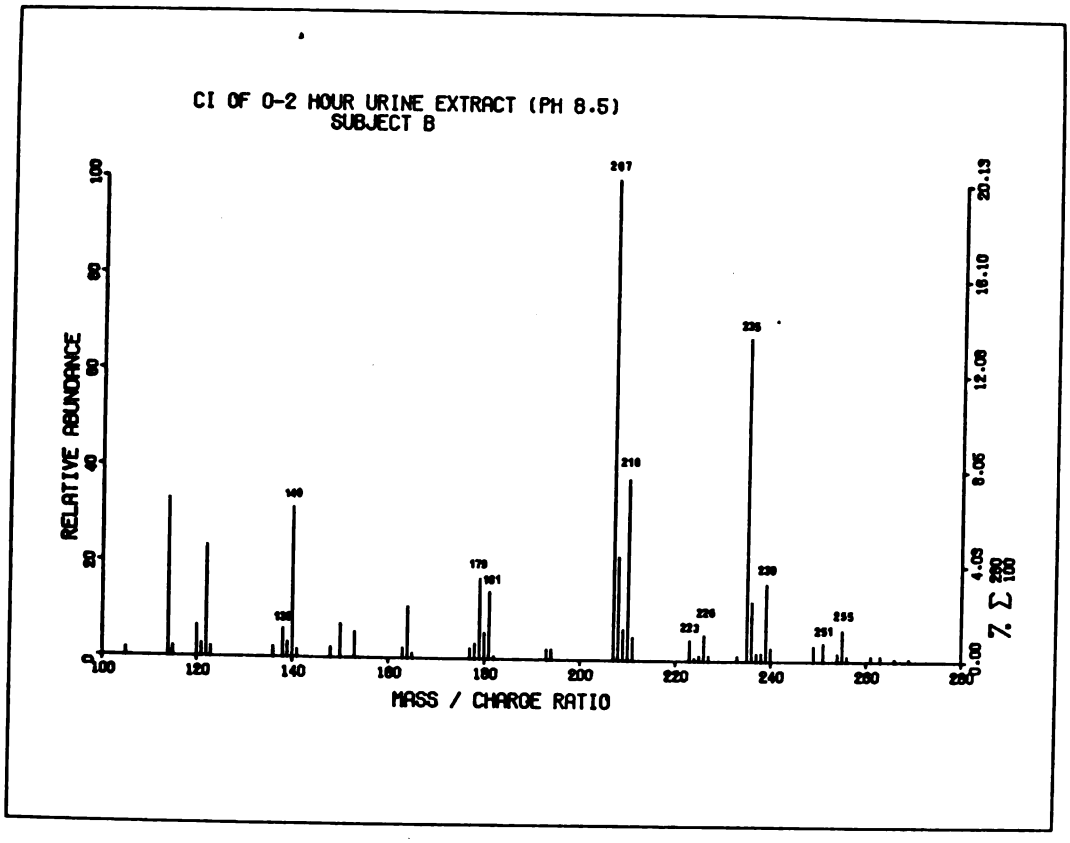


	R_1	R_2	
Lidocaine	**Et**	**Et**	**N,N-diethylglycine**
MEGX	**Et**	**H**	**Monoethylglycine**
GX	**H**	**H**	**Glycine**

Little information has been reported concerning these possible metabolites probably because of inherent difficulties in isolation and confirmation of the structures of water-soluble glycine derivatives. Geddes[16,17] reported that rat liver slices hydrolyzed lidocaine to

Figure I-11. Comparative CI mass spectra of the methylene chloride extracts from basified (pH 8.5) 0-2 and 8-24 hour urine samples of subject B. The labeled peaks represent the following metabolites and their deuterated standards:

1) m/e = 138, 4-hydroxy-2,6-dimethylaniline
   m/e = 140, 4-hydroxy-2,6-dimethylaniline-d_2
2) m/e = 179, GX and m/e = 181, GX-d_2
3) m/e = 207, MEGX and m/e = 210, MEGX-d_3
4) m/e = 223, m- and/or p-hydroxyMEGX
   m/e = 226, m-hydroxyMEGX-d_3
5) m/e = 235, lidocaine and m/e = 239, lidocaine-d_4
6) m/e = 251, m- and/or p-hydroxylidocaine
   m/e = 255, m-hydroxylidocaine-d_4


CI OF 0-2 HOUR URINE EXTRACT (PH 8.5)
SUBJECT B
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
207
216
236
140
179
181
239
226
223
255
251
100
120
140
160
180
200
220
240
260
280
20.13
16.10
12.08
8.05
4.03
0.00

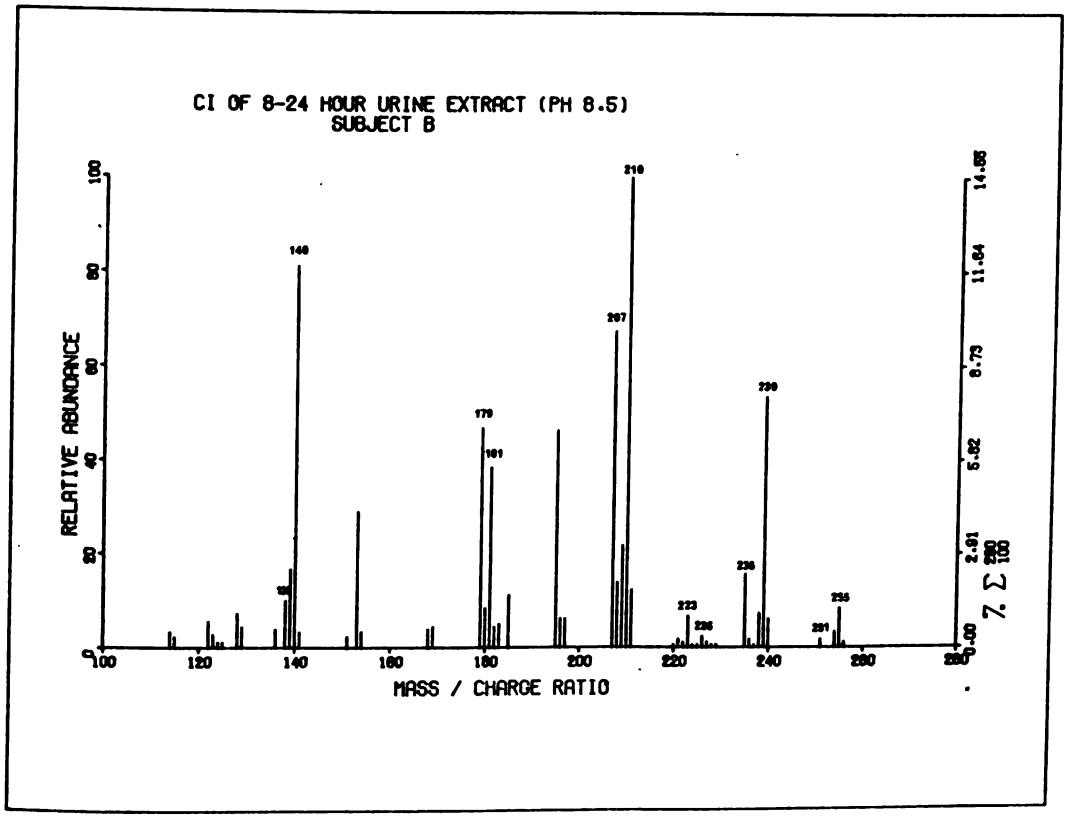
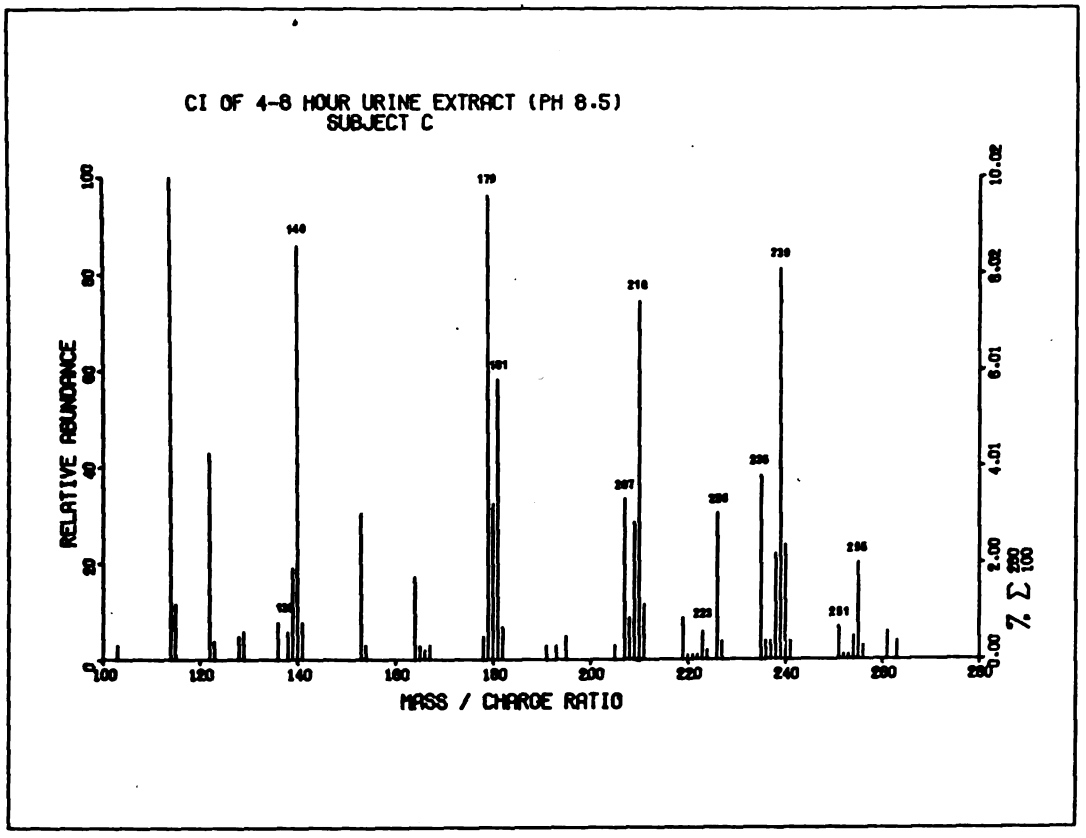
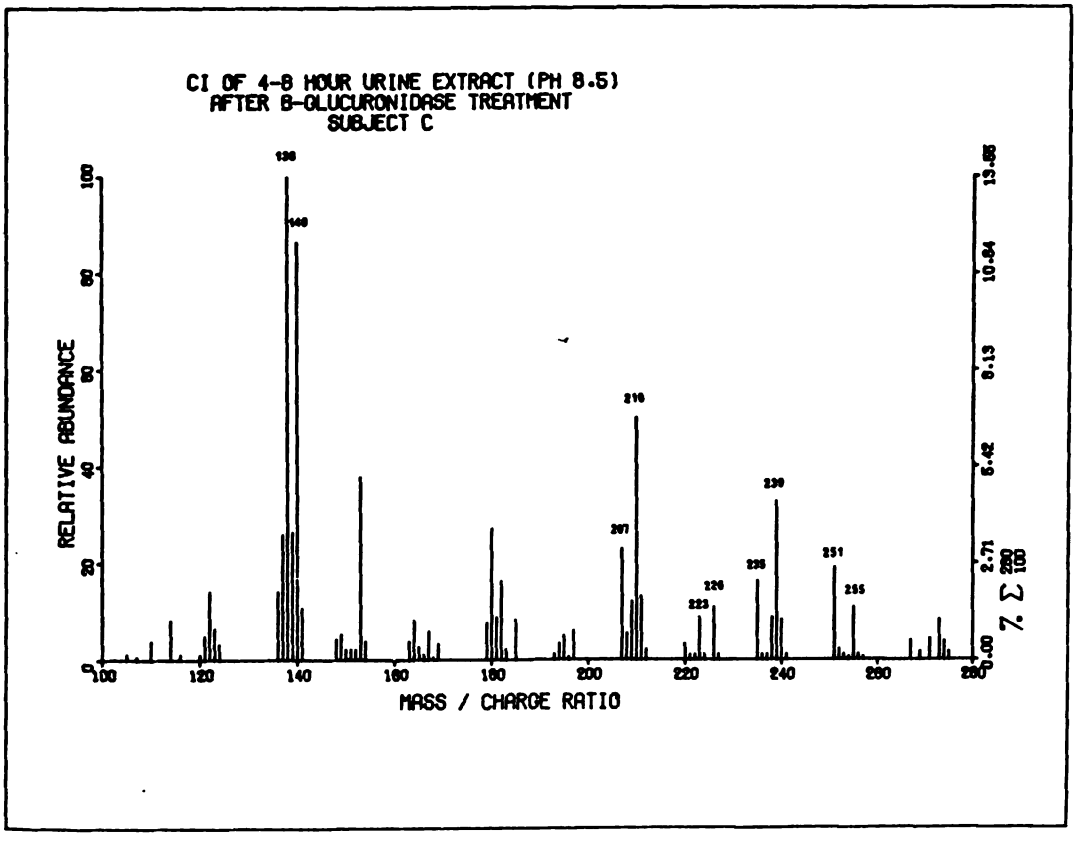

CI OF 8-24 HOUR URINE EXTRACT (PH 8.5)
SUBJECT B
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
216
140
207
179
181
239
235
223
226
255
251
100
120
140
160
180
200
220
240
260
280
14.68
11.84
8.79
5.82
2.91
0.00

Figure I-12. Comparative CI mass spectra of the methylene chloride extracts from basified (pH 8.5) 4-8 hour urine samples (subject C) before and after β-glucuronidase-sulfatase treatment. The labeled peaks represent the following metabolites and their deuterated standards:

1) m/e = 138, 4-hydroxy-2,6-dimethylaniline
   m/e = 140, 4-hydroxy-2,6-dimethylaniline-d_2
2) m/e = 179, GX and m/e = 181, GX-d_2
3) m/e = 207, MEGX and m/e = 210, MEGX-d_3
4) m/e = 223, m- and/or p-hydroxyMEGX
   m/e = 226, m-hydroxyMEGX-d_3
5) m/e = 235, lidocaine and m/e = 239, lidocaine-d_4
6) m/e = 251, m- and/or p-hydroxylidocaine
   m/e = 255, m-hydroxylidocaine-d_4


CI OF 4-8 HOUR URINE EXTRACT (PH 8.5)
SUBJECT C
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
% Σ


CI OF 4-8 HOUR URINE EXTRACT (PH 8.5)
AFTER B-GLUCURONIDASE TREATMENT
SUBJECT C
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
% Σ

N, N-diethylglycine and 2, 6-dimethylaniline based on radio-chromatographic data. Hollunger[19] partially purified an amidase from rabbit liver microsomes that preferentially hydrolyzed MEGX to yield monoethylglycine. From this work Hollunger suggested that the rats used in Geddes study may have also metabolized lidocaine to MEGX followed by amide hydrolysis since the two N-substituted glycines have almost identical rf values in the chromatographic system employed by Geddes.

During the course of the research presented in this thesis, isolation and characterization by chromatographic and mass spectral methods was made of both monoethyl- and N, N-diethylglycine from the urine of a Rhesus monkey treated with lidocaine, and N, N-diethylglycine from human urine. Although these two amino acids were isolated and characterized, quantification using the CIMS-stable isotope technique was not accomplished. One of the important features of the CIMS method was its simplicity, i.e., minimal purification prior to analysis. However, even with extensive treatment of the urine involving urease, lyophylization, and tlc separation, the CIMS technique proved inadequate due to the presence of large amounts of interfering water soluble material in urine. Ion exchange chromatography will probably be required to separate the amino acids from the other water soluble materials. A combination of radio-labeled monoethyl- and N, N-diethylglycine to follow the course of ion-exchange elution, and stable-isotope labeling for CIMS quantification should provide a useful procedure.

Some rather inexact quantitative data on the excretion of these metabolites was obtained in humans after an oral dose of 500 mg of

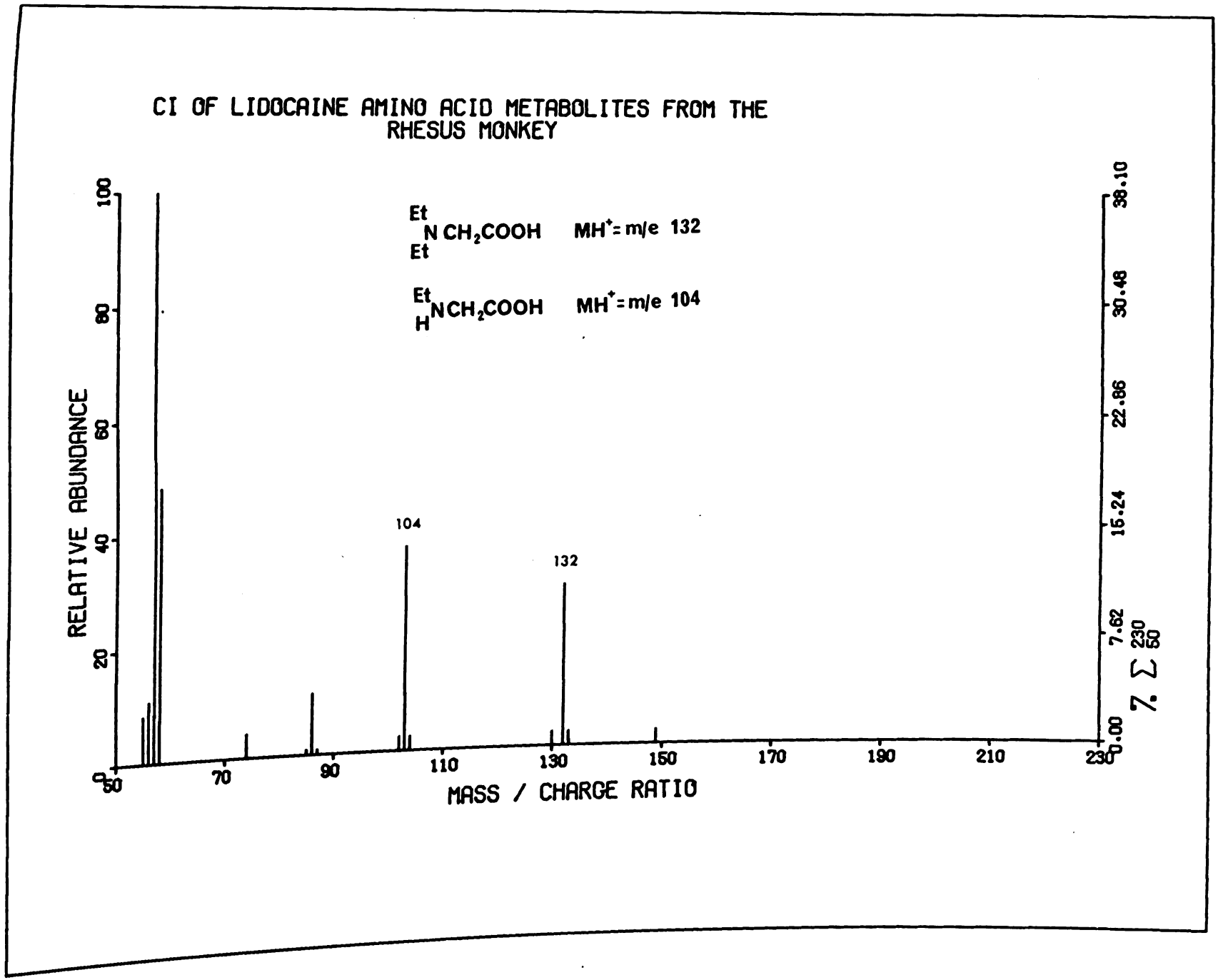
randomly tritiated lidocaine.[22] The non-specific tritium labeling technique used in the synthesis of the radioactive compound only allows us to report that minimally N, N-diethylglycine represents 35% of the excreted dose in two human subjects. This was determined by standard isotope dilution methods reported in the experimental section. Although, indications are that monoethylglycine is also present, based on some inexact CIMS data, this amino acid eluded quantification. However, in a Rhesus monkey, both amino acids were quantified after using an ion exchange combined with a glc and CIMS analysis. After receiving an i. v. dose of 100 mg lidocaine this monkey excreted a minimum of 15% N, N-diethylglycine and 19% of monoethylglycine based on radioactivity data combined with glc and a CIMS analysis which is shown in Figure 13.

4. Discussion

a. The CIMS-Stable Isotope Dilution Technique

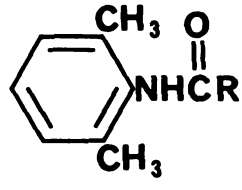
The results of this work show the extreme usefulness of the CIMS technique employed in both clinical analysis and in drug metabolism studies. Using this procedure in a study of human lidocaine metabolism, several metabolites and their deuterated internal standards could be simultaneously monitored and quantified in a single temperature-programmed analysis. The technique combines speed, accuracy, and specificity in determining and quantifying metabolites, as well as safety for the investigator. Drawbacks of the method include cost and upkeep of the instrument, time and expense required to synthesize the necessary

Figure I-13. CI mass spectrum of the lidocaine amino acid metabolites isolated by ion exchange chromatography from the urine of a Rhesus monkey.


CI OF LIDOCAINE AMINO ACID METABOLITES FROM THE RHESUS MONKEY
Et
N CH2COOH MH+= m/e 132
Et
Et
H NCH2COOH MH+= m/e 104
RELATIVE ABUNDANCE
100
80
60
40
20
0
104
132
50
70
90
110
130
150
170
190
210
230
MASS / CHARGE RATIO
38.10
30.48
22.86
15.24
7.62
0.00
7. Σ 230 50

deuterated standards, and inability to provide information necessary to differentiate similar structural isomers of a metabolite such as m- and p-hydroxylidocaine.

General application of the technique for determining minor metabolites relative to background ions will require the use of computer analysis at conditions of moderate mass resolution. For example certain ions not present in control urine were detected in small amounts including ions at m/e = 177, 221 and 249 which might represent the following compounds, respectively:



1 R, CH=N:OH 2 R, $CH_2\overset{H}{N}COCH_3$ 3 R, $CH_2N\overset{Et}{\underset{COCH_3}{}}$

The first is an oxime which could arise from oxidation and reduction of GX, the second might come from acetylation of GX, and the third, acetylation of MEGX. All of these pathways are known to be operative in man. Computer acquisition of CI data at moderate resolution might support or refute the presence of such minor metabolites.

b. Pathways of Lidocaine Metabolism

Using the CIMS-stable isotope dilution technique, we have been able to account for 70-80% of lidocaine metabolism in man. The results tend to support the metabolic sequence outlined by Keenaghan and Boyes[24] (Scheme 2). As in most species studied, the major primary pathway of lidocaine biotransformation in man appears to be N-dealkylation followed by secondary oxidations, conjugations, and hydrolysis. We have ruled out any major contributions to lidocaine metabolism by oxidation of the aromatic methyl groups. However, whereas Keenaghan and Boyes suggested only hydrolysis of the amide linkage after deethylation, our results indicate that a substantial amount of direct hydrolysis of lidocaine takes place.

This hydrolytic pathway, which appears to operate rapidly in man, may bear some relation to the spectrum of toxic manifestations of lidocaine therapy. One severe, although not prevalent, side effect of lidocaine therapy is convulsive seizures.[43-45] The cause of the seizures seems to be CNS excitation as a result of selective blockade of inhibitory cortical synapses.[46-49] Although, a priori, lidocaine itself has been implicated in the blockade of the inhibitory synapse, another possibility should be examined. Either of the amino acid metabolites may act as inhibitors at the synapses since previous work by Paine and Heinz[50] and Vidaver, et al.,[51] has shown that monoethylglycine competitively inhibits glycine transport across certain membranes. Glycine is normally a transmitter at these synapses. More work should be carried out on

quantifying the amino acid metabolites in man and on their possible role in blockading CNS inhibitory synapses.

E. EXPERIMENTAL

1. Synthesis of Suspected Lidocaine Metabolites and Specifically Deuterated Analogs

a. Materials

Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Ir spectra were recorded on either a Perkin-Elmer 337 or Varian IR-5A spectrophotometer; nmr on a Varian A-60A (δ) or T-60 (δ^T); EI mass spectra were obtained on an AEI MS 902

(direct inlet; 70 eV); CI mass spectra were obtained on an AEI MS-9 double focusing mass spectrometer modified for CI using isobutane as the reagent gas and a specially constructed ion source. The electron gun voltage was set at 510 volts, the ion repeller was variable between 0.0 and 1.0 volts while source and analyzer ion gauges read 1.8×10^{-4} torr and 7.6×10^{-6} torr respectively. The chamber source pressure was 0.51 torr as read from a calibrated (3.1 mm = 1 mm ion source pressure) McLeod gauge some distance from the ion chamber. Both sample and reagent gas were introduced via a specially designed direct insertion probe. All mass spectra were run at an accelerating voltage of 8KV and a mass resolution of $m/\Delta m = 3500$ (5% valley definition). All samples were dissolved in either Gold Shield ethanol or methanolic-HCl, then applied to the ceramic probe tip and evaporated with a gentle stream of nitrogen. C, H, N analyses were performed by Huffman Laboratories, Wheatridge, Colorado.

b. ω-Diethylamino-2,6-dimethylacetanilide or Lidocaine

This compound was a gift from Astra Pharmaceutical Products, Inc., Worcester, Mass. and was recrystallized from purified pet ether (bp 30-60°) to obtain colorless needles, mp 67-68°.[52] The CI mass spectrum showed that the MH^+ ion at m/e = 235 and its ^{13}C satellite represented 84.8% of the total ion current (TIC) and m/e = 164 represents 15.2% TIC from m/e = 100 to 300.

c. ω-[α,α-d_4-Diethylamino]-2,6-dimethylacetanilide or Lidocaine-d_4

1) A mixture of freshly distilled acetonitrile (4.10 g, 0.1 mole) and 200 mg of Adam's catalyst in 30 ml of freshly distilled acetic anhydride was hydrogenated (30 psi, D_2 gas) for 4 hours. An additional 100 mg of catalyst was added and the hydrogenation continued an additional 2 hours. After removal of reduced Pt by suction filtration, the filtrate was distilled through a short-path fractionating column to yield N-ethylacetamide-d_2, bp 204-207° (7.1 g, 80%).

2) To an ice bath cooled solution of AlD_3 (~46 mmoles generated from $LiAlD_4$ and sulfuric acid) in THF was added dropwise a THF solution of N-ethylacetamide-d_2 (1.90 g, 23 mmoles) over a 60 min period. The solution was slowly allowed to come to room temperature and stirred an additional 18 hours under nitrogen. Excess AlD_3 was destroyed with 4 ml of 1:1 THF:water followed by hydrolysis of aluminate complexes with 8 ml of 15% NaOH. The liberated diethylamine -d_4 was co-distilled with THF at 59-60°.

3) To the 60 ml of THF solution containing diethylamine-d_4 was added ω-chloro-2,6-dimethylacetanilide[52] (1.97 g, 10 mmoles) and the solution stirred at reflux for 3 hours. To the solution was added 450 mg NaOH in 2 ml water and the reaction refluxed an additional 2 hours. Rotary evaporation of solvent yielded a pale yellow liquid which was taken up in ether, washed with water and extracted into 10% HCl. The acid extract was basified with 5N NaOH and back extracted into ether. The

ether back extract was washed with water, filtered through anhydrous sodium sulfate and dried over Drierite. Removal of solvent and three recrystallizations from pet ether yielded 1.4 g, mp 65-67°; nmr $\delta(CDCl_3, TMS)$ 1.13 [broad s, $N(CD_2CH_3)_2$] 2.23 (s, aryl Me's), 3.20 (s, $O{=}CCH_2N$), 7.07 (s, aromatics) and 8.90 ppm (broad s, HNC=O). High resolution mass spectrum calcd for $C_{14}H_{18}N_2O$ ($-d_4$) 230.14191, found 230.14148.

A CI mass spectral analysis showed the following isotopic abundances: lidocaine-d_4 71.5% (m/e = 239), lidocaine-d_3 19.0% (m/e = 238), lidocaine-d_2 4.4% (m/e = 237), lidocaine-d_1 5.1% (m/e = 236) and lidocaine 0% (m/e = 235).

d. ω-Ethylamino-2,6-dimethylacetanilide or MEGX

To a solution of ω-chloro-2,6-dimethylacetanilide[52] (51.5 g, 0.25 mole) in 250 ml of benzene was added ethylamine, 60 ml of a 70% solution (0.5 mole) and the two-phase system refluxed for 5 hours. After cooling, the reaction mixture was poured into 1 volume of water and the benzene layer separated and extracted with two 0.5 volumes of 10% HCl. Combined acid extracts were back-washed with 1 volume of benzene, basified with NaOH to pH 11, and back extracted with three 1/3 volumes of purified methylene chloride.[53] Combined organic extracts were washed with 1 volume of water and dried (sodium sulfate) and solvent removed by rotary evaporation at room temperature to yield a crystalline mass.

Recrystallization from purified hexane gave MEGX (25 g. ~50%) as white needles, mp 49-50.5° (lit[52] 47-49°); hydrochloride mp 285-288° (lit[52] 289-290°); nmr of free base δ ($CDCl_3$, TMS) 1.18 (t, J = 7Hz, $NCH_2\underline{CH_3}$), 1.63 (s, NH), 2.25 (s, aryl Me's), 2.82 (q, J = 7Hz, $N\underline{CH_2}CH_3$), 3.48 (s, O = CCH_2N), 7.1 (s, aromatics) and 8.8 ppm (broad s, HNC = O). High resolution mass spectrum calcd for $C_{12}H_{18}N_2O$ 206.14191, found 206.14228; mass fragments m/e 191, 163, 148, 132, 120, 105, 91, 77, 53.

The CI mass spectrum showed that the MH^+ ion at m/e = 207 of MEGX and its ^{13}C satellite represent 96.5% TIC and m/e = 164 is 3.5% TIC.

Anal for $C_{12}H_{18}N_2O$, calcd: C 69.87, H 8.80, N 13.58; found: C 69.69, H 8.97, N 13.39.

e. ω-[β-d_3-Ethylamino]-2,6-dimethylacetanilide or MEGX-d_3

1) Ethylamine-d_3 hydrochloride was synthesized according to the procedure of Ross, et al.,[54] by $LiAlH_4$ reduction of acetonitrile-d_3.

2) To a magnetically stirred mixture of 295 mg (2 mmoles) of ω-chloro-2,6-dimethylacetanilide and 254 mg (3 mmoles) of d_3-ethylamine hydrochloride in 20 ml dioxane and 5 ml of water, was added 420 mg (3 mmoles) anhydrous potassium carbonate in 20 mg portions over a 2 hour period while heating the reaction at 60° in an oil bath. After addition was complete, the reaction was stirred an additional 3 hours. Rotary evaporation of solvents yielded a white crystalline mass which was worked

up as described for MEGX. Removal of solvent gave a pale yellow liquid which was taken up in 20 ml purified hexane and recrystallized to yield 350 mg small needles, mp 49-51°. The hydrochloride was made by dissolving the free base in absolute ethanol and bubbling through anhydrous HCl. Light needles formed and were collected by suction filtration, mp 285-288° with decomposition.

A CI mass spectral analysis showed the following isotopic abundances: MEGX-d_3 66.0% (m/e = 210), MEGX-d_2 22.4% (m/e = 209), MEGX-d_1 8.0% (m/e = 208) and MEGX 3.6% (m/e = 207).

f. ω-Amino-2,6-dimethylacetanilide or GX

1) To a magnetically stirred solution of freshly distilled 2,6-dimethylaniline (1.21 g, 0.01 mole) and N-carbobenzyloxyglycine (Aldrich, 2.10 g, 0.01 mole) in 50 ml methylene chloride was added DCCI (Aldrich, 2.25 g, 0.011 mole) dissolved in 25 ml of methylene chloride. Addition was accomplished over a 20-minute period. During the addition a heavy white precipitate formed. An additional 75 ml of methylene chloride was added and the slurry stirred for 2 hours at room temperature.

2) The white precipitate, containing both dicyclohexylurea and ω-carbobenzyloxyamino-2,6-dimethylacetanilide, was collected by suction filtration and dissolved in 75 ml of aldehyde free ethanol.[55] The mixture was hydrogenated (25 psi, H_2) on a Paar shaker over 300 mg of 10% Pd/C for 15 hours at room temperature. Rotary evaporation of ethanol

gave a pale yellow liquid which was dissolved in 50 ml purified chloroform[53] and extracted with two 25 ml portions of 10% HCl. Combined acid extracts were dried over anhydrous sodium sulfate and evaporated to yield a semi-solid mass. Recrystallization from purified hexane-isopropanol gave 750 mg white needles, mp 80-81° (lit[35] 79-80°); ir (KBr) 3400 cm^{-1} (NH_2), 3300 cm^{-1} (amide NH), 1660 cm^{-1} (amide C=O); nmr δ^T($CDCl_3$, TMS) 1.55 (broad m, NH_2), 2.20 (s, aryl Me's), 3.50 (t, J = 7 Hz, $O=CCH_2NH_2$), 7.05 (s, aromatics), and 8.80 ppm (broad s, HNC=O); addition of D_2O showed loss of the broad multiplet at 1.65 ppm and collapse of the triplet at 3.50 ppm to a singlet.

The isobutane CI mass spectrum of GX showed that the MH^+ ion at m/e = 179 and its ^{13}C satellite represent 96.8% TIC and m/e = 164 is 3.2% TIC. For a published EI spectrum see ref. 35.

g. α-d_2-ω-Amino-2,6-dimethylacetanilide or GX-d_2

To a solution of ω-amino-2,6-dimethylacetanilide (750 mg) in 20 ml 99.7% D_2O and 5 ml freshly distilled THF, was added 0.5 g anhydrous potassium carbonate and the solution heated at 90° for 24 hours. The solution was cooled, lyophilized, and the procedure repeated three times. After the final lyophilization, the white crystalline residue was taken up in 50 ml purified chloroform and 30 ml water and shaken vigorously. The chloroform extract was washed with two additional 10 ml of water and dried over anhydrous sodium sulfate. Rotary evaporation yielded 650 mg pale yellow crystalline solid which was recrystallized

from purified hexane-isopropanol to give 510 mg white needles, mp 79-80.5°; nmr δ^{T}(CDCl$_3$, TMS) 1.55 (broad s, NH$_2$), 2.20 (s, aryl Me's), 7.05 (s, aromatics) and 8.80 ppm (broad s, HNC=O).

The CI mass spectrum showed the following isotopic abundances: GX-d$_2$ 99.0% (m/e = 181), GX-d$_1$ 1.0% (m/e = 180) and GX 0% (m/e = 179).

h. 3-Hydroxy-ω-diethylamino-2,6-dimethylacetanilide or m-Hydroxylidocaine

This compound was synthesized in 52% overall yield according to the method of Keenaghan and Boyes,[24] by nitration of lidocaine, reduction to the aromatic amino compound, diazotization and hydrolysis to yield the phenol, white hexagonal plates from benzene-EtOAc, mp 203-204°. The CI mass spectrum is shown in Figure 3.

i. 3-Hydroxy-ω-[α,α-d$_4$-diethylamino]-2,6-dimethylacetanilide or m-Hydroxylidocaine-d$_4$

This compound was synthesized in 57% overall yield according to the method of Keenaghan and Boyes[24] starting with lidocaine-d$_4$, mp 202-204° after sublimation (140°, 0.3 mm Hg); nmr δ(CDCl$_3$, TMS) 1.13 [broad s, N(CD$_2$CH$_3$)$_2$], 2.05 and 2.17 (s, aryl Me's), 3.30 (s, O=CCH$_2$N), 6.75 (AB m, J=9 Hz, aromatics) and 8.90 ppm (broad s, HNC=O).

The CI mass spectrum showed the following isotopic abundances: m-hydroxylidocaine-d$_4$ 71.0% (m/e = 255), m-hydroxylidocaine-d$_3$

22.9% (m/e = 254), m-hydroxylidocaine-d_2 4.0% (m/e = 253), m-hydroxylidocaine-d_1 2.1% (m/e = 252) and m-hydroxylidocaine 0% (m/e = 251).

j. 3-Hydroxy-ω-ethylamino-2,6-dimethylacetanilide or m-HydroxyMEGX

This compound was synthesized in 38% overall yield according to the method of Keenaghan and Boyes,[24] using the same reaction sequence as that described for m-hydroxylidocaine but starting with MEGX, off-white small plates from purified chloroform, mp 147-149°; nmr δ^T($CDCl_3$, TMS) 1.18 (t, J=7 Hz, $NCH_2\underline{CH_3}$), 2.05 and 2.17 (s, aryl Me's), 2.80 (q, J=7 Hz, $N\underline{CH_2}CH_3$), 3.40 (s, $O{=}CH_2N$), 4.60 (broad s, OH and NH), 6.75 (AB m, J=9 Hz, aromatics) and 8.90 ppm (broad s, HNC=O).

k. 3-Hydroxy-ω-[β-d_3-ethylamino]-2,6-dimethylacetanilide or m-HydroxyMEGX-d_3

This compound was synthesized in 28% overall yield according to the method of Keenaghan and Boyes[24] starting with MEGX-d_3, mp 147-149°; nmr δ^T($CDCl_3$, TMS) 2.05 and 2.17 (s, aryl Me's), 2.80 (broad s, NCH_2CD_3), 3.40 (s, $O{=}CCH_2N$), 4.60 (broad s, OH and NH), 6.75 (AB m, J=9 Hz, aromatics) and 8.90 ppm (broad s, HNC=O).

The CI mass spectrum showed the following isotopic abundances: m-hydroxyMEGX-d_3 64.8% (m/e = 226), m-hydroxyMEGX-d_2 22.5% (m/e = 225), m-hydroxyMEGX-d_1 9.0% (m/e = 224) and m-hydroxyMEGX 3.7% (m/e = 223).

l. 2-Amino-3-methyl benzyl alcohol

A solution of 2-amino-3-methylbenzoic acid (Aldrich, 151 mg, 1 mmole) in 10 ml THF (freshly distilled from LAH) was added dropwise to a magnetically stirred slurry of $LiAlH_4$ (60 mg, 1.7 mmoles) in 10 ml THF over a 20 minute period. The reaction was stirred an additional hour at room temperature. To the slurry was added in succession 0.06 ml water, 0.06 ml 15% NaOH, and 0.18 ml water. After stirring for 15 min the granular white precipitate was removed by suction filtration. Rotary evaporation of the filtrate yielded 120 mg of off-white crystalline solid which was recrystallized from a 3:2 chloroform:hexane mixture to give white prisms, mp 69-70° (lit[56] 71°); ir (KBr) 3450 cm^{-1} (OH), 3300 cm^{-1} (NH_2); nmr δ($CDCl_3$, TMS), 2.05 (s, aryl Me's), 3.58 (broad s, NH_2 and OH), 4.41 (s, benzyl CH_2) and 6.80 ppm (A_2B m, aromatics).

CIMS showed that the MH^+ ion at m/e = 138 and its C^{13} satellite represent 46.6% TIC while (MH^+ - 18) at m/e = 120 is 53.4% TIC.

m. 2-Amino-3-methyl-α-d_2-benzyl alcohol

This compound was synthesized in 92% yield using the same procedure outlined for the undeuterated analog using $LiAlD_4$ (Stohler); mp 69-70°; nmr δ ($CDCl_3$, TMS) showed the same peaks as the undeuterated compound except for the loss of resonance at 4.41 ppm for the methylene hydrogens of the benzyl carbon.

CIMS showed the following isotopic abundances: benzyl alcohol-d_2 91.8% (m/e = 140), benzyl alcohol-d_1 8.2% (m/e = 140), benzyl alcohol 0% (m/e = 138).

n. d_2-2-Methyl-6-methylaniline or 2,6-Dimethylaniline-d_2

A solution of 2-amino-3-methyl-α-d_2-benzyl alcohol in 50 ml of 95% ethanol containing 1 ml concentrated HCl was hydrogenolyzed (30 psi, H_2) over 150 mg 10% Pd/C for 4 hours on a Paar hydrogenator at room temperature. The catalyst was removed by suction filtration and the filtrate rotary evaporated to yield a semi-solid residue which was taken up in 20 ml water, basified with 10% sodium carbonate, and the free amine extracted into ether. The extract was washed with water and dried over Drierite. Solvent was removed and the product vacuum-distilled to give 950 mg clear liquid (bp 37-41°, 0.3 mm Hg); nmr δ ($CDCl_3$, TMS) 2.07 (s, aryl Me's), 3.42 (broad s, NH_2) and 6.8 ppm (A_2B complex m, aromatics) integration ratio 4:2:3, respectively.

CIMS showed the following isotopic abundances: 2,6-dimethylaniline-d_2 77.2% (m/e = 124), 2.6-dimethylaniline-d_1 22.8% (m/e = 123) and 2,6-dimethylaniline 0% (m/e = 122).

o. ω-Diethylamino-2-hydroxymethyl-6-methylacetanilide or Lidocaine benzyl alcohol

1) To an ice-bath cooled, magnetically stirred solution of 2-amino-3-methyl-benzyl alcohol (2.6 g, 20 mmoles) in a mixture of 30 ml THF

and 20 ml benzene, was added dropwise a solution of chloroacetic anhydride (Aldrich, 3.4 g, 20 mmoles) in 30 ml THF. After $\frac{1}{2}$ hour a white crystalline precipitate formed and continued to form over the entire addition period. The slurry was warmed to room temperature and stirred an additional 2 hours. After cooling again in ice, the slurry was suction filtered and the filter-pak washed with three 30 ml portions of water. After air drying, the white crystals were recrystallized from 40 ml chloroform to yield 3.7 g of ω-chloro-2-hydroxymethyl-6-methylacetanilide, mp 130-132°; ir (KBr) 3400 cm^{-1} (OH), 3300 cm^{-1} (amide NH) and 1665 cm^{-1} (amide C=O); nmr δ ($CDCl_3$, TMS) 2.17 (s, aryl Me), 2.77 (broad s, OH), 4.20 (s, $O=CH_2Cl$), 4.58 (s, $\underline{CH_2}$-OH), 7.20 (s, aromatics) and 8.38 ppm (broad s, HNC=O).

Anal for $C_{10}H_{12}NO_2Cl$, calcd: C 56.20, H 5.66, N 6.56; found: C 56.15, H 5.61, N 6.52.

2) A solution of ω-chloro-2-hydroxymethyl-6-methylacetanilide (641 mg, 3 mmoles) and 0.78 ml diethylamine (550 mg, 7.5 mmoles) in 20 ml dry p-dioxane was heated at 70-75° for 3 hours during which period a white precipitate of diethylamine hydrochloride formed. The mixture was cooled to 15°, suction filtered to remove the amine salt, and rotary evaporated to yield a pale yellow liquid which was recrystallized from 30 ml of an 80:20 benzene:pet ether mixture to give cubic white crystals, mp 113-115°; ir (KBr) 3400 cm^{-1} (OH), 3300 cm^{-1} (amide NH), 1660 cm^{-1} (amide C=O); nmr δ ($CDCl_3$ TMS) 1.17 [t, J=7 Hz, N

($CH_2\underline{CH_3})_2$], 2.15 (s, aryl Me), 2.85 [q, J=7 Hz, $N(\underline{CH_2}CH_3)_2$], 3.38 (s, $O{=}CH_2N$), 3.71 (broad s, OH), 4.57 (s, CH_2-OH), 7.21 (s, aromatics) and 8.60 ppm (broad s, HNC=O).

CIMS showed that the MH^+ ion at m/e 251 and its ^{13}C satellite represent 64.0% TIC, (MH^+ - 16) at m/e = 235 represents 11.5% TIC, m/e = 132 is 13.0% TIC, and other 11.5% TIC from m/e 100-300.

Anal for $C_{14}H_{22}N_2O_2$, calcd: C 67.17, H 8.86, N 11.19; found: C 67.41, H 8.90, N 11.41.

p. ω-Ethylamino-[α-d_2-2-hydroxymethyl]-6-methylacetanilide or MEGX benzyl alcohol-d_2

This compound was synthesized in 79% yield using the same procedure as described for the diethylamino derivative using a 3-fold excess of ethylamine and 2-amino-3-methyl-α-d_2-benzyl alcohol; mp 81-83° from chloroform; ir (KBr) 3400 cm^{-1} (OH and NH), 3300 cm^{-1} (amide NH) and 1660 cm^{-1} (amide C=O); nmr δ ($CDCl_3$, TMS) 1.13 (t, J=7 Hz, $NCH_2\underline{CH_3}$), 2.21 (s, aryl Me), 2.68 (q, $N\underline{CH_2}CH_3$), 3.38 (s, $O{=}CH_2N$), 3.68 (broad s, OH) 7.20 (s, aromatics) and 8.60 ppm (broad s, HNC=O). High resolution EIMS calcd for $C_{12}H_{16}N_2O_2(-d_2)$ 220.12120, found 220.12100.

CIMS showed that the MH^+ ion at m/e = 225 and its ^{13}C satellite represent 52.8% TIC, (MH^+ - 18) at m/e = 207 is 38.1% TIC, m/e = 122 is 5.2% TIC and m/e = 104 is 3.9% TIC.

q. 4-Hydroxy-2,6-dimethylaniline

This compound was synthesized according to known procedures, mp 179-181°.[57]

r. 4-Hydroxy-2-d_2-methyl-6-methylaniline or 4-Hydroxy-2,6-dimethylaniline-d_2

This deuterated analog was synthesized by the reaction shown in Scheme 3 as outlined by Wepster.[58]

1) To a magnetically stirred solution of 2,6-dimethylaniline-d_2 (2.4 g, 0.02 mole) in 8 ml dry pyridine, was added p-TsCl (4.4 g, 0.023 mole) and the solution refluxed for 2 hours. The solution was cooled, poured into a beaker containing 80 ml ice-cold 2N HCl, and the slurry of white crystals stirred for 10 minutes prior to suction filtration. The 5.1 g of pale yellow crystals were washed thoroughly with water before the nitration step.

2) To a magnetically stirred solution of 6 ml fuming nitric acid (90%) in 45 ml water, was added in succession the 5.1 g crude p-toluenesulfonamido-2,6-dimethylaniline-d_2, 45 ml glacial acetic acid, and 0.17 g sodium nitrite. The slurry was brought to reflux and stirred for 1 hour during which period all material went into solution. The reaction mixture was cooled and poured into a beaker containing 90 ml of ice water, stirred and 5.3 g pale yellow crystals collected by suction filtration. After thorough washing with water the crystals were air dried.

3) A solution of the p-toluenesulfonamido-4-nitro-2,6-dimethylaniline-d_2 (2.40 g, 7.5 mmoles) in 100 ml 95% ethanol was hydrogenated (20 psi, H_2) over 400 mg 10% Pd/C in a Paar apparatus at room temperature for 2 hours. Catalyst was removed by suction filtration and the filtrate rotary evaporated to yield 1.90 g of off-white needles, mp 203-205°; ir (KBr) 3400 and 3500 cm^{-1} (NH_2), 3300 cm^{-1} (sulfonamide NH), 1295 and 1145 cm^{-1} (O=S=O); nmr δ (d_2-DMSO, TMS) 1.76 (s, aryl m-Me's), 2.37 (s, p-Me), 4.8 (broad s, NH_2 and NH), 6.19 (s, aromatic meta-H's) and 7.43 ppm (A_2B_2 q, p-toluenesulfonamido-aromatics).

4) To a magnetically stirred slurry of p-toluenesulfonamido-4-amino-2,6-dimethylaniline-d_2 (1.45 g, 5 mmoles) in 20 ml 10% HCl, was added a solution of sodium nitrite (350 mg, 5.1 mmoles) in 10 ml water. The reaction was held at 4-6° throughout addition by cooling in an ice bath. The insoluble diazonium salt that formed was collected by suction filtration and hydrolyzed by stirring in 30 ml water at room temperature for 3 days.

5) The product was collected from the cooled solution and the sulfonamide hydrolyzed at 90° in 11 ml 90% sulfuric acid solution which had been previously degassed for one hour with nitrogen bubbled through a pyrogallol trap to remove oxygen. The black mixture was poured into a 250 ml beaker cooled in ice and 60 ml ice cold 5N NaOH added slowly to bring the solution to pH 7.0. The mixture was extracted with three 50 ml portions of ether, combined extracts washed with two 10 ml portions

of water, and dried over Drierite. Removal of solvent under a gentle stream of nitrogen yielded 285 mg crude purple product, mp 165-180° with decomposition. The material was sublimed (80-90°, 3 mm Hg) to give 155 mg very pure white powder crystals, mp 179-181°, and 110 mg slightly impure light purple crystals, mp 175-180°; nmr δ ($CDCl_3$, TMS) 2.06 (s, aryl Me's), 4.22 (broad s, NH_2), 6.47 (s, OH) and 6.59 ppm (s, aromatics) integration ratio 4:2:1:2, respectively. High resolution EIMS calcd for $C_8H_9NO(-d_2)$ 135.0684, found 135.0650.

CIMS showed that MH^+ ions represented 100% TIC with the following isotopic abundances: 4-hydroxy-2,6-dimethylaniline-d_2 60.2% (m/e = 140), 4-hydroxy-2,6-dimethylaniline-d_1 22.4% (m/e = 139) and 4-hydroxy-2,6-dimethylaniline 17.4% (m/e = 138).

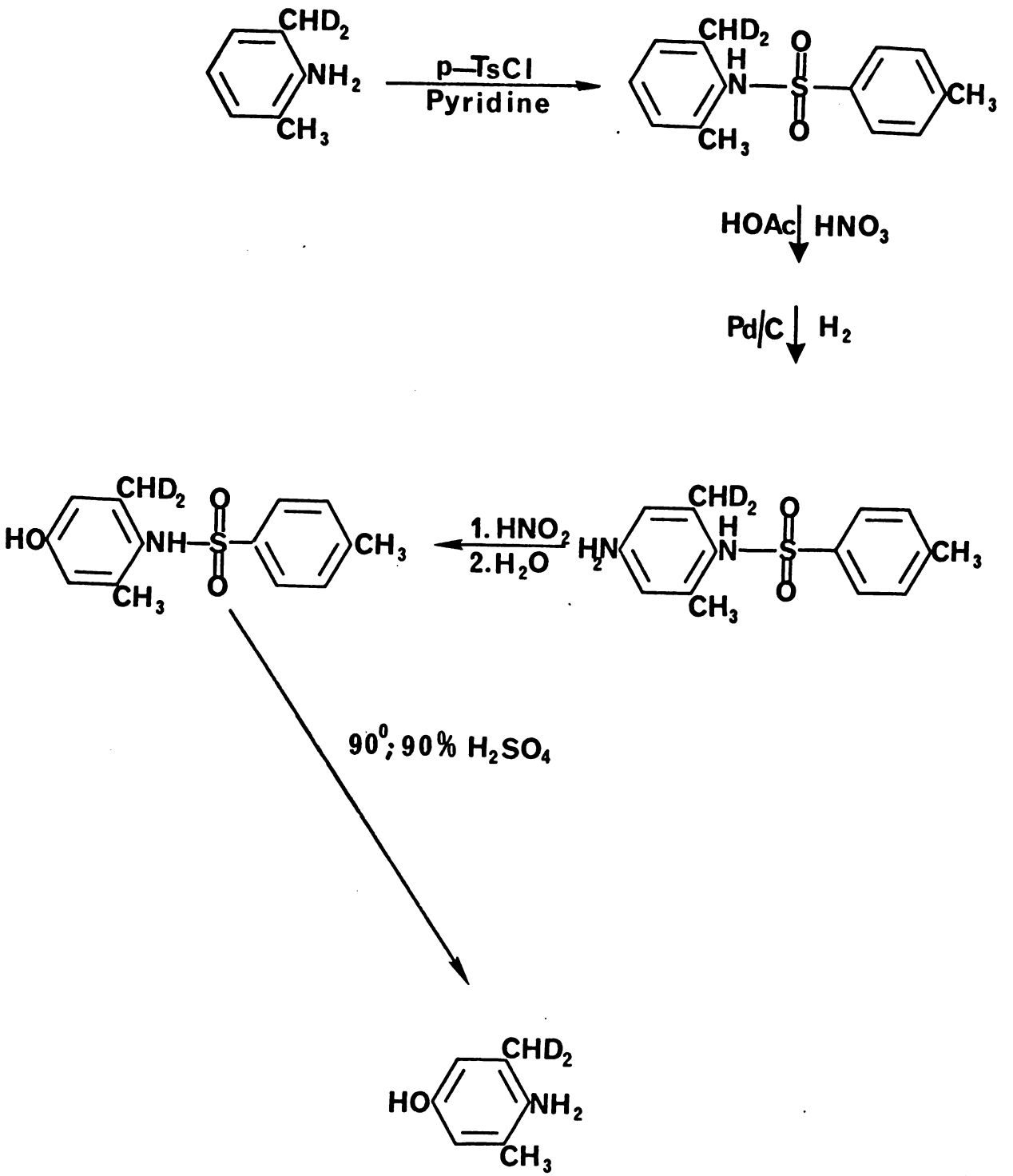
s. N,N-Diethylglycine

This substituted amino acid was prepared by the method of Bowman and Stroud,[59] using reductive alkylation of glycine with excess acetaldehyde and H_2, mp 133-135°.

t. Deuterated N,N-Diethylglycine

A mixture of glycine (3.8 g, 0.05 mole) and freshly distilled acetaldehyde (9.0 g, 0.2 mole) in 70% $EtOD/D_2O$ was hydrogenated (20 psi, D_2) over 1.5 g of 10% Pd/C for 14 hours at room temperature. Catalyst was removed by suction filtration and the filtrate rotary evaporated to yield a viscous pale yellow liquid which was recrystallized as the

Scheme I-3. The synthesis of 4-hydroxy-2,6-dimethylaniline-d_2


CHD2
NH2
CH3
p–TsCl
Pyridine
CHD2
H
N
S
O
O
CH3
CH3
HOAc
HNO3
Pd/C
H2
HO
NH
1. HNO2
2. H2O
H2N
90°; 90% H2SO4

hydrochloride from 75 ml of 1:4 methanol:acetone to give 5.7 g white crystalline solid, mp 124-126° (lit[60] 126-127°).

CIMS showed that deuterium scrambling had occurred during the reductive alkylation with incorporation ranging from zero to 8 deuteriums into the N-alkyl side chains. The resultant deuterium envelope showed greatest deuterium incorporation for N,N-diethylglycine-d_3 21.0% (m/e = 135). Parent $MH(D)_x^+$ ions represent 70.1% TIC, (MH^+ - $C_2H_2O_2$) ions are 22.4% TIC.

u. N-Ethylglycine or Monoethylglycine

This compound was prepared by the method of Hanke.[61]

1) To an ice-bath cooled solution of ethylamine hydrochloride (40 g, 0.5 mole) dissolved in 70 g of 35% aqueous formaldehyde, was added, with vigorous stirring, small portions of 33 g KCN in 50 ml water. Carbon dioxide was bubbled through the mixture while being stirred for 1 hour. Upon warming to room temperature, the mixture produced a yellow liquid as a separate layer which was extracted into three 100 ml portions of ether. Combined extracts were washed with 5% sodium bicarbonate, followed by saturated sodium chloride solution, and finally dried over anhydrous sodium sulfate. Rotary evaporation of solvent yielded 39.1 g of pure N-ethylamino-acetonitrile.

2) N-Ethylaminoacetonitrile (0.87 g, 0.01 mole) was refluxed in 10% HCl for 12 hours. The solution was lyophilized after neutralizing to pH 6.5 with 5N NaOH, and the lyophilate sublimed (0.3 mm, 120-130°)

to give 340 mg of white flakey solid which was recrystallized from absolute ethanol in the cold (-15°), mp 180-182° with decomposition.

v. β-d_3-N-Ethylglycine or Monoethylglycine-d_3

This deuterated analog was synthesized by the procedure outlined for monoethylglycine using ethylamine hydrochloride-d_3,[54] mp 180-182° with decomposition.

CIMS showed the following isotopic abundances: monoethylglycine-d_3 57.9% (m/e = 107), monoethylclycine-d_2 20.8% (m/e = 106), monoethylglycine-d_1 13.2% (m/e = 105) and monoethylglycine 8.1% (m/e = 104).

2. Quantification of Lidocaine and its Metabolites in Urine and Plasma

a. Materials

1) Collection bottles for urine and pyrex culture tubes for plasma were thoroughly washed with steaming water and Lab-tone, rinsed three times with hot water, then cold water, and finally rinsed five times with distilled water.

2) A 200 ml stock solution containing 1.60 mg m-hydroxylidocaine-d_4, 8.00 mg lidocaine-d_4, 1.60 mg m-hydroxyMEGX-d_3, 20.00 mg MEGX-d_3, 4.00 mg GX-d_2, and 2.06 mg 2,6-dimethylaniline-d_2 was made up the morning of the *in vivo* experiment. All weighings were performed on a Cahn Electrobalance Model M-10.

3) All solvents used for extractions (benzene, methylene chloride) were purified[53] and distilled prior to use.

4) Mass spectra were determined on an AEI MS-9 modified for CIMS as previously described, p. 39.

5) Sigma Type II β-glucuronidase-sulfatase from Helix Pomatia was used for hydrolysis of conjugates.

b. Methods

Two healthy male volunteers (A = 80 Kg, B = 70 Kg) received a 250 mg oral dose of encapsulated lidocaine hydrochloride monohydrate (Xylocaine, Astra Pharmaceutical Products, Inc., Worcester, Mass.); a third subject (C = 75 Kg) received a molar equivalent (202 mg) of lidocaine as the free base. All doses were received in the morning with no restrictions on diet, smoking, etc. Prior to the experiment, 24-hour urine controls were collected from each subject and stored in a freezer (-15°). Immediately prior to dosing, a 15 ml "0-time" blood sample was drawn into a heparinized tube. Successive 15 ml blood samples were taken 30, 60, 90 and 180 min following administration of the drug, and the plasma was immediately separated by centrifugation at 800 x g for 15 minutes. To 2 ml samples of each plasma fraction was added 2 ml x 1.01 μg/ml lidocaine-d_4, 1 ml x 1.00 μg/ml MEGX-d_3, and 0.5 ml x 1.00 μg/ml GX-d_2. Samples were then stored frozen until further work-up.

Serial urine samples were collected 0-2, 2-4, 4-8 and 8-24 hours following administration of the drug. One-third of each sample was

transferred at the time of collection to a glass bottle containing 10 ml of the stock solution of deuterated standards. To two-tenths of this sample containing the deuterated standards (2/10 x 1/3) was added 1.0 mg of 4-hydroxy-2,6-dimethylaniline-d_2 and the samples stored in a freezer (-15°) until further work-up. To one-twentieth (1/20) of the 0-24 hour urine aliquots from subjects A, B and C was added 1.0 mg each of the deuterated amino acids, N,N-diethylglycine-d_x and monoethylglycine-d_3.

c. Isolation and Analysis

1) Plasma

All plasma samples were extracted the day following collection. To the 5.5 ml of sample, containing 2 ml of plasma and 3.5 ml of deuterated standards solution, was added 0.5 ml of 5N NaOH and the basified mixture extracted with 10 ml of benzene in 30 ml pyrex culture tubes fitted with Teflon caps. The sample was then centrifuged at 800xg for 5 minutes and the benzene layer removed and rotary evaporated at 20°. The residue was dissolved in 0.05 ml of ethanol and placed on the ceramic tip of the direct insertion probe. The probe was inserted into the mass spectrometer and the CI mass spectrum determined at 120°.

2) Urine

Urine samples, including urine controls, were extracted within 2 days following collection. One-half of each urine sample, containing all deuterated standards (i.e., $\frac{1}{2}$ x 2/10 x $\frac{1}{3}$ = 1/30 of each serial

urine sample), was basified to pH 8.5 ± 0.2 with 5N NaOH and extracted with 2 volumes of purified methylene chloride. The extract was filtered through anhydrous sodium sulfate and rotary evaporated at 20°. The residue was transferred to a 1 dram vial with 1 ml of purified methylene chloride. The solvent was evaporated under a gentle stream of nitrogen and samples stored in a dessicator in a freezer until CIMS analysis was performed.

The other half of each urine sample was diluted with an equal volume of pH 5.0 Walpole's acetate buffer and hydrolyzed for 24 hours on a shaker-incubator set at 37.0° and 100 oscillations/min. β-glucuronidase-sulfatase was added in two 0.5 ml portions, once at the beginning of the incubation, and again 12 hours after the incubation was started. Extraction of each β-glucuronidase treated sample was carried out in the same manner as untreated urine. All of these samples, however, required a primary suction filtration through glass wool to break difficult emulsions before filtration through anhydrous sodium sulfate. All residues of the hydrolyzed urine were dark, foul-smelling, and tarry.

CIMS analysis was accomplished by dissolving each residue in 0.05 ml of methanolic-HCl, evaporating the sample on the probe tip with a gentle stream of nitrogen, and temperature programming each sample from 90-220°, taking spectra approximately every 20°. Temperature programming was necessary to determine the more volatile 2,6-dimethylaniline (90-110°) and 4-hydroxy-2,6-dimethylaniline (120-150°); and also to rid the spectrum of some interfering peaks near m/e = 180

so that GX could be determined above 170°. The methanolic-HCl treatment served two purposes: a) it converted the metabolites to their less volatile amine hydrochlorides b) combined with temperature programming, the treatment destroyed any N-hydroxyamides that might have been present by causing Bamberger[62]-type reactions so that these compounds would not interfere with quantification of lidocaine and MEGX.

d. Calculations

Peak heights were measured using a calipher. Standard procedure was followed in subtracting isotope contributions from the preceeding two peaks. The contributions of undeuterated or unreacted ionic species present in the reference solution (internal standard) were then subtracted. A comparison of the now corrected peak heights of labeled and unlabeled compound was made, and the concentration of drug or metabolite initially present in the plasma or urine sample was determined.

e. Standardization Experiments

1) Plasma

Previous work has shown that extraction and mass spectral response to a compound and a deuterated analog of that compound are equivalent.[39,40] To test this under the CIMS conditions used, lidocaine and lidocaine-d_4 (1 μg/ml), MEGX and MEGX-d_3 (0.5 μg/ml), and GX and GX-d_2 (0.25 μg/ml) were added to 2 ml blank plasma samples and extracted according

to the procedures outlined previously.[34] CIMS analysis was performed on duplicate samples and the corrected peak height ratios of undeuterated vs. deuterated standards showed good agreement between known and calculated concentrations, the largest variance being 2.2% for lidocaine vs. lidocaine-d_4.

2) Urine

To ten X 10 ml blank urine samples in 50 ml pyrex culture tubes, was added 1.0 mg of 4-hydroxy-2,6-dimethylaniline-d_2 plus 1 ml of the deuterated standards stock solution. To these samples was added 1 ml of stock solutions containing the same undeuterated compounds so as to obtain five sets of duplicate samples with concentrations of undeuterated/deuterated analogs ranging from 1/5 to 5/1. The 12 ml of solution was basified to pH 8.5 with 5N NaOH and extracted with 20 ml of purified methylene chloride. CIMS analysis with temperature programming was performed as described previously. Calculations showed that response was linear over the range analyzed and that the lidocaine derived compounds and their deuterated analogs behaved identically. (See Figure 4)

3. Quantification of Amino Acid Metabolites

a. Materials

All radioactive determinations (M.S.E. = 1.5%) were made using a Tri-Carb Model 3375 scintillation counter. All determinations were made in 10 ml Aquasol (NEN) and corrected for quenching using internal

standardization with either toluene-^{3}H standard (NEN) or toluene-^{14}C standard (NEN). Glc was performed on a Varian Aerograph Model 2100 gas chromatograph using flame ionization detectors with N_2 carrier gas flow rate at 40 ml/min. Separation was obtained at 55° on 3% OV-17 on Chromasorb W 100-120 mesh in a 5.5 ft X 0.25 in X 2 mm i.d. glass U column equipped with a 10:1 effluent splitter.

Sigma Type III Urease from Jack Beans was used to hydrolyze urea. Tlc separation was accomplished on Eastman plates 20X20 cm with silica gel or cellulose adsorbent.

b. Methods

1) Using Radioactivity Measurements

a) Two human subjects received an oral dose of 500 mg of randomly tritiated lidocaine hydrochloride monohydrate.[22] An aliquot of the urine (0-72 hours) was treated with urease and after filtration was lyophilized. The lyophilate was extracted (Soxhlet) with successive portions of CH_2Cl_2, iPrOH and MeOH to constant activity. The iPrOH extract, accounting for 70% of the activity in the lyophilate, was evaporated under reduced pressure, taken up in 10 ml water and placed on a 250 ml (20 x 4 cm) Dowex AG50WX4 hydrogen form cation exchange column, which was then washed with 1.5 l water, 2 l 8N HOAc, and 4 l water. Very small amounts of activity were eluted in these fractions. Finally, a linear gradient from water to 2N HCl was run (2 l total volume) and 10 ml fractions collected. Eighty percent of the activity applied to the

column was found between fractions 105-130. The fractions were combined and lyophilized repeatedly to remove excess HCl and the white residue washed repeatedly with iPrOH. Combined extracts were evaporated to a small volume and streaked on two 20 x 20 cm Silica Gel HR tlc plates (Analtech, 250 μ). The plates were developed twice in butanol: HOAc:water 4:1:1. Autoradiographic scanning showed only one radioactive band, rf = 0.3, which was scraped from the plates and eluted with ethanol. Evaporation of ethanol yielded a residue which was treated with diazomethane in ether and gas chromatographed. One peak was found (ret. time 6.2 min) and collected, and determined to be radioactive by scintillation counting. EIMS of the TFA salt of the methylated amino acid showed peaks at m/e = 145, 86, 69 and 58. N,N-Diethylglycine, prepared by the method of Bowman and Stroud,[59] and methylated with thionyl chloride in methanol, showed identical glc and mass spectral characteristics. Isotopic dilution of samples of the isolated amino acid in the synthetic amino acid, with continuous recrystallization to constant specific activity, confirmed the identification of the metabolite.

b) Quantification of N,N-Diethylglycine from Human Urine

Aliquots (100 ml) of urine from two subjects receiving 500 mg of randomly tritiated lidocaine hydrochloride monohydrate[22] were incubated with urease, filtered, and 50 ml of the filtrate lyophilized with 362 mg of non-radioactive synthetic N,N-diethylglycine. The material was lyophilized twice to insure removal of any exchangeable tritium. The lyophilate was triturated with three 50 ml portions of boiling benzene,

filtered, and the benzene carefully evaporated under nitrogen at room temperature. The residue was repeatedly recrystallized from boiling benzene to constant specific activity.

Subject A Activity of the original 50 ml urine 4.63×10^5 dpm

Activity of purified N,N-diethylglycine 1.63×10^5 dpm

= 35.5% of the activity in the original urine

Subject B = 34.9% of the activity in the original urine was found in the recrystallized N,N-diethylglycine.

c) Isolation and Quantification of Amino Acids from Monkey Urine

A 4.8 Kg Rhesus monkey was administered i.v. a total of 100 mg of lidocaine (labeled with ^{14}C in an N-ethyl group, synthesis described in Chapter III) over a 20-hour period and urine was collected throughout and an additional 24 hours. A 150 ml aliquot of the pooled urine, containing 5.50×10^5 dpm ^{14}C activity, was adjusted to pH 6.5 with 20% NaOH and lyophilized on a rotary evaporator. The lyophilate was successively triturated with benzene, methylene chloride, and methanol. Half of the methanol extract (1.5×10^5 dpm) was evaporated to dryness and eluted from 60 gm of Bio-Rad analytical cation exchange resin, AG50WX8 100-200 mesh, packed in a 30 x 2 cm column. Successive elution of the column with 6 volumes (360 ml) of water, 6 volumes of 8 N HOAc, and 16 volumes of water yielded less than 1% of the activity applied to the column. A continuous gradient elution from water to 2N HCl (a total of

1 l) was collected in 10 ml fractions with an automatic fraction collector. Of the activity applied to the column 77% was found in fractions 35-45, which were combined, adjusted to pH 6.5 with 20% NaOH, and lyophilized. The white lyophilate was triturated with five 10 ml portions of isopropanol which were combined and evaporated to dryness. The solid residue was taken up in 2 ml of methanol and 1 ml was chromatographed on a 20 x 20 cm silica gel GF plate (Analtech) developed in 4:1:1 butanol:HOAc:water. An area with rf = 0.2-0.3 contained most of the activity and was scraped off and eluted with methanol. Overall radioactivity in the extract was 3.1×10^4 dpm.

Part of the extract was subjected to on-column methylation with Meth-L-Ute (Pierce) on a 3% OV-17 column. Samples of the two major peaks had identical retention times as the synthetic methyl esters of N,N-diethylglycine and monoethylglycine, 6.2 min and 4.1 min, respectively. The two peaks were collected from the column using the effluent splitter and found to be radioactive. Another sample of the eluted tlc zone was subjected to CIMS. The spectrum (Figure 13) showed only the two major peaks at m/e = 132 (MH^+ of N,N-diethylglycine) and m/e = 104 (MH^+ of monoethylglycine) in a ratio of 3.0/3.8 respectively.

2) Using the CIMS-Stable Isotope Dilution Technique

The 1/20th aliquots or urine from subjects A, B and C, containing 1.0 mg each of the deuterated amino acids, were treated with urease (100 mg) at 30° with shaking for 72 hours in an equal volume of pH 7.0 sodium phosphate buffer. Each sample was then lyophilized to yield

approximately 600 mg of light brown powder which was extracted with 10 ml of hot methanol. The methanol extract was rotary evaporated and the residue sublimed (130°, 0.3 mm Hg) for 3 hours. The sublimate was taken up in a few drops of methanol and chromatographed on silica gel plates using 8:3:2 n-butanol:water:acetic acid as the developing solvent. The appropriate area (rf = 0.2-0.4), as determined by co-chromatography with standards, was scraped off and eluted with methanol followed by rechromatography in the same system. Tlc on silica gel of a sample of the final extract showed a ninhydrin sensitive area corresponding to the rf region for both N,N-diethylglycine and monoethylglycine. Tlc on cellulose using 6:3:1 95% ethanol:isopropanol:water also indicated ninhydrin sensitive areas for N,N-diethylglycine (rf = 0.51, very pale pink spot) and monoethylglycine (rf = 0.27, purple-pink spot).

CIMS of each extract showed small peaks at both m/e = 104 and m/e = 132 corresponding to the amino acids, along with envelopes of peaks attributable to their deuterated internal standards. However, due to the large number of peaks in the same mass ranges, meaningful quantification was virtually impossible.

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CHAPTER II

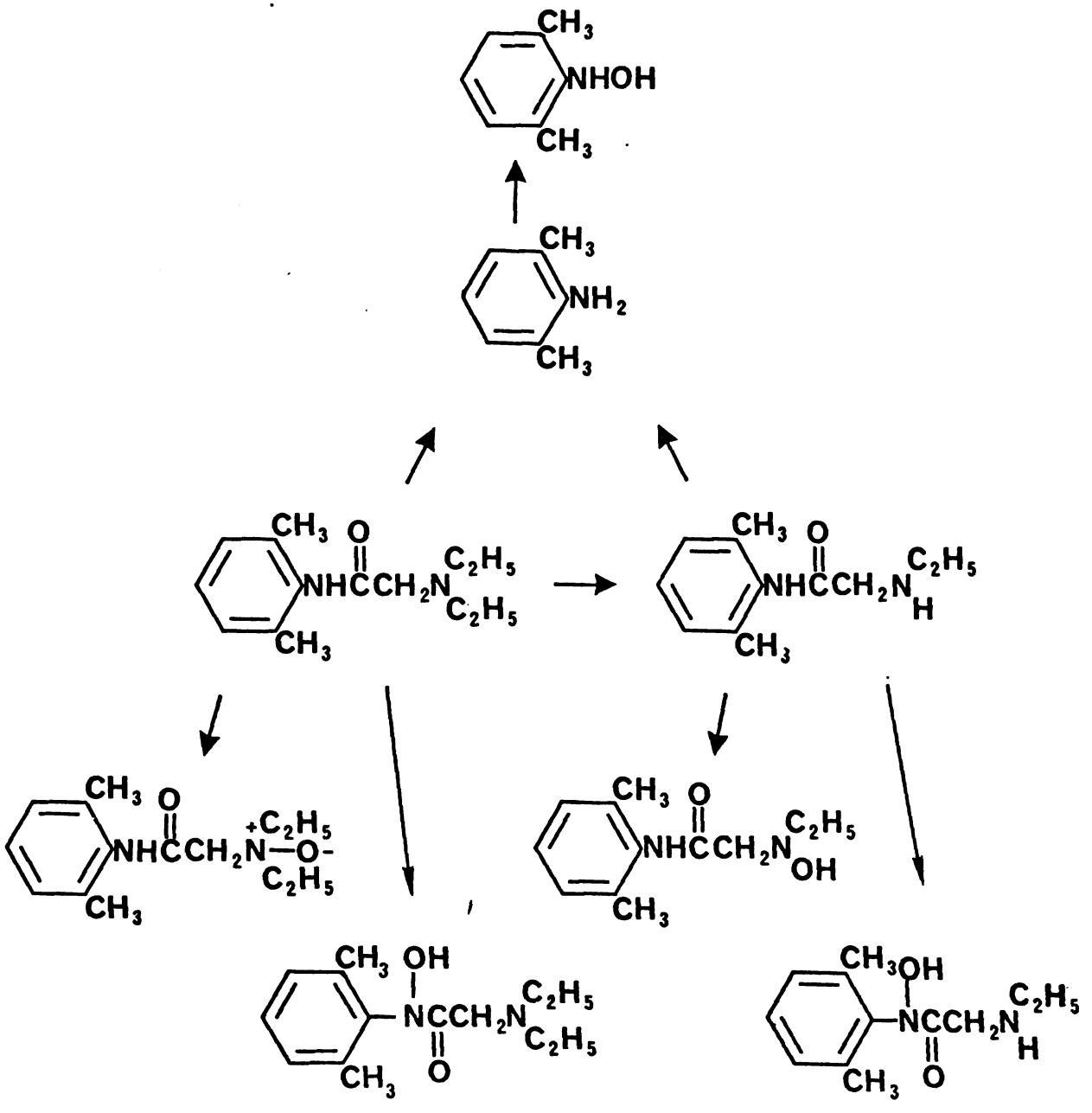
N-OXIDATIONS AS POSSIBLE PATHWAYS IN LIDOCAINE METABOLISM

A. INTRODUCTION

In the last five years a wealth of information has been published on the significance of the oxidation of nitrogen in biological systems and its relation to carcinogenesis (for reviews see ref. 1, 2, and 3). All of the information available implicates a variety of oxidized nitrogen compounds, such as tertiary amine N-oxides, hydroxylamines, and N-hydroxyamides, as proximate carcinogens. As stated in the reviews, quantitatively N-hydroxylation appears to be a minor reaction, but the intermediates formed are often quite reactive as electrophiles which, contrary to most biotransformation processes, leads to toxic rather than detoxified products.

Lidocaine has incorporated in its structure a tertiary amino group and an aromatic amido group, both of which are susceptible to oxidation. N-deethylation of lidocaine produces MEGX which is a secondary amine susceptible to further oxidation to a hydroxylamine. Hydrolysis of lidocaine and MEGX by amidases produces 2,6-dimethylaniline which may be N-hydroxylated as well as hydroxylated in the aromatic ring. Since only 70-80% of the metabolism of lidocaine has been accounted for, these other possible pathways, illustrated in Scheme 1, were looked into further. Since the inception of this work, Mather and Thomas[4] have presented

Scheme II-1. Possible N-hydroxylation pathways of lidocaine.


CH3
NHOH
CH3
CH3
NH2
CH3
CH3
O
NHCCH2N
C2H5
C2H5
CH3
CH3
O
NHCCH2N
C2H5
H
CH3
CH3
O
NHCCH2N
+C2H5
O-
C2H5
CH3
CH3
O
NHCCH2N
C2H5
OH
CH3
CH3
OH
NCCH2N
C2H5
C2H5
O
CH3
CH3OH
NCCH2N
C2H5
H
O
CH3

preliminary evidence that the N-hydroxyamides of lidocaine and MEGX may account for as much as 25% of an oral dose of lidocaine. Work presented in this chapter indicates that this figure may actually be much less.

B. ALIPHATIC AND AROMATIC AMINE OXIDATIONS

1. Lidocaine N-Oxide

Many biologically important tertiary amines, such as nicotine,[5] methadone,[6] chlorcyclizine,[7] and others,[8] undergo metabolic N-oxidation to yield N-oxide metabolites. Tertiary amine N-oxides are important metabolites since some appear to promote the development of certain neoplasms.[9,10] Therefore, the synthesis of lidocaine N-oxide was carried out, and studies were undertaken to establish both its chemical characteristics and likelihood as an *in vivo* metabolite of lidocaine.

Recent work by Bickel[11] and Ziegler[12] indicates that N-oxides may be major primary *in vivo* metabolites in humans with secondary reactions of the N-oxide leading eventually to the N-dealkylated product which is analyzed in the urine. These secondary metabolic reactions of tertiary amine N-oxides are analogous to chemical reactions that N-oxides undergo when heated near their melting point. At this point concerted intramolecular rearrangements can occur and/or decomposition into radicals which recombine by multiple pathways to yield a number of products.[13]

Lidocaine N-oxide is no exception. Heating the compound at 100° for a few minutes resulted in the formation of six products distinguishable by tlc and seven products distinguishable using two different glc columns. The identity of three of the major products has been established unequivocally by comparative tlc and glc with synthetic compounds. These include lidocaine, MEGX, and cyclic metabonate. Figure 1 shows the tlc and glc data for the thermal rearrangement products of lidocaine N-oxide. Some of the remaining reaction products have been tentatively identified on the basis of MH^+ ions present in the CI mass spectrum of lidocaine N-oxide. CIMS proved to be a powerful tool in studying the N-oxide thermal rearrangements since the ions are formed as a result of heating the N-oxide on the ceramic tip of the direct insertion probe. Figure 2 shows the CIMS of lidocaine N-oxide and Scheme 2 indicates the possible thermal rearrangement pathways for lidocaine N-oxide.

To determine if lidocaine N-oxide was a significant urinary metabolite of lidocaine in either the Rhesus monkey or man, control urine from each, containing 0.1% w/v lidocaine N-oxide, was concentrated to a small volume and extracted with purified chloroform. Tlc of the extracts on silica gel showed the presence of the N-oxide visualized by uv light. The same procedure was followed with urine from a Rhesus monkey and a human subject who had received radio-labeled lidocaine. The tlc plate was scraped 0.5 cm above and below the appropriate rf region. Scintillation counting showed that no N-oxide was detectable in the urine of either Rhesus monkey or human subject.

Figure II-1. Thin-layer chromatography (tlc) and gas-liquid chromatography (glc) of the thermal rearrangement products of lidocaine N-oxide.

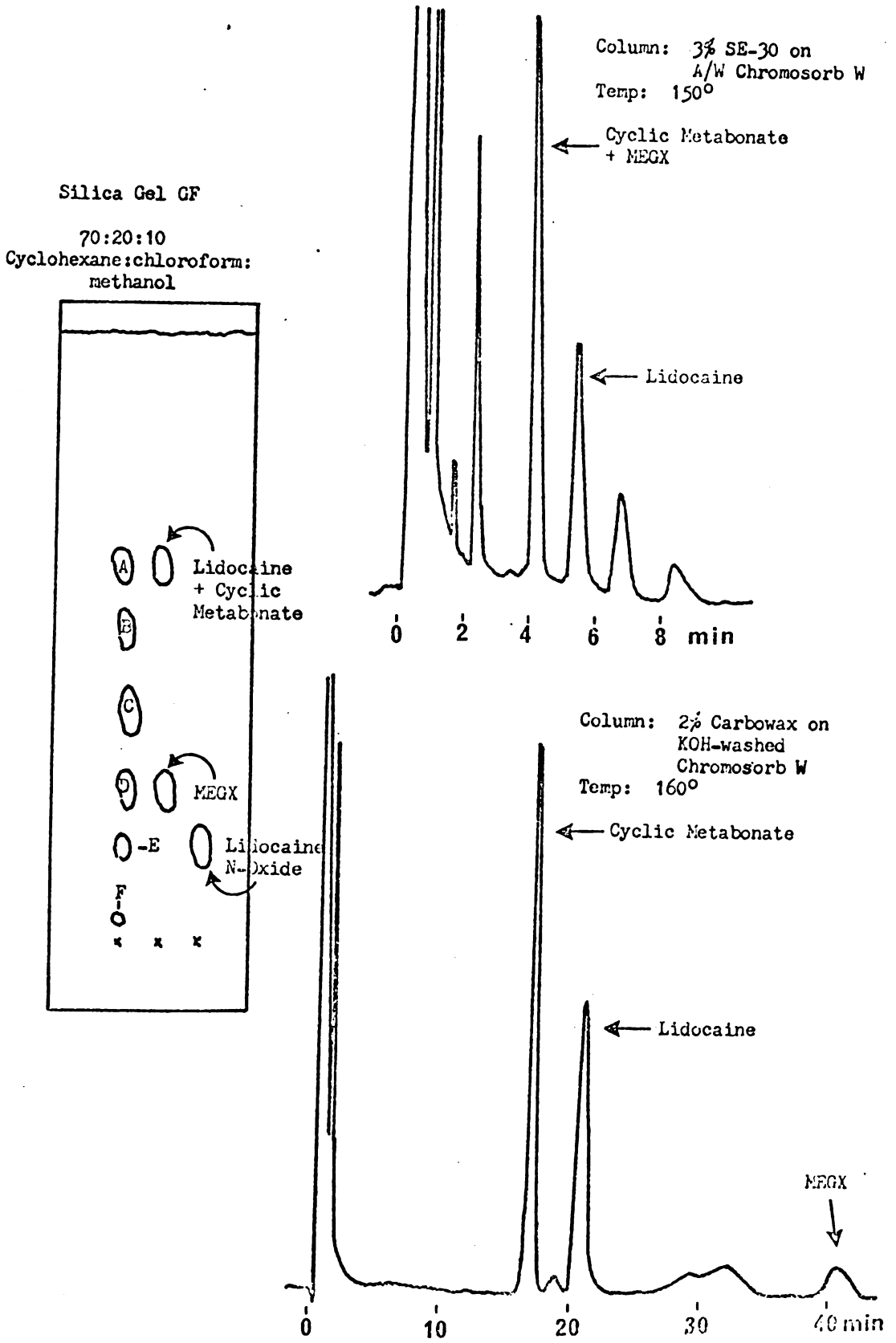
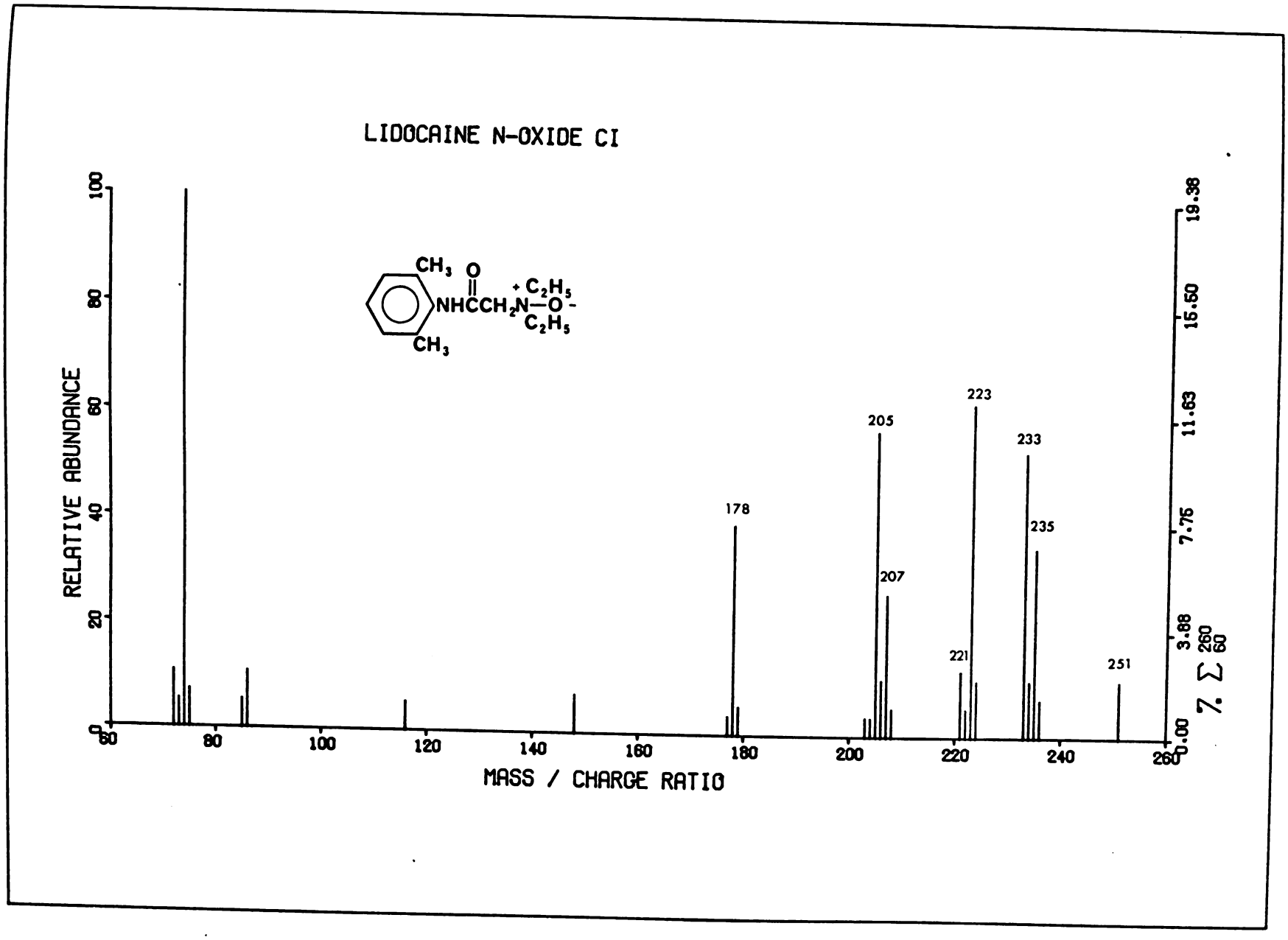
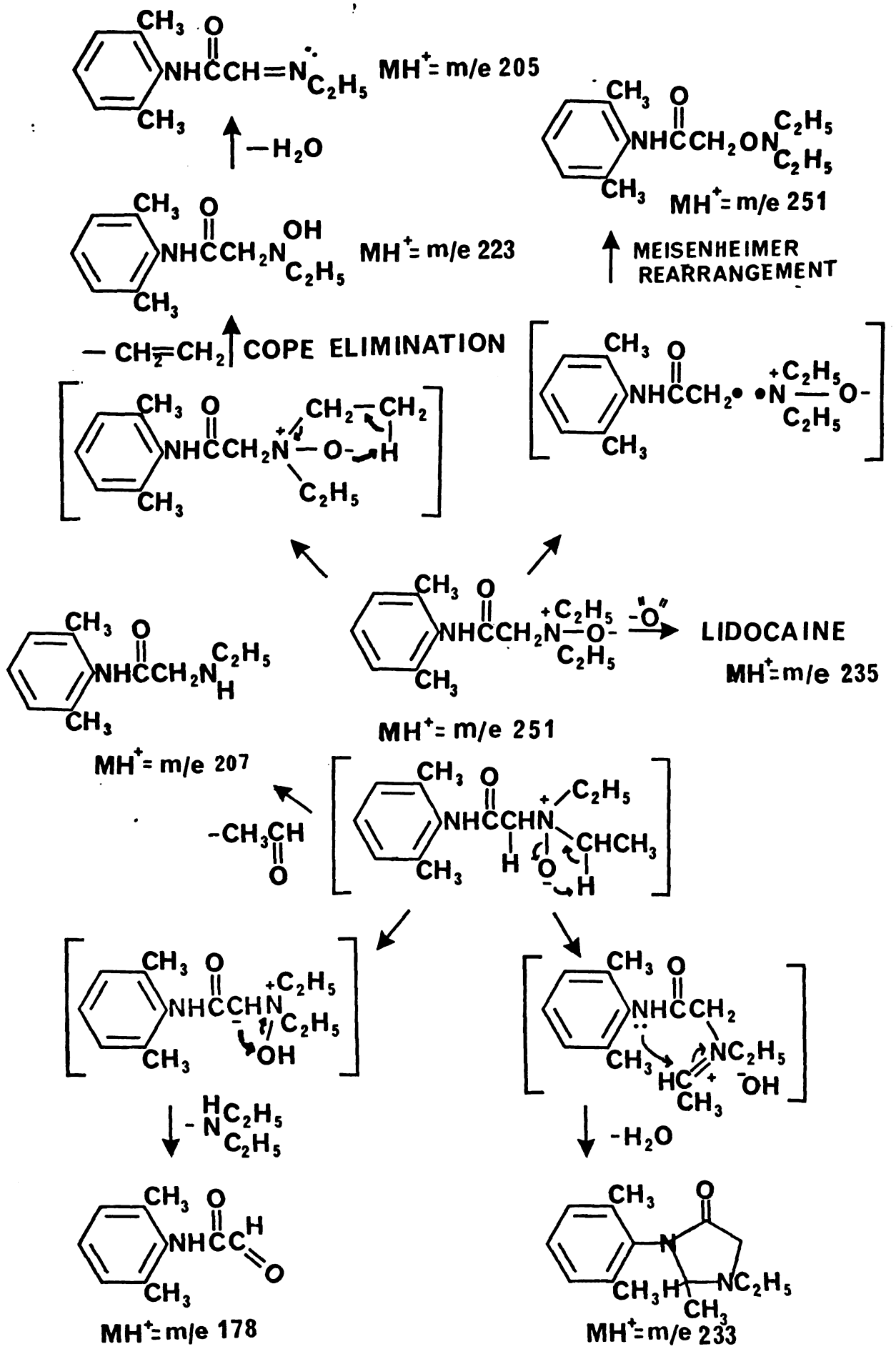

Silica Gel GF
70:20:10
Cyclohexane:chloroform:
methanol
A
B
C
D
-E
F
Lidocaine
+ Cyclic
Metabonate
MEGX
Lidocaine
N-Oxide
Column: 3% SE-30 on
A/W Chromosorb W
Temp: 150°
Cyclic Metabonate
+ MEGX
Lidocaine
0
2
4
6
8 min
Column: 2% Carbowax on
KOH-washed
Chromosorb W
Temp: 160°
Cyclic Metabonate
Lidocaine
MEGX
0
10
20
30
40 min

Figure II-2. CI mass spectrum (100°) of lidocaine N-oxide.


LIDOCAINE N-OXIDE CI
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
178
205
207
221
223
233
235
251
7. Σ 260 60

Scheme II-2. Possible rearrangement pathways for lidocaine N-oxide.


MH+= m/e 205
−H2O
MH+= m/e 223
MH+= m/e 251
MEISENHEIMER REARRANGEMENT
− CH2=CH2
COPE ELIMINATION
LIDOCAINE
MH+=m/e 235
MH+= m/e 251
MH+= m/e 207
-CH3CH=O
-HN(C2H5)2
-H2O
MH+=m/e 178
MH+=m/e 233

Although no lidocaine N-oxide was found in urine, some lidocaine N-oxide may be formed in vivo and then further biotransformed by reduction back to lidocaine, rearrangement and subsequent dealkylation to MEGX, and/or rearrangement and dehydration to cyclic metabonate. It is noteworthy that these three compounds are not only formed by mild thermal rearrangement of lidocaine N-oxide, but are also detectable in the urine and plasma of subjects receiving lidocaine.

2. MEGX-hydroxylamine

Beckett and Al-Sarraj[14] have reported the metabolism of several secondary and primary alkyl amines to hydroxylamines. Attempts were made to synthesize MEGX-hydroxylamine by mild m-chloroperoxybenzoic acid oxidation of MEGX using the procedure of Beckett and Salami.[15] Only a very small amount of the desired product could be detected as a black spot (rf = 0.42, 6:4:1 cyclohexane:chloroform:methanol, silica gel) on tlc using an ammoniacol silver nitrate spray reagent. Attempts to purify the product led to further decomposition. Due to the apparent instability of the hydroxylamine no further attempts were made to synthesize or detect the material in biological fluids.

3. 2,6-Dimethylphenylhydroxylamine

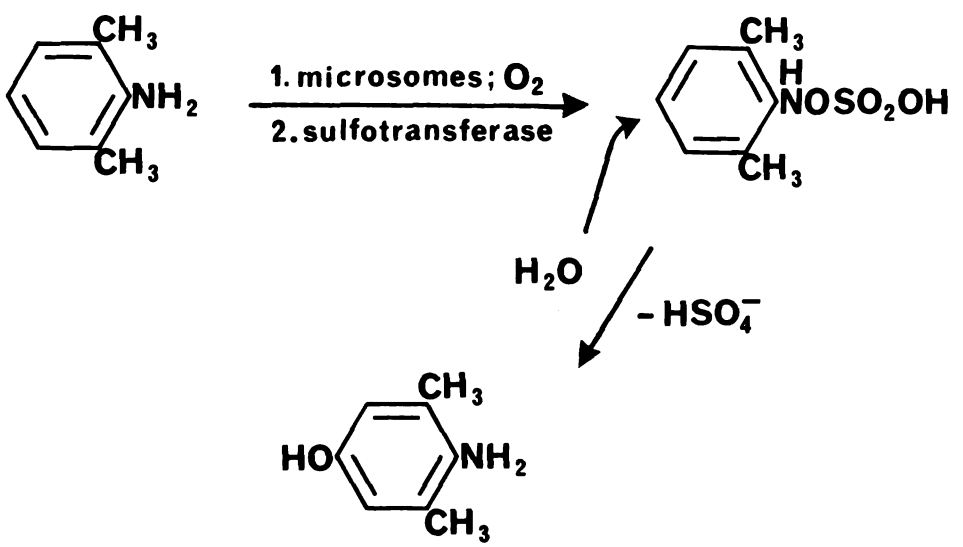
The biological conversion of aniline and substituted anilines to aromatic hydroxylamines yields products which are responsible for the in vivo formation of methemoglobin.[3,16] Since the hydrolysis of lidocaine

and MEGX to amino acids and 2,6-dimethylaniline appears to be a major metabolic pathway in humans, the possibility of further oxidation of 2,6-dimethylaniline to the hydroxylamine was investigated.

The quantitative results reported in Chapter I indicate that most of the 2,6-dimethylaniline (60-70%) is oxidized and further conjugated to give sulfates and/or glucuronides of 4-hydroxy-2,6-dimethylaniline. Keenaghan and Boyes[17] used Lindstrom's[18] procedure of acid hydrolysis of urine to quantify these conjugates. They reported finding 70-75% of the metabolite in human urine and suggested that the 2,6-dimethylaniline is directly oxidized to the 4-hydroxy product. Based on the chemical reactivity of 2,6-dimethylphenylhydroxylamine, the proposal of Keenaghan and Boyes may or may not be correct.

Synthetic 2,6-dimethylphenylhydroxylamine was found to be reasonably stable in crystalline form, but very unstable in aqueous solution, especially in acid. In dilute HCl a good yield of p-chloro-2,6-dimethylaniline is formed along with small amounts of 4-hydroxy-2,6-dimethylaniline. After 6 hours in human urine (pH 6.5) tlc and nmr of an ether extract indicated the presence of the hydroxylamine, 4-hydroxy-2,6-dimethylaniline, and 3 other unidentified products derived from the hydroxylamine. Glc analysis on 3% OV-17 (150°) showed breakdown of the hydroxylamine to 4 major products, the primary product ($\sim$75%) being 2,6-dimethylaniline. These preliminary studies agree with studies on other aromatic hydroxylamines where Bamberger rearrangements[19] and other reactions have been shown to occur.[3]

The problem of establishing the formation of the hydroxylamine metabolites may be very difficult. The hydroxylamine may be formed initially in vivo followed by conjugation with sulfate which converts the N-hydroxy group into an excellent leaving group. This conjugate may then react rapidly in a Bamberger-type rearrangement to yield the more thermodynamically stable p-hydroxy derivative. In other words, some or all of the 4-hydroxy-2,6-dimethylaniline quantified in human urine may be arising as shown below. The sequence will probably have to be established using $^{18}O_2$ and purified enzymes.



To determine if any free 2,6-dimethylphenylhydroxylamine was present in human urine following an oral dose of lidocaine, titanium trichloride ($TiCl_3$) treatment of urine was carried out. This reagent is known to reduce the N-O bond in N-hydroxy compounds as well as N-oxides and nitro groups.[20] A comparison of quantities of 2,6-dimethylaniline was therefore made before and after $TiCl_3$ treatment using the CIMS-stable isotope technique. No increase could be found. The same procedure could not be carried out on β-glucuronidase-sulfatase treated samples because of some unknown change in 2,6-dimethylaniline rendering it unquantifiable after the enzyme treatment as pointed out in Table I-3. Thus, the problem of whether the hydroxylamine is a metabolite of lidocaine still remains unresolved.

C. OXIDATIONS OF THE AMIDE GROUP

1. Previous Work

In 1960, the Millers[21] showed that metabolic N-hydroxylation of N-fluorenylacetamide produced the intermediate responsible for the high carcinogenic activity of the compound. Since that time, many investigations have been carried out on this and other hydroxamic acid derivatives,[3] a number of which have been found to have carcinogenic and possibly mutagenic activity.

More recently, Mather and Thomas[4] found substantial increases in the amounts of both lidocaine and MEGX in human urine aliquots treated with $TiCl_3$, the urine collected from three subjects who had received oral

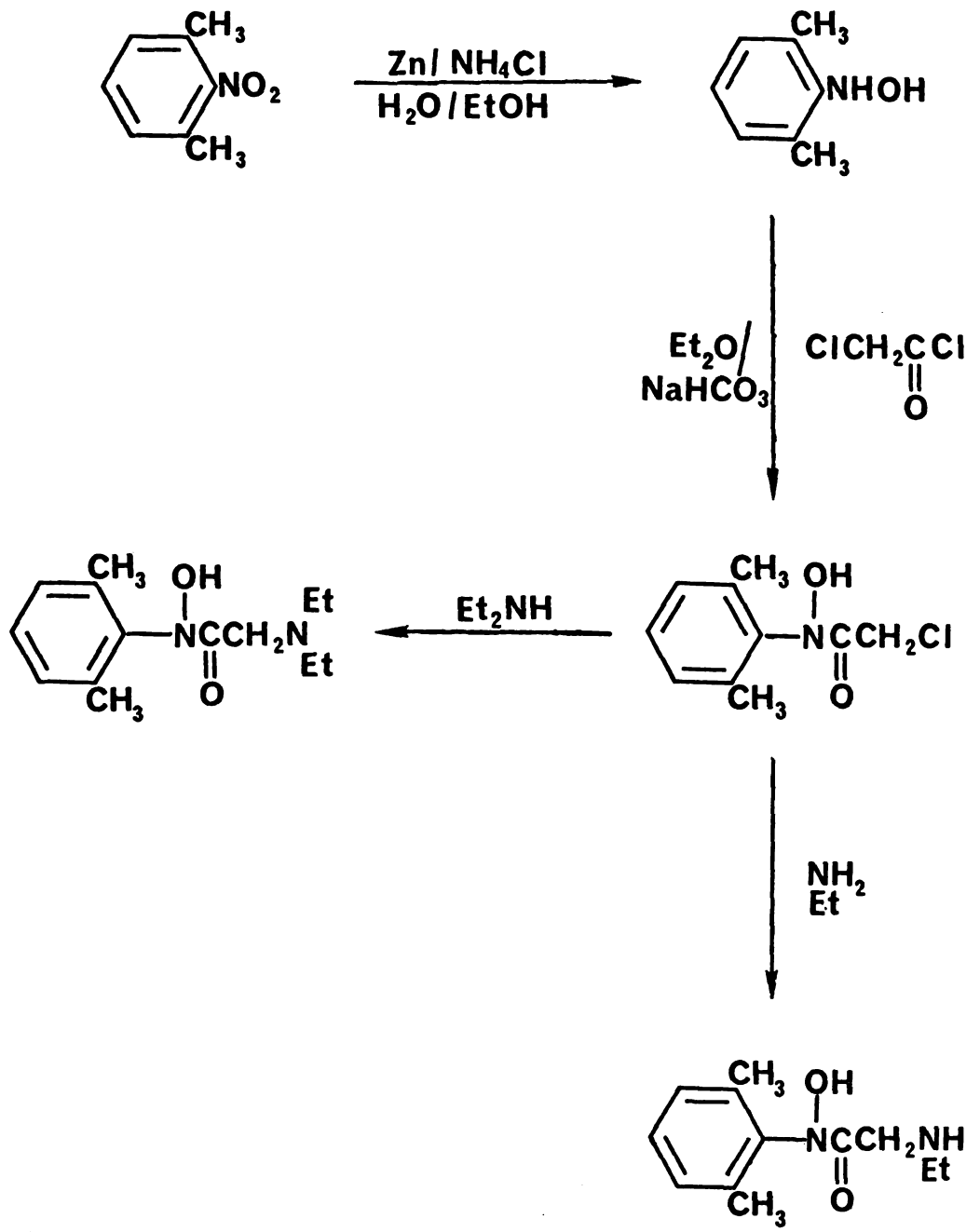
doses of lidocaine (115 mg). Since the increases were even greater under conditions of constant acidic urinary pH (5.0-5.5), the authors suggested that oxidation had occurred at the amide nitrogen and not on the more basic amino group. In the case of lidocaine, an even greater amount of lidocaine was found after β-glucuronidase treatment followed by $TiCl_3$ treatment, further implicating the N-hydroxyamide rather than the N-oxide which does not form conjugates.

2. Synthesis and Properties of the N-Hydroxyamides of Lidocaine and MEGX

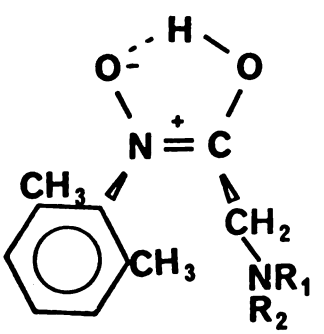
In order to substantiate the human in vivo formation of the N-hydroxyamides of lidocaine and MEGX, and to determine their carcinogenic potential, the synthesis of N-hydroxylidocaine and N-hydroxyMEGX was carried out according to Scheme 3. Controlled reduction of 2,6-dimethylnitrobenzene using zinc dust and ammonium chloride yielded the hydroxylamine which was acylated in the cold (-5°) with chloroacetyl chloride using a slurry of aqueous sodium bicarbonate in ether to trap the hydrochloric acid released in order to prevent interaction with the remaining hydroxylamine.[22a] The ω-chloro-N-hydroxyamide produced was then reacted with the appropriate amine to produce the desired N-hydroxyamide.

The N-hydroxyamides reacted to give a red color in ferric chloride in ethanol and produced a violet color with vanadium (V) in chloroform.[23] Reaction of N-hydroxylidocaine, N-hydroxyMEGX, and their hydrochloride salts with $TiCl_3$ yielded the reduced compounds lidocaine and MEGX, respectively. C, H and N analyses and mass spectral analyses (CI, EI

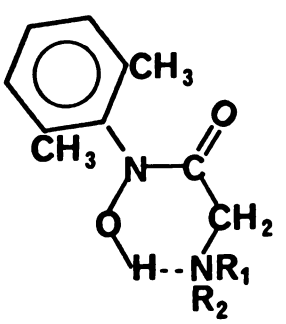
Scheme II-3. Syntnesis of tne N-hydroxyamides of lidocaine and MEGX.


CH3
NO2
CH3
Zn/ NH4Cl
H2O / EtOH
CH3
NHOH
CH3
Et2O/
NaHCO3
ClCH2CCl
O
CH3 OH
NCCH2N
Et
Et
O
CH3
Et2NH
CH3 OH
NCCH2Cl
O
CH3
NH2
Et
CH3 OH
NCCH2NH
Et
O
CH3

and high resolution mass determinations) were all consistent with the N-hydroxyamide structure. N-Hydroxylidocaine and N-hydroxyMEGX show interesting (M-17) and small (M-16) ions under EI, and (MH^+ - 16) and (MH^+ - 18) ions under CI conditions. This as well as nmr evidence possibly indicates the presence of the two tautomeric forms shown below, with loss of 16 coming from the imino-enol (1) while loss of 17 in EIMS comes from OH radical departure from the hydroxamic acid structure (2). The loss of 16 in CIMS is probably a thermal cleavage of the N-O bond from 1, and the loss of 18 is the loss of water from the protonated hydroxyl group of 2. Both EI and CI mass spectra of N-hydroxylidocaine and N-hydroxyMEGX are shown in Figures 3a and 3b.

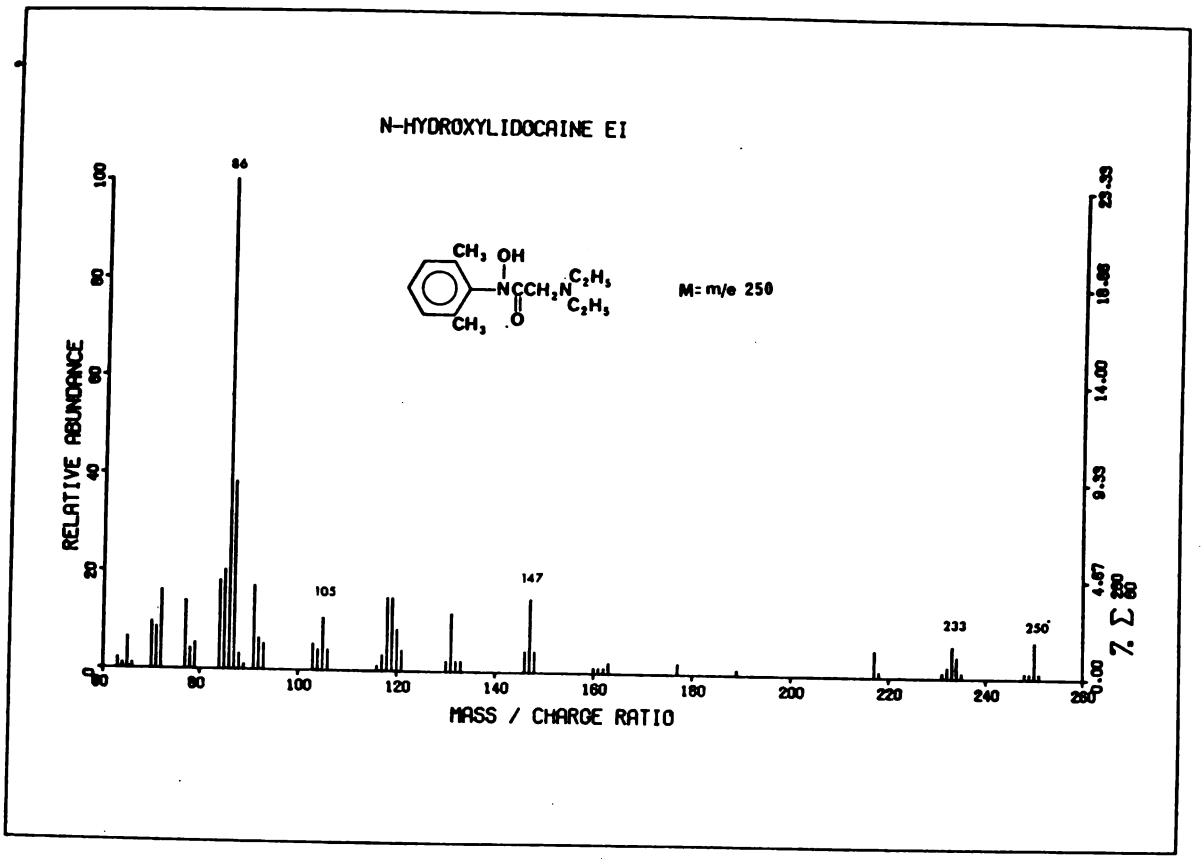


1



2

Figure II-3a. Comparative EI and CI mass spectra of N-hydroxylidocaine.


N-HYDROXYLIDOCAINE EI
M= m/e 250
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
86
105
147
233
250

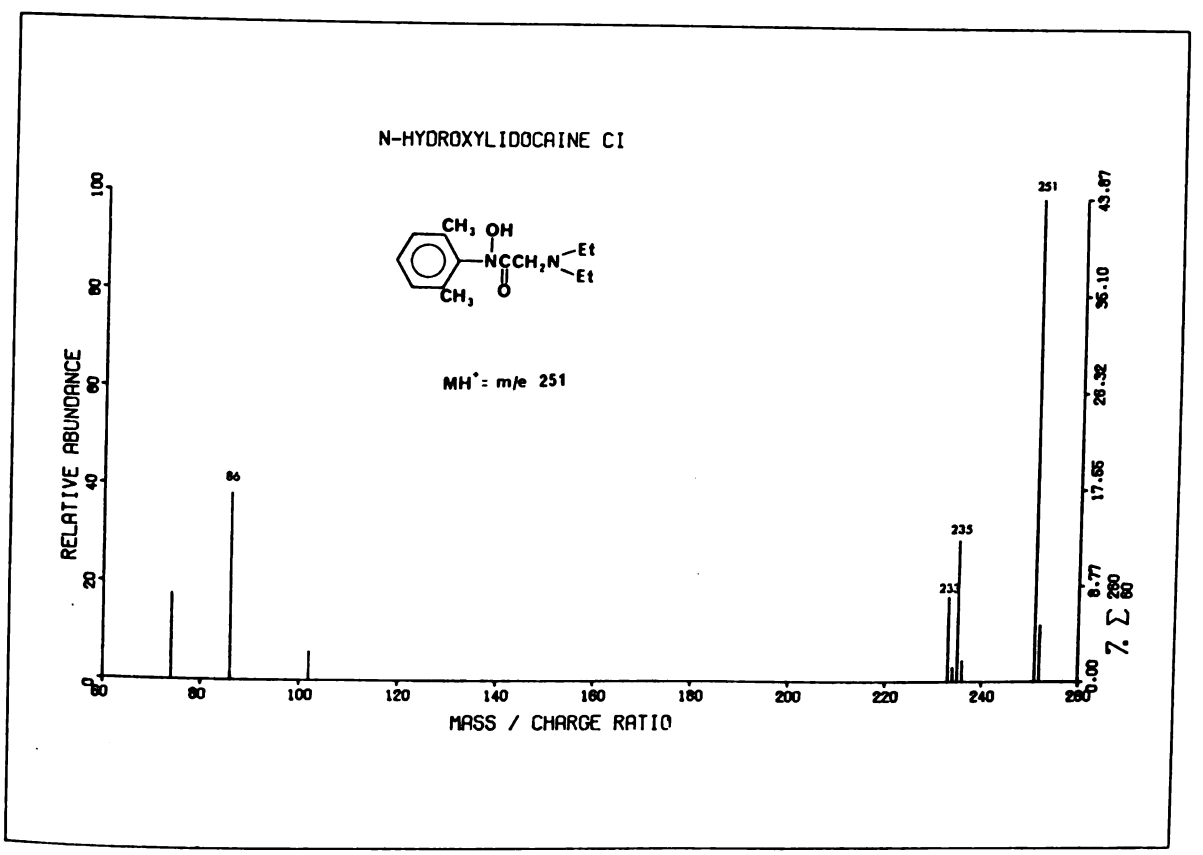
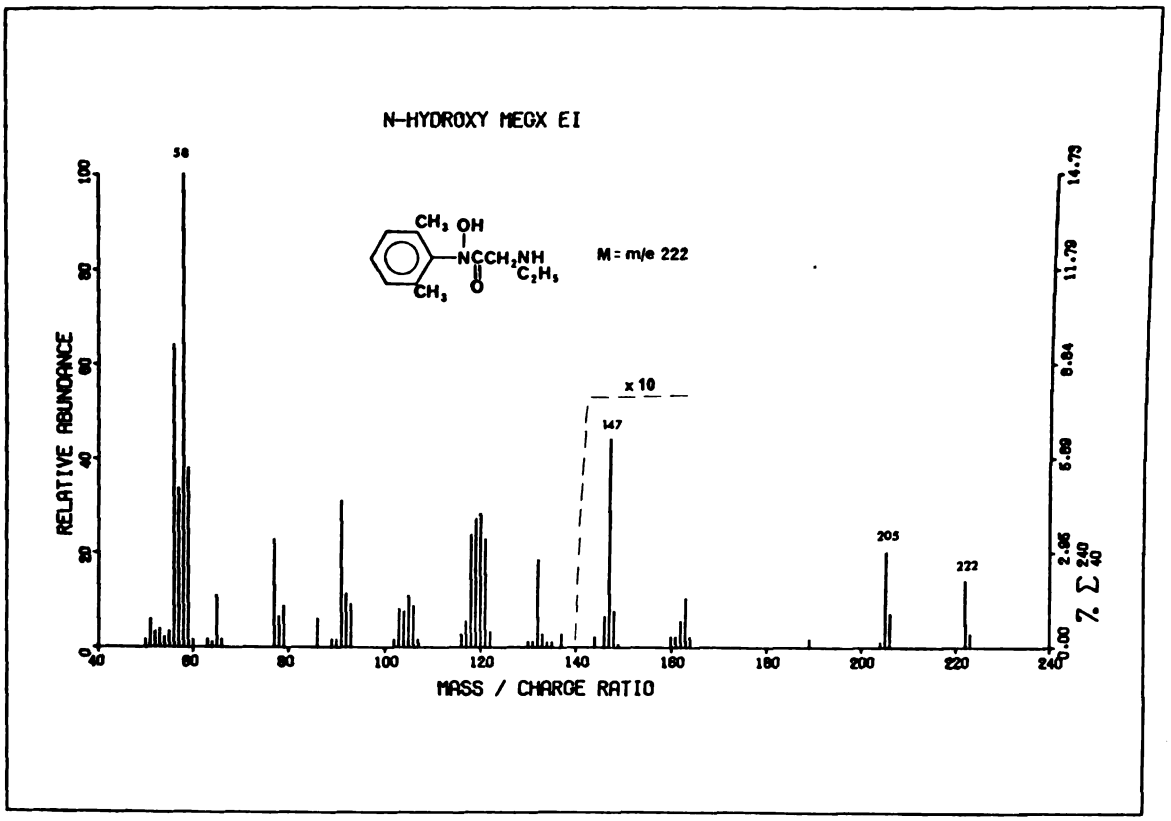
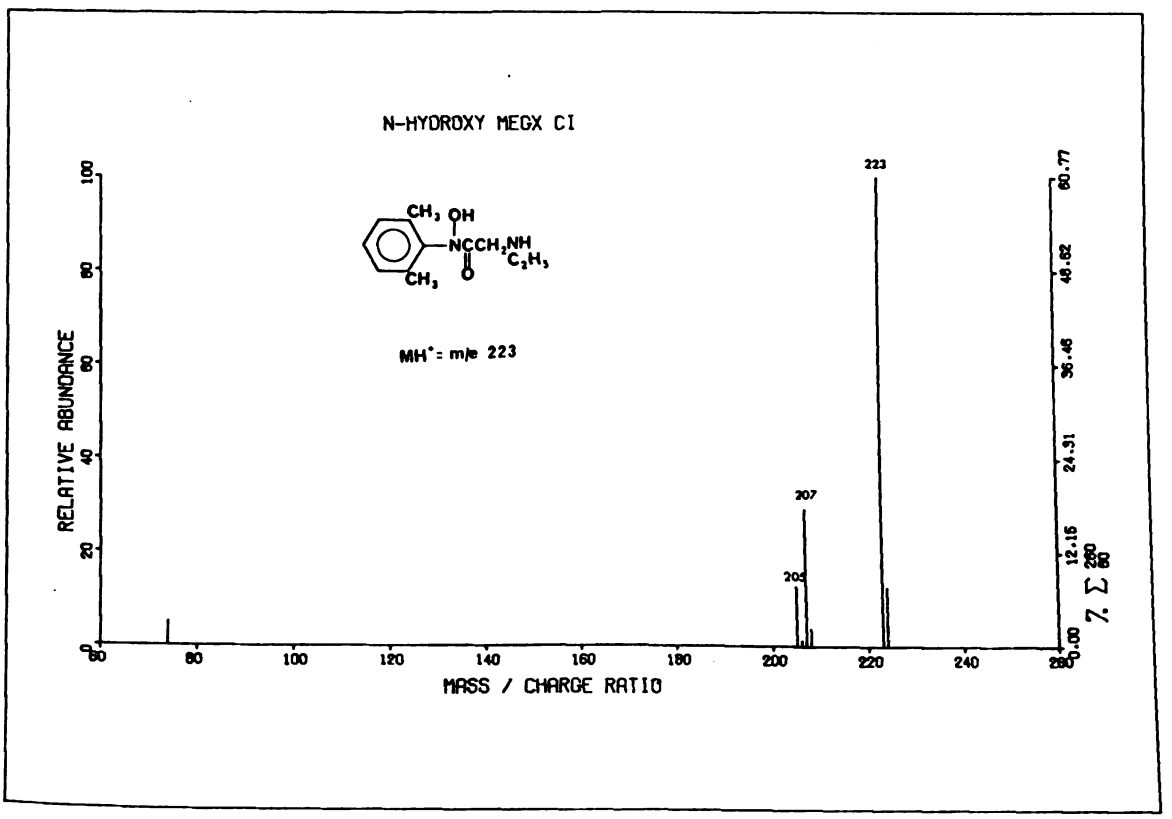

N-HYDROXYLIDOCAINE CI
MH⁺= m/e 251
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
86
233
235
251

Figure II-3b. Comparative EI and CI mass spectra of N-hydroxyMEGX.


N-HYDROXY MEGX EI
58
147
205
222
x 10
M = m/e 222
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
7. Σ 240 40


N-HYDROXY MEGX CI
223
207
205
MH⁺ = m/e 223
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
7. Σ 280 80

Although the ir and nmr spectra of the N-hydroxyamide hydrochlorides are consistent with the N-hydroxyamide structure, the spectra taken of the free amines in organic solvents suggests the presence of two different isomers. This probably results from strong intramolecular hydrogen bonding between the enol hydroxyl group and the nitrone-like oxygen as illustrated for tautomer 1, and between the N-hydroxyl proton and the terminal amino group as shown for tautomer 2. In effect a five- and six-membered ring are produced which results in different magnetic environments in the nmr for otherwise equivalent protons. This is especially true for the methylene hydrogens between the carbonyl group and amine function where the chemical shift difference is 1.05 ppm between isomers in N-hydroxylidocaine, and 0.85 ppm in N-hydroxyMEGX.* The downfield shift probably occurs in tautomer 1 as a result of deshielding of the methylene group by both the adjacent imminium ion structure and by the magnetic anisotropy of the aromatic ring. An nmr temperature study and possibly ^{13}C-nmr might provide useful information to help substantiate or disprove this interpretation.

All of these N-hydroxyamides were pure compounds with sharp melting points. The two tautomeric forms observed in organic solvents

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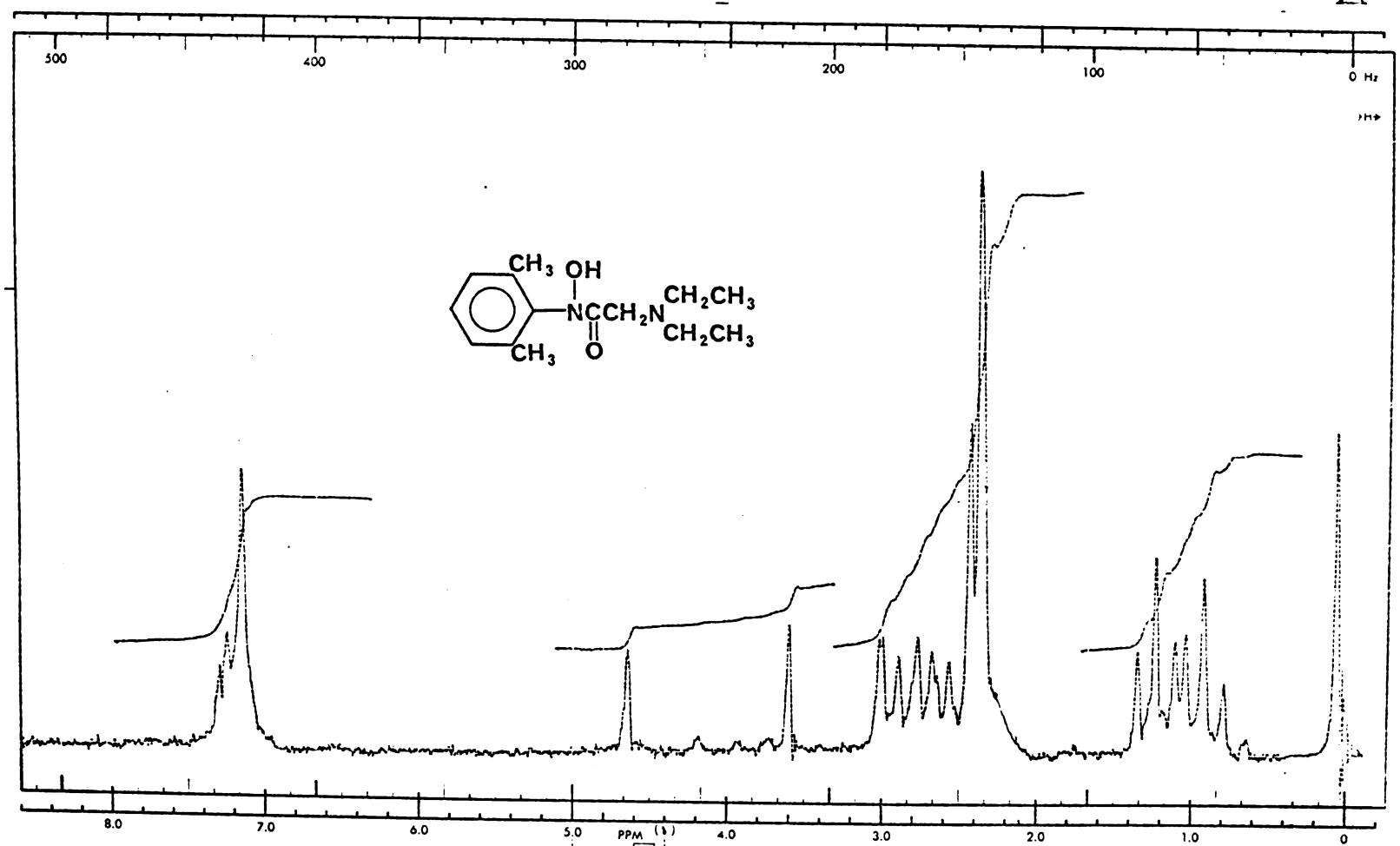
*The same downfield shift is observed with the methylene hydrogens in ω-chloro-2,6-dimethylphenylacetohydroxamic acid, the precursor to N-hydroxylidocaine and N-hydroxyMEGX. Here, the imino-enol can still form the five-membered ring, but the hydroxamic acid tautomer will not necessarily form a six-membered hydrogen-bonded ring structure since there is no terminal amino group for hydrogen bonding.

could also be observed from the hydrochlorides of the N-hydroxyamides after neutralization to give the free amines. Nmr spectra of the free amines and their hydrochloride salts can be seen in Figures 4 and 5.

Preliminary glc analysis of N-hydroxylidocaine and N-hydroxyMEGX on 3% OV-17 at 185° showed degradation to 5 or 6 compounds, the primary products being lidocaine and MEGX, respectively. This is noteworthy since both N-hydroxyamides are extractable with methylene chloride using the procedure of Keenaghan and Boyes[17] who then analyzed the extract with a 3% OV-17 column. Their reported levels of lidocaine and MEGX may, therefore, include levels of the corresponding N-hydroxyamides.

Figure II-4a. Nmr spectrum of N-hydroxylidocaine (60 MHz, $CDCl_3$, TMS).



SWEEP OFFSET (Hz): 0
SPECTRUM AMPLITUDE: 20
INTEGRAL AMPLITUDE: 5
SPINNING RATE (RPS): 30

RECORDING CHARTS
GRAPHIC CONTROLS CORPORATION
BUFFALO, NEW YORK
PRINTED IN U.S.A.
No. VN 1009 (S-60T)

MANUAL
SWEEP TIME (SEC): 50
SWEEP WIDTH (Hz): 25 50 100 250 500
FILTER: 1 2 3 4 5 6 7 8
RF POWER LEVEL: 0.08

AUTO ☐
(250)
(500)
(2)
(.05)

SAMPLE:

N-Hydroxylidocaine

REMARKS:

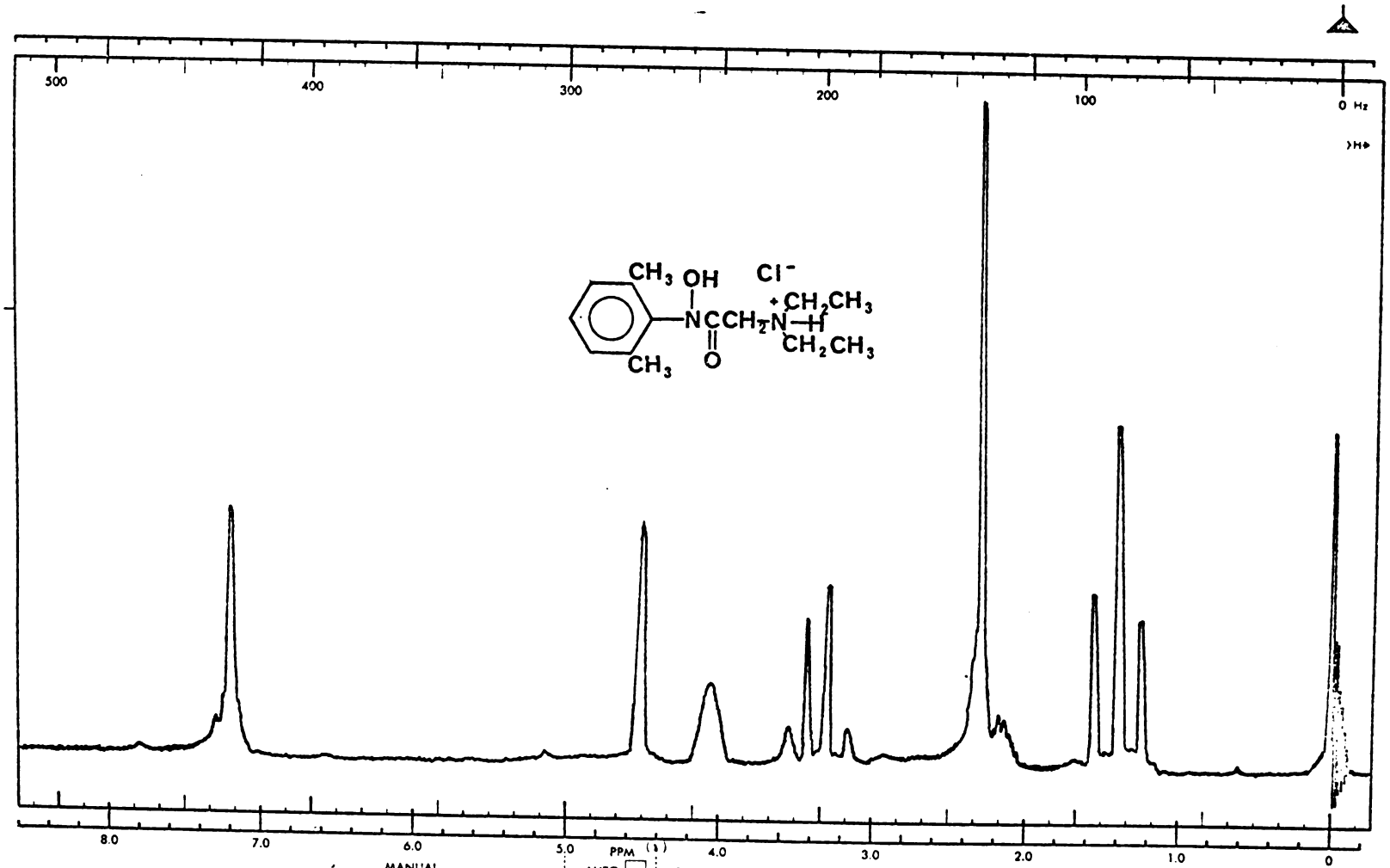
SOLVENT: $CDCl_3$

DATE:

OPERATOR: S.D.N.

60 MHz NMR
SPECTRUM NO. SB-45C-2

Figure II-4b. Nmr spectrum of N-hydroxylidocaine hydrochloride (60 MHz, Acetone-d_6, TMS).



SWEEP OFFSET (Hz): 0
SPECTRUM AMPLITUDE: 16.9
INTEGRAL AMPLITUDE: ←
SPINNING RATE (RPS): 25
RECORDING CHARTS
GRAPHIC CONTROLS CORPORATION
BUFFALO, NEW YORK
PRINTED IN U.S.A.
No. VN 1009 (S-60T)

MANUAL
SWEEP TIME (SEC): 50 ✓5
SWEEP WIDTH (Hz): 25 50 100 250 ✓500
FILTER: ✓2 3 4 5 6 7 8
RF POWER LEVEL: 0.08

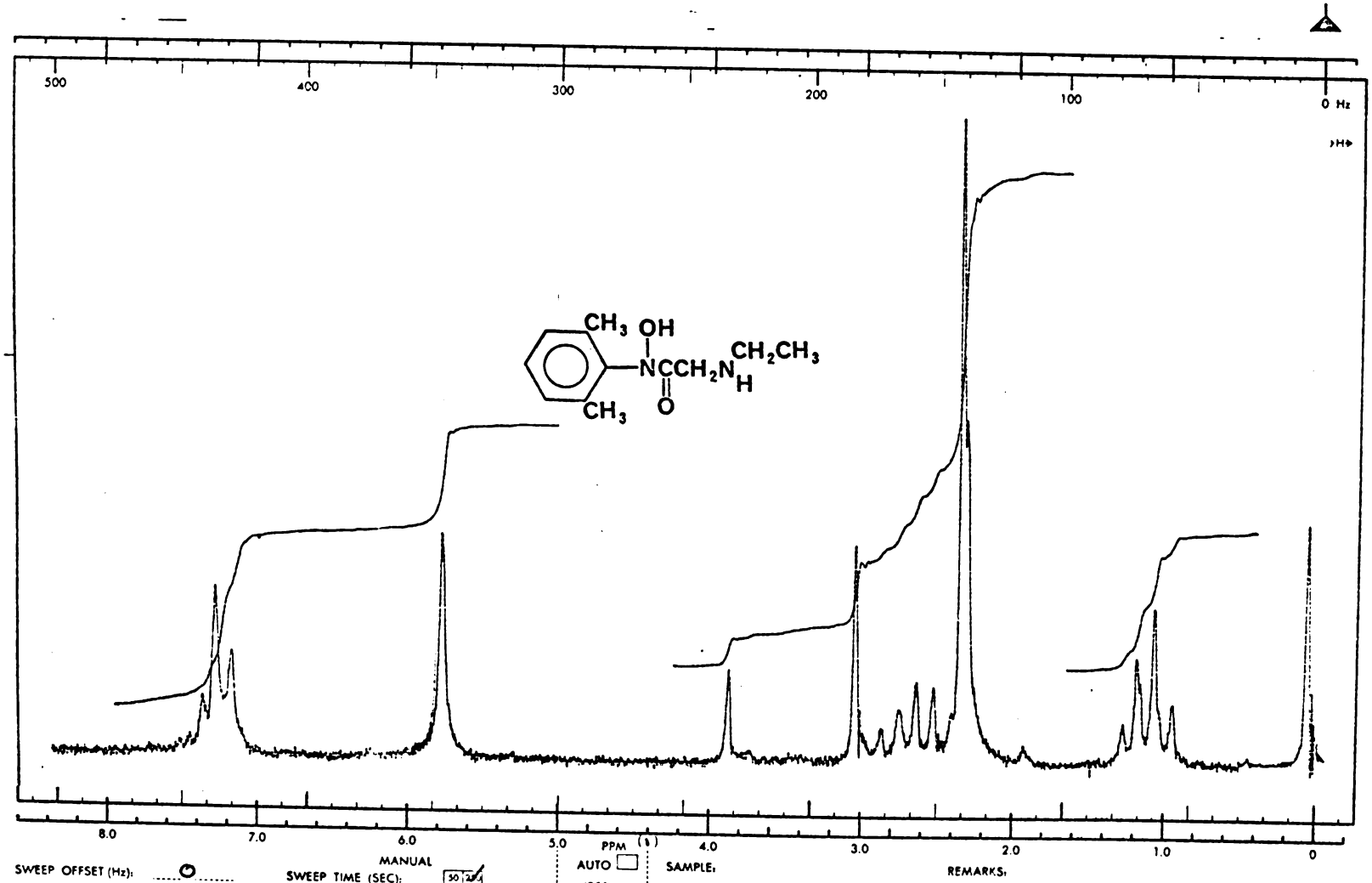
AUTO ☐
(250)
(500)
(2)
(.05)

SAMPLE: N-Hydroxylidocaine · HCl
REMARKS:

SOLVENT: d_6- Acetone

DATE:
OPERATOR: S.D.N.
60 MHz NMR
SPECTRUM NO. Sn-37C-1

Figure II-5a. Nmr spectrum of N-hydroxyMEGX (60 MHz, $CDCl_3$, TMS).



SWEEP OFFSET (Hz): 0
SPECTRUM AMPLITUDE: 16.0
INTEGRAL AMPLITUDE: 6.0
SPINNING RATE (RPS): 25
RECORDING CHARTS
GRAPHIC CONTROLS CORPORATION
BUFFALO, NEW YORK
PRINTED IN U.S.A.
No. VN 1009 (S-60T)

MANUAL
SWEEP TIME (SEC): 50 [20]
SWEEP WIDTH (Hz): 25 50 100 250 [500]
FILTER: [1] 2 3 4 5 6 7 8
RF POWER LEVEL: 0.08

AUTO ☐
(250)
(500)
(2)
(.05)

SAMPLE:

REMARKS:
N-Hydroxy MEGX

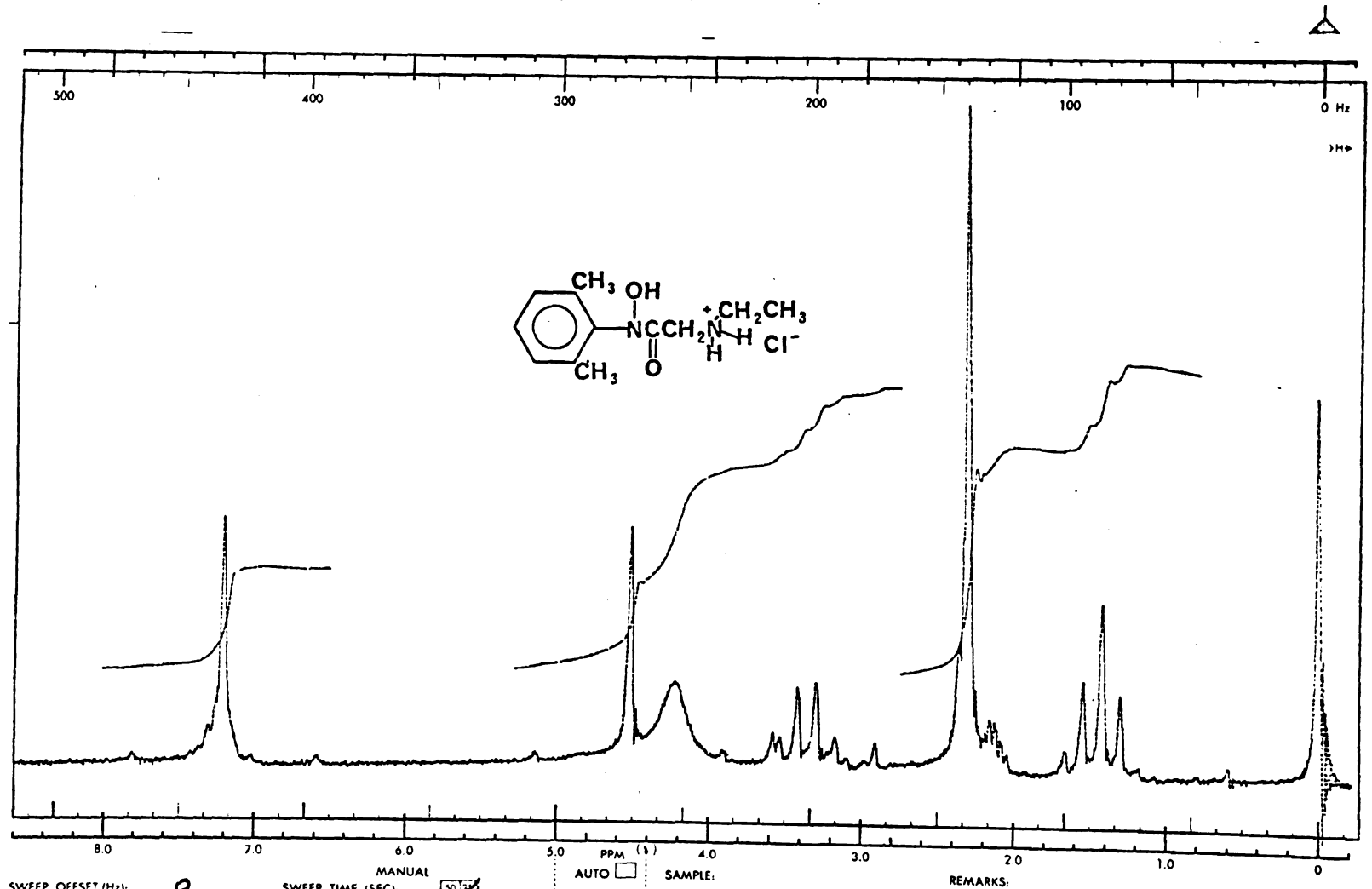
SOLVENT: $CDCl_3$

DATE:

OPERATOR: S.D.N.

60 MHz NMR
SPECTRUM NO. Sn-40C-1

Figure II-5b. Nmr spectrum of N-hydroxyMEGX hydrochloride (60 MHz, Acetone-d_6, TMS).



SWEEP OFFSET (Hz): 0
SPECTRUM AMPLITUDE: 10.0
INTEGRAL AMPLITUDE: 6
SPINNING RATE (PPS): 30

GRAPHIC CONTROLS CORPORATION
BUFFALO, NEW YORK
PRINTED IN U.S.A.
No. VN 1009 (S-60T)

MANUAL
SWEEP TIME (SEC): 50 ✓
SWEEP WIDTH (Hz): 25 50 100 250 ✓
FILTER: ✓ 2 3 4 5 6 7 8
RF POWER LEVEL: 0.08

AUTO ☐
(250)
(500)
(2)
(.05)

SAMPLE:

REMARKS:
N-HydroxyMEGX·HCl

SOLVENT: d6-Acetone

DATE:

OPERATOR: S.D.N.

60 MHz NMR
SPECTRUM NO. Sn-45 C-3

3. Quantification of N-Hydroxyamides in Human Urine

a. Procedure

Aliquots of the 24-hour urine from subjects A and B (Chapter I) were analyzed for N-hydroxylidocaine and N-hydroxyMEGX using the combined CIMS-stable isotope technique and $TiCl_3$ treatment. A third subject (C) was treated by the procedure of Mather and Thomas[4] with ammonium chloride dosage prior to and during oral lidocaine treatment to maintain a constant acidic urinary pH (5.0-5.3). To aliquots of each urine sample from the three subjects was added known amounts of lidocaine-d_2, MEGX-d_2, N-hydroxylidocaine-d_4, and N-hydroxyMEGX-d_3. A portion of each aliquot was basified to pH 12 and extracted with purified methylene chloride, and a second portion was treated first with $TiCl_3$ then basified and extracted. The same procedure was followed after treating the remaining aliquot with β-glucuronidase-sulfatase at 37° for 24 hours in an equal volume of pH 5.0 Walpole's acetate buffer.

The pKa of the N-hydroxy group of aromatic hydroxyamides is approximately that of a phenol[22b] so that CI mass spectra of the basified extracts without prior $TiCl_3$ treatment showed only MH^+ ions corresponding to MEGX (m/e = 207) and MEGX-d_2 internal standard (m/e = 209), and lidocaine (m/e = 235) and lidocaine-d_2 internal standard (m/e = 237). Pretreatment of urine samples with $TiCl_3$ reduced the N-hydroxyMEGX-d_3 standard quantitively to MEGX-d_3 (m/e = 210), and the N-hydroxylidocaine-d_4 standard to lidocaine-d_4 (m/e = 239). This

showed that the $TiCl_3$ treatment worked and at the same time allowed for quantification of any N-hydroxyamide metabolites by differences in ratios of MEGX (MH^+ = m/e 207) vs. MEGX-d_2 (MH^+ = m/e 209) and lidocaine (MH^+ = m/e 235) vs. lidocaine-d_2 (MH^+ = m/e 237) before and after $TiCl_3$ treatment.

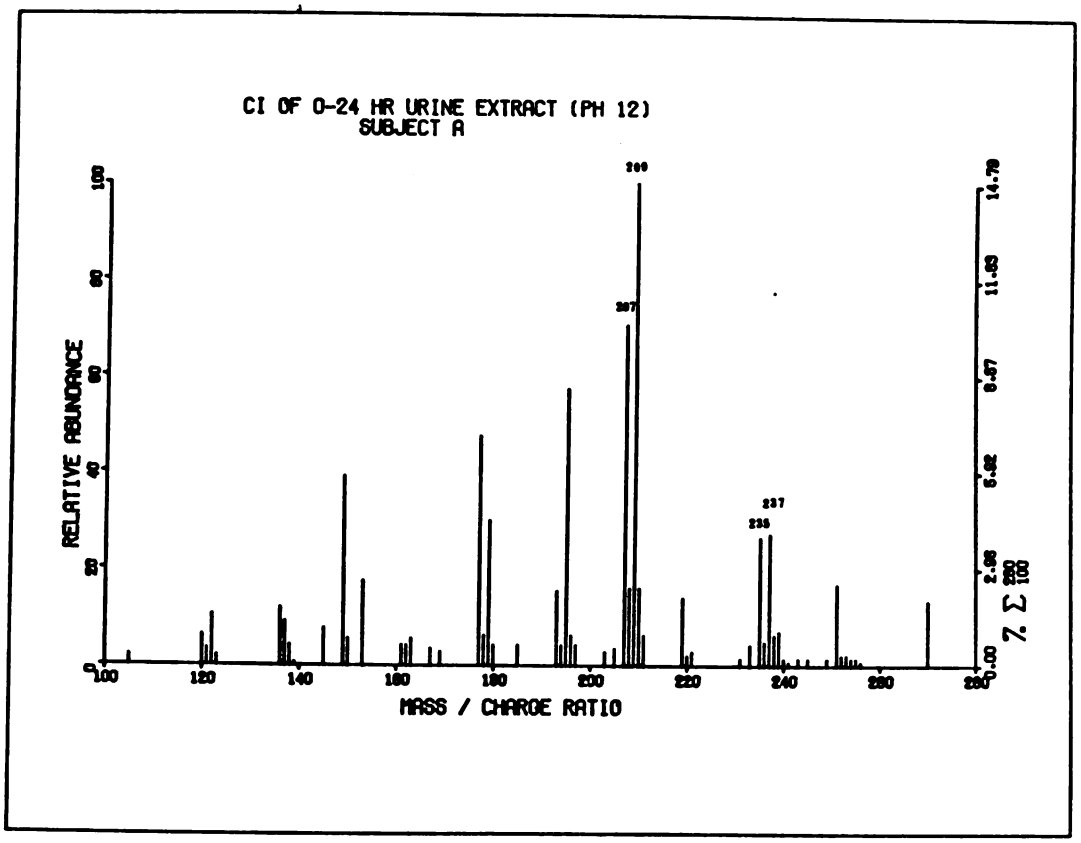
b. Results

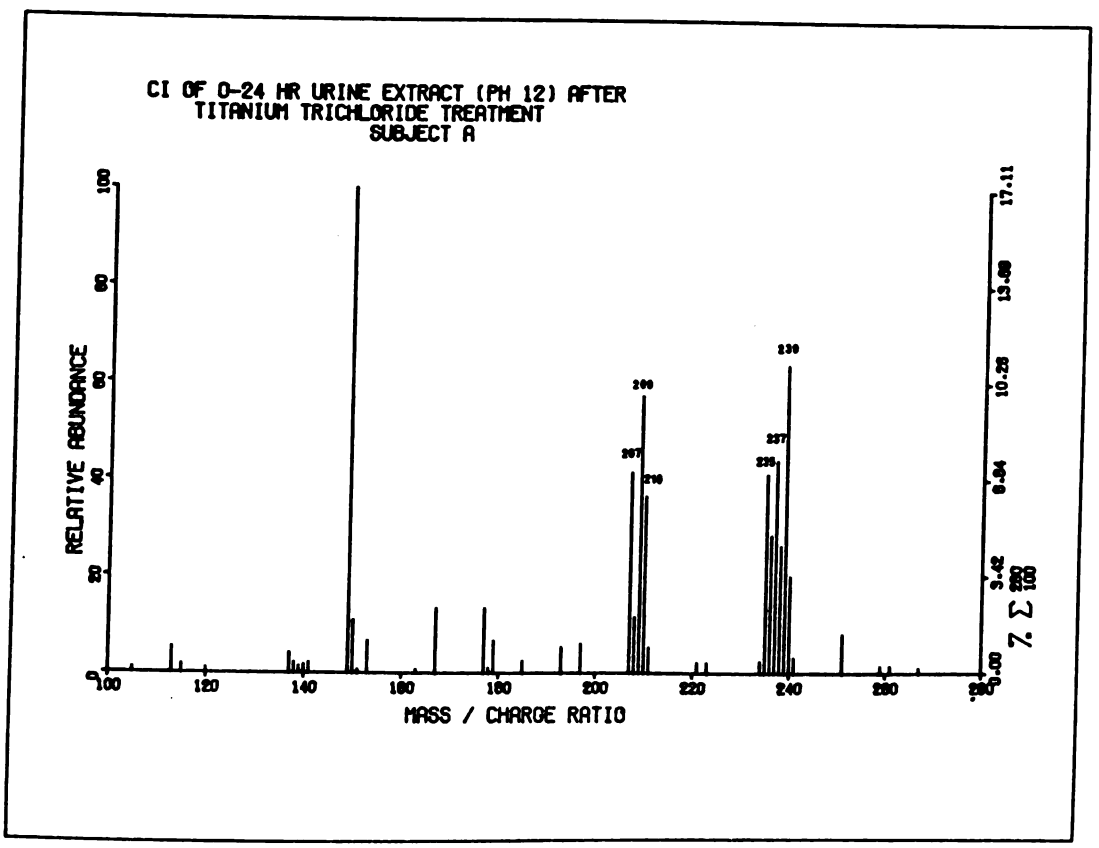
Contrary to the results of Mather and Thomas,[4] virtually no increase (> 5%) in the amounts of either lidocaine or MEGX was found after $TiCl_3$ treatment of urine either before or after β-glucuronidase treatment. Figure 6 shows the results of subject A before β-glucuronidase treatment of the urine. Figure 6a shows that direct extraction of basified urine yielded only MH^+ ions at m/e = 207 and 209 for MEGX metabolite and MEGX-d_2 internal standard, and MH^+ ions at m/e = 235 and 237 for lidocaine and lidocaine-d_2 internal standard. Figure 6b shows that prior $TiCl_3$ treatment of the urine produced a new peak at m/e = 210 due to the reduction of N-hydroxyMEGX-d_3 to MEGX-d_3, and a new peak at m/e = 239 due to the reduction fo N-hydroxylidocaine-d_4 to lidocaine-d_4. However, no substantial increase (> 5%) in the amounts of MEGX or lidocaine MH^+ ions relative to that of their deuterated standards can be determined.*

*Determinations were made by calculating the peak height ratios as described under "Calculations" in the Experimental Section of Chapter I.

Figure II-6a. The CI mass spectrum of the methylene chloride extract of basified urine (pH 12) from subject A. The ion at m/e = 207 is MEGX metabolite, m/e = 209 represents added MEGX-d_2 standard, m/e = 235 is lidocaine metabolite, and m/e = 237 represents added lidocaine-d_2 standard.

Figure II-6b. The CI mass spectrum of the methylene chloride extract of basified urine (pH 12) from subject A after $TiCl_3$ treatment of the urine. The ion at m/e = 207 is MEGX metabolite, m/e = 209 represents added MEGX-d_2 standard, m/e = 210 represents MEGX-d_3 which comes from reduction of added N-hydroxyMEGX-d_3 standard, m/e = 235 is lidocaine metabolite, m/e = 237 represents added lidocaine-d_2 standard, and m/e = 239 represents lidocaine-d_4 which comes from reduction of added N-hydroxylidocaine-d_4 standard.


CI OF 0-24 HR URINE EXTRACT (PH 12)
SUBJECT A
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
100
120
140
160
180
200
220
240
260
209
207
237
235


CI OF 0-24 HR URINE EXTRACT (PH 12) AFTER
TITANIUM TRICHLORIDE TREATMENT
SUBJECT A
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
100
120
140
160
180
200
220
240
260
209
207
210
239
237
235

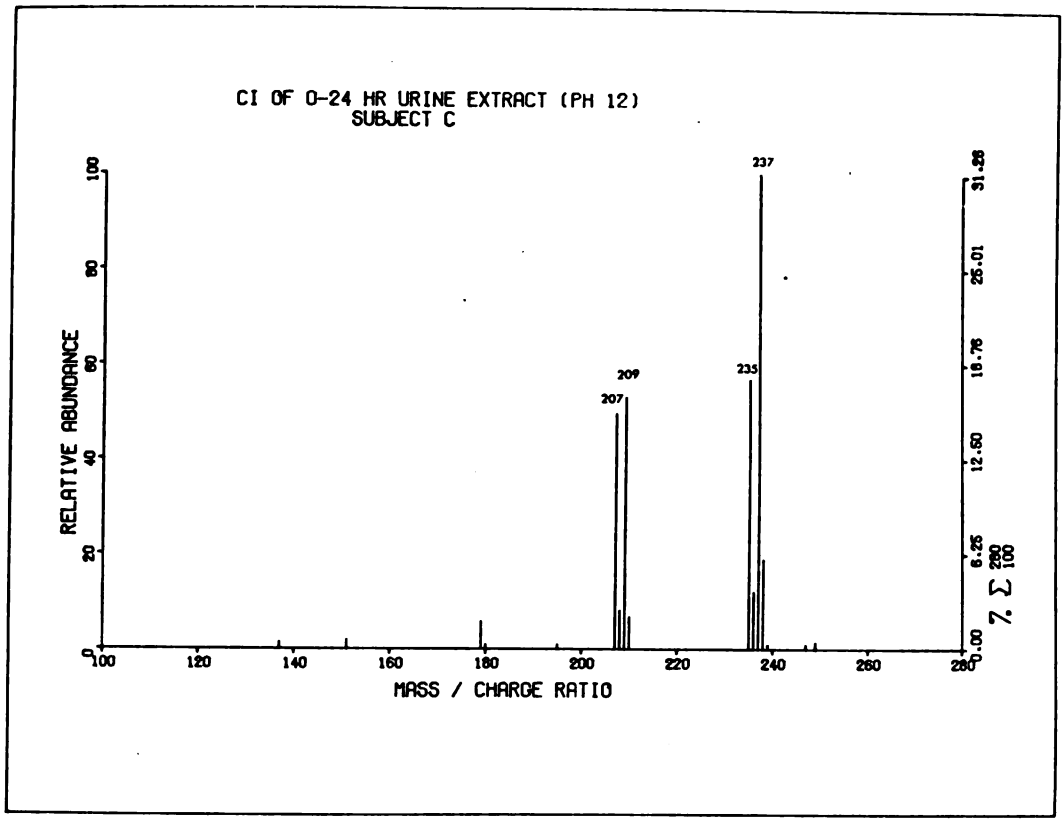
Figures 7 and 8 of the urine samples collected under conditions of constant acidic urinary pH, both before and after β-glucuronidase treatment, show identical results. Another portion of this urine sample was extracted, and the N-hydroxyamides separated by tlc using two different developing systems. CIMS of the separated material showed only the presence of the deuterated N-hydroxyamide standards. No peaks corresponding to the MH^+ ions of the suspected N-hydroxyamide metabolites could be found above background. The same procedure was carried out with β-glucuronidase-sulfatase treated urine and showed the same results.

Mather and Thomas,[4] using a glc procedure, found in one subject, without prior ammonium chloride treatment, that levels of lidocaine and MEGX increased ~100% after $TiCl_3$ treatment. No data was reported after β-glucuronidase treatment of this urine. Under conditions of constant acidic urinary pH using the ammonium chloride treatment, levels of lidocaine increased 30% and those of MEGX ~500% after $TiCl_3$ treatment. Following β-glucuronidase treatment, MEGX levels unexplicably decreased while lidocaine levels increased 200-300% over controls without $TiCl_3$ treatment.

c. Discussion

There now exists a sizable body of knowledge on molecular interactions between N-hydroxy derivatives and cellular nucleophiles.[3] Recently, Mitchell, et al.,[24] reported that acetaminophen-induced hepatic necrosis is

Figure II-7. CI mass spectra of basified (pH 12) urine extracts (subject C) before and after $TiCl_3$ treatment. The urine was collected under conditions of constant acidic urinary pH 5.0-5.3 as described in the text and experimental. The labeled ions are represented by metabolites and standards as described for Figures 6a and 6b.


CI OF 0-24 HR URINE EXTRACT (PH 12)
SUBJECT C
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
237
209
207
235
100
120
140
160
180
200
220
240
260
280

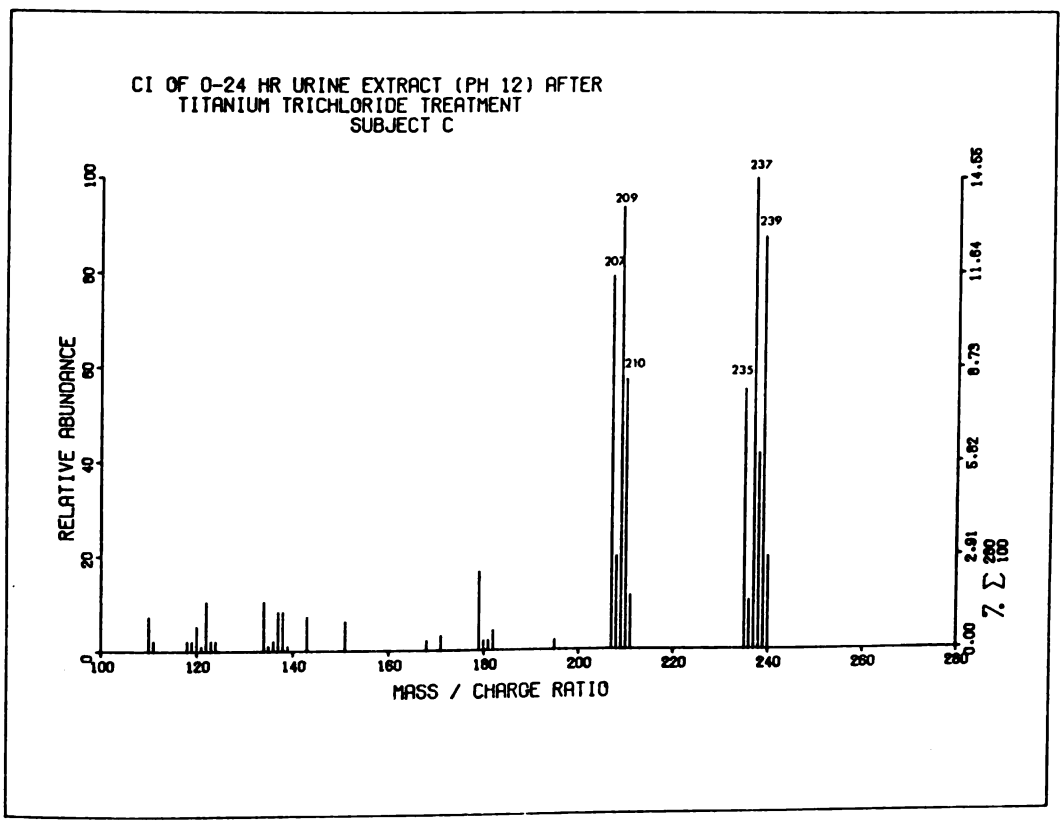
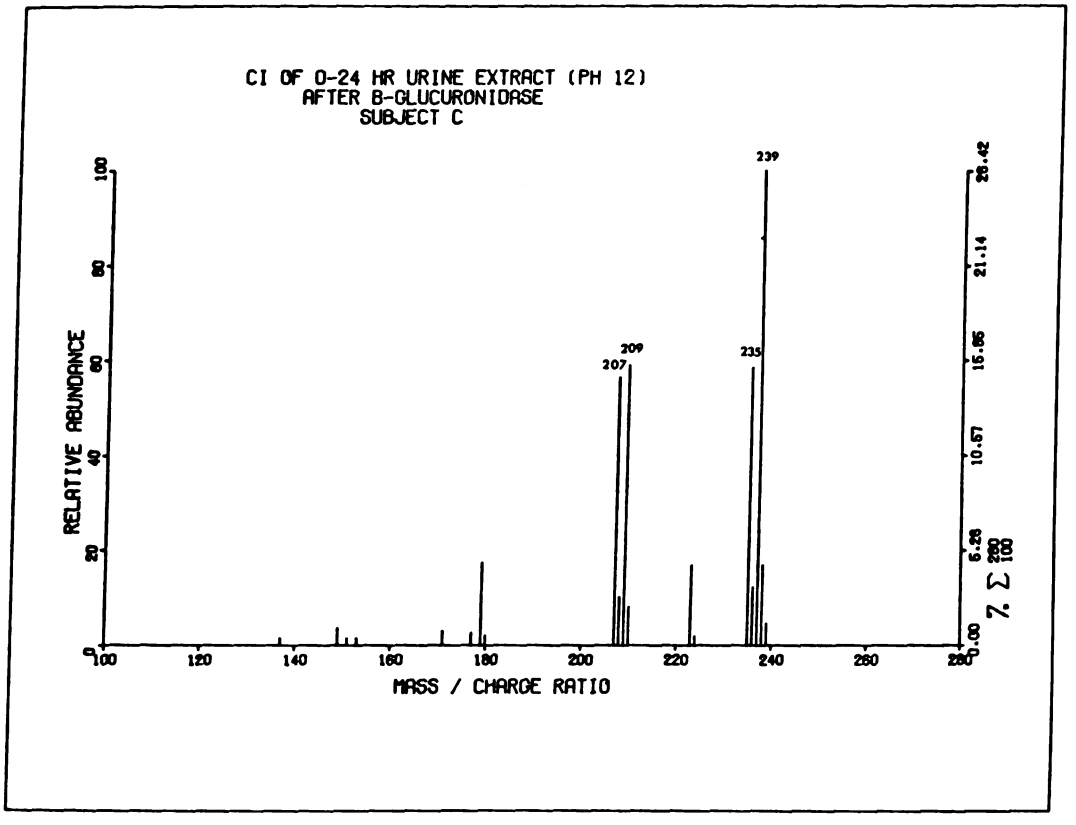
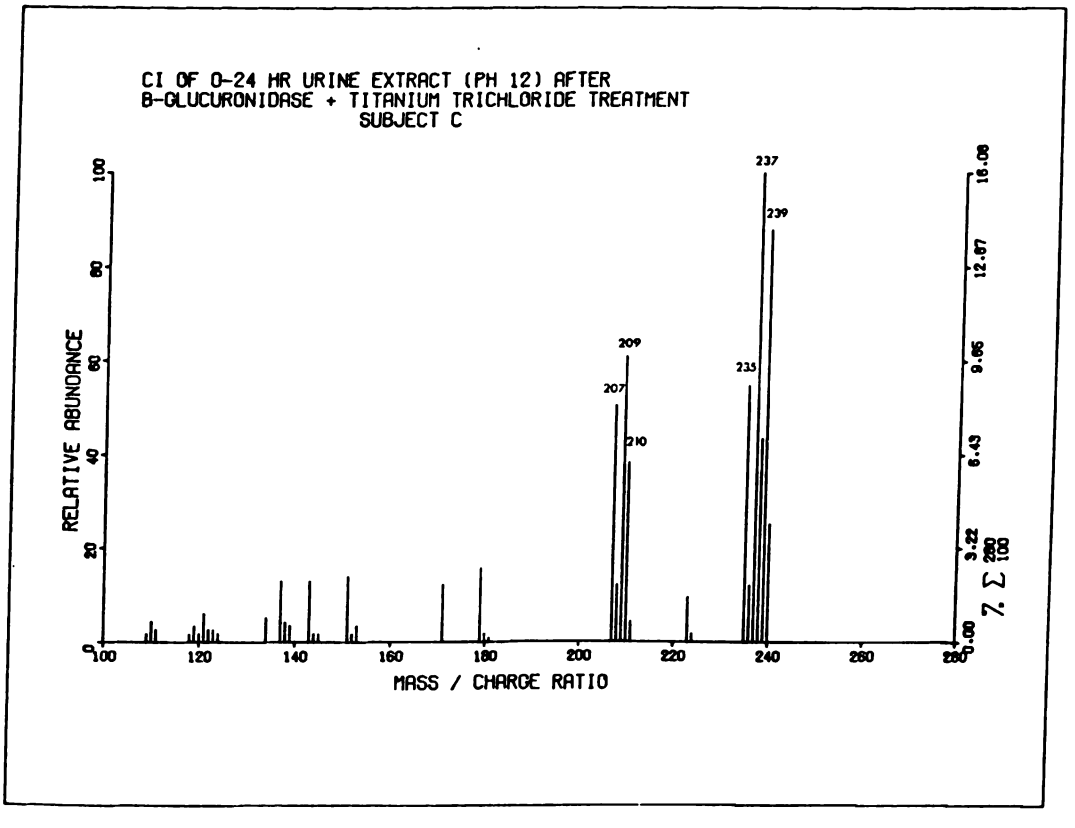

CI OF 0-24 HR URINE EXTRACT (PH 12) AFTER
TITANIUM TRICHLORIDE TREATMENT
SUBJECT C
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
237
209
239
202
210
235
100
120
140
160
180
200
220
240
260
280

Figure II-8. CI mass spectra of basified (pH 12) urine extracts (subject C) before and after $TiCl_3$ treatment of urine samples treated for 24 hours with β-glucuronidase-sulfatase at pH 5.0. The urine was collected under conditions of constant acidic urinary pH 5.0-5.3 as described in the text and experimental. The labeled ions are represented by metabolites and standards as described for Figures 6a and 6b.

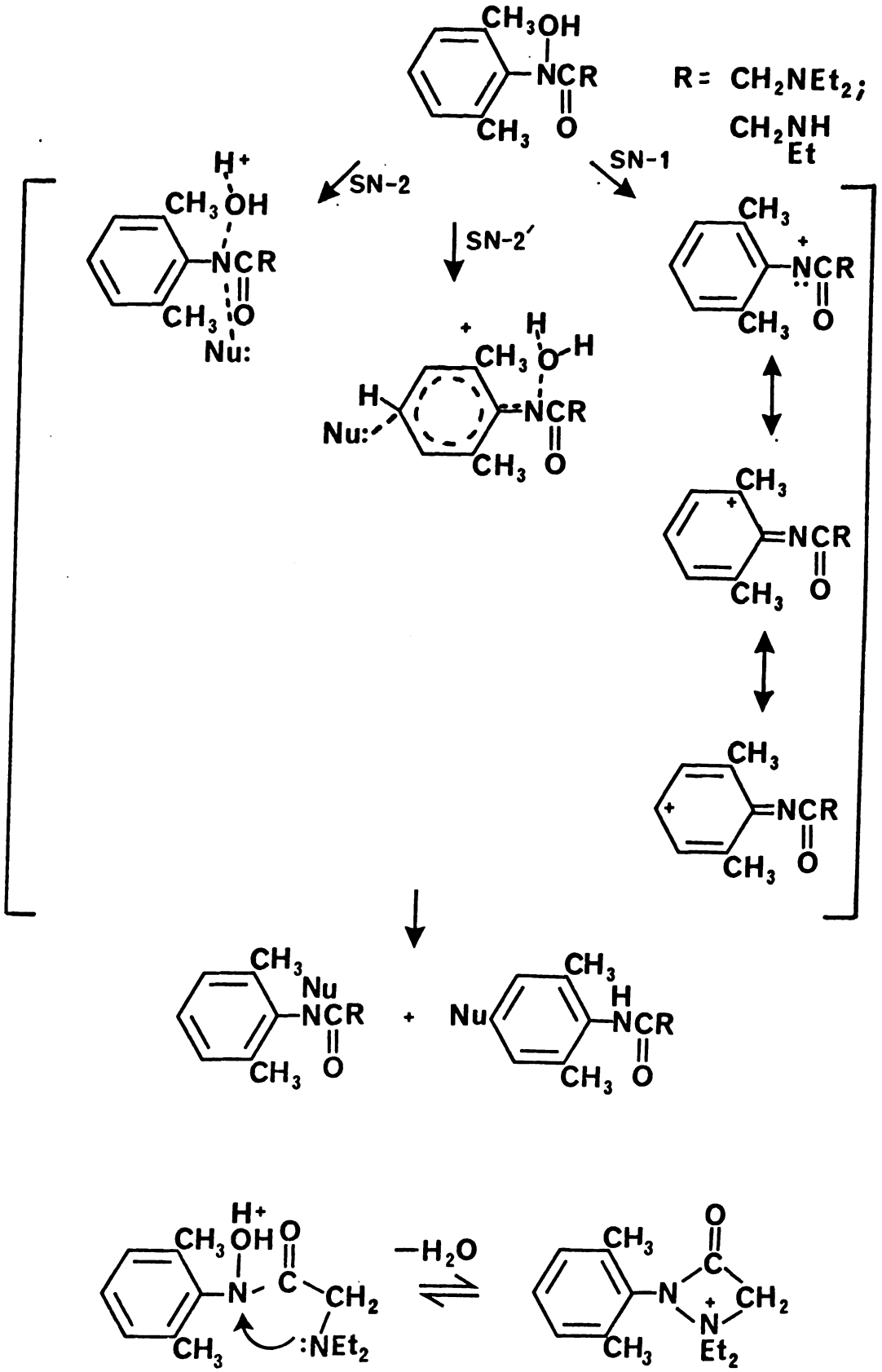

CI OF 0-24 HR URINE EXTRACT (PH 12)
AFTER B-GLUCURONIDASE
SUBJECT C
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
239
209
207
235
100
120
140
160
180
200
220
240
260
280
0
20
40
60
80
100
28.42
21.14
15.85
10.57
6.28
0.00
7. Σ 280 100


CI OF 0-24 HR URINE EXTRACT (PH 12) AFTER
B-GLUCURONIDASE + TITANIUM TRICHLORIDE TREATMENT
SUBJECT C
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
237
239
209
207
235
210
100
120
140
160
180
200
220
240
260
280
0
20
40
60
80
100
16.08
12.87
9.65
6.43
3.22
0.00
7. Σ 280 100

caused by reactions between cellular proteins and N-hydroxyacetaminophen when large doses of acetaminophen are given. However, smaller doses of acetominophen within therapeutic range (100-225 mg/Kg in mice) do not lead to necrosis even though the N-hydroxyamide is formed. Evidence was presented that glutathione, a sulfhydryl-containing polypeptide, protected against the necrosis by reacting as a nucleophile with the electrophilic N-hydroxyacetaminophen. When glutathione levels were depleted at higher doses (375 mg/Kg in mice) other tissue nucleophiles reacted with the metabolite and necrotic lesions formed.

Compared to N-hydroxyacetaminophen, N-hydroxylidocaine and N-hydroxyMEGX may be either more or less reactive towards cellular nucleophiles depending on the reaction mechanism. If the reaction involves rate-determining formation of an electrophilic radical or amidonium ion, the N-hydroxyamides of lidocaine and MEGX will be more reactive since the aryl methyl groups will help stabilize the intermediate and thus lower the activation energy for its formation. This intermediate (Scheme 4) can then be attacked by nucleophiles in an SN-1 or SN-1' reaction.[19] However, if the reaction involves bimolecular displacement (SN-2 reaction) the aryl methyl groups will sterically hinder attack at the amide nitrogen, although SN-2' attack can occur at the para position on the aromatic ring. Both N-hydroxyamide amines may also be more reactive if the amine group anchimerically assists departure of the N-hydroxyl group as shown. Future in vitro chemical and microsomal work with the N-hydroxyamides of lidocaine and MEGX should resolve these questions.

Scheme II-4. Possible reactions of N-hydroxylidocaine and N-hydroxyMEGX.


R = CH2NEt2; CH2NHEt
SN-1
SN-2
SN-2'
Nu:
-H2O

The basic question that still remains to be resolved in the case of lidocaine therapy is whether or not these N-hydroxyamide metabolites are formed in man at all. Sterically, because of the ortho-methyl groups, one might reason that enzymatic hydroxylation of the amide nitrogen might be more difficult compared to other aromatic amides. Our results in 3 human subjects indicate that very little, if any, of these metabolites can be found in human urine following oral lidocaine administration. These results were obtained using $TiCl_3$ treatment combined with a CIMS-stable isotope technique, and were confirmed using a tlc separation of the N-hydroxyamides and subsequent CIMS analysis. On the other hand, Mather and Thomas[4] have reported large amounts of $TiCl_3$ reducible material in human urine after oral lidocaine therapy using a more indirect $TiCl_3$ and glc analysis. One possible, but unlikely explanation, is variability from one set of subjects to another. Another suggestion is that the standard used by Mather and Thomas for glc quantification was affected by the $TiCl_3$ treatment, or that other material is formed during $TiCl_3$ treatment which has the same retention times on the glc column employed as lidocaine and MEGX. The CIMS-stable isotope technique averts all of these problems by using internal standards which are identical in all respects to the compounds being analyzed, except for the stable isotope, and the use of mass spectroscopy insures that only the compounds in question are being quantified.

Although our in vivo results indicate little, if any, of the N-hydroxyamides present in human urine following an oral dose of 250 mg

of lidocaine, these possible metabolites may be formed within the body and then react with cellular nucleophiles and/or form glutathione conjugates. Since lidocaine therapy in the management of ventricular arrhythmias may involve a total dose exceeding 5 grams in a 24-hour period,[25] the possibility of liver necrosis should be thoroughly investigated. The glutathione protective mechanism outlined for N-hydroxyacetaminophen may be saturated in man at such dosage levels if the metabolites are formed. Preliminary testing is now being undertaken through Dr. Elizabeth Weisburger at the National Cancer Institute, Bethesda, Md., using the N-hydroxylidocaine and N-hydroxyMEGX synthesized during the course of this research.

D. EXPERIMENTAL

1. Materials

General instrumentation was as previously described, Chapter I. Glc analyses were obtained on a Varian 2100 gas chromatograph equipped with flame ionization detectors using a 6 ft X 0.25 in X 2 mm i.d. glass U column packed with one of three packing materials; 2% Carbowax 20M on 80-100 mesh KOH-washed Chromosorb W, 3% SE-30 on 80-100 mesh A/W Chromosorb W, or 3% OV-17 on 80-100 mesh A/W Chromosorb W. Conditions employed were: column temperature, variable; injector 250°; detector 250°; N_2 carrier gas flow 40 ml/min; hydrogen and air adjusted to give maximum flame response; chart speed (Varian A-25 recorder) variable from 0.1-0.25 in/min.

2. Synthesis

a. Lidocaine N-Oxide

To an ice-bath cooled, magnetically stirred solution of lidocaine (2.34 g, 0.01 mole) in 20 ml methylene chloride, was added a cold solution of m-chloroperoxybenzoic acid (Aldrich, 85%, 2.23 g, 0.011 mole) in 30 ml methylene chloride. After a dropwise addition period of 30 min the reaction mixture was allowed to come to room temperature and stirred overnight. The reaction mixture was then poured through a 150 g basic alumina column and unreacted lidocaine eluted with 100 ml chloroform, followed by 200 ml 95:5 chloroform:methanol which yielded a pale yellow

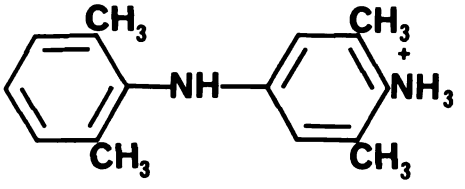
liquid after rotary evaporation of solvent. Recrystallization from 70:30 ether:methylene chloride in the cold gave 1.8 g of white hygroscopic crystalline solid, mp 105-107°; ir (KBr) 3100-3400 cm^{-1} (OH of hydrate and amide NH), 1690 cm^{-1} (amide C=O); nmr δ ($CDCl_3$, TMS) 1.37 [t, J = 7 Hz, $N(CH_2\underline{CH_3})_2$], 2.25 (s, aryl Me's), 2.92 (broad s, HNC = O and H_2O), 3.45 [q, J = 7 Hz, $N(\underline{CH_2}CH_3)_2$], 3.90 (s, $O{=}CCH_2N$) and 7.06 ppm (s, aromatics).

Anal for $C_{14}H_{22}N_2O_2 \cdot H_2O$, calcd: C 62.66, H 9.01, N 10.44; found: C 62.37, H 8.81, N 10.43.

b. 2,6-Dimethylphenylhydroxylamine

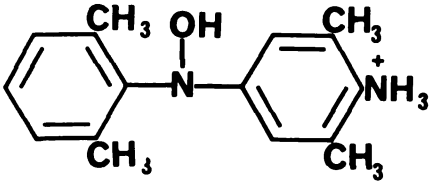
To a vigorously stirred solution of 2,6-dimethylnitrobenzene (Aldrich, 15.1 g, 0.1 mole) in 30 ml absolute ethanol, was added in one portion a solution of ammonium chloride (1 g) in 15 ml of water followed by small portions of zinc dust (Merck, 94%, 15.0 g). The temperature of the reaction mixture was maintained between 65 and 70° by the portion-wise addition of zinc dust until all had been added. The greyish-white slurry was then allowed to slowly cool to 55° with continued vigorous stirring. The warm mixture was suction-filtered and the filter-pak washed with 30 ml anhydrous ether. Rotary evaporation of the filtrate gave a pale yellow precipitate which was recrystallized twice from purified pet ether:benzene 1:1 to yield 12.2 g white needles, mp 103-104° (lit.[26] 99°); ir (KBr) 3300 cm^{-1} (NH stretch), 3200 cm^{-1} (OH) and 860 cm^{-1} (N-O stretch); nmr δ ($CDCl_3$, TMS) 2.35 (s, aryl Me's), 6.50 (broad s, NH and OH) and 7.05 ppm (s, aromatics).

CIMS showed that the MH^+ ion at m/e = 138 and its ^{13}C satellite represent 23.0% TIC, (MH^+-16) at m/e = 122 is 56.0% TIC, m/e 243 is 18.0% TIC, and m/e 259 is 3% TIC. The ion at m/e = 243 may result from the reaction of 2,6-dimethylaniline and 2,6-dimethylphenylhydroxylamine to give,



m/e **243**

or loss of oxygen from



m/e **259**

Anal for $C_8H_{11}NO$, calcd: C 70.04, H 8.08, N 10.21; found: C 69.95, H 8.11, N 10.04.

c. ω-Chloro-2,6-dimethylphenylacetohydroxamic acid or ω-Chloro-N-hydroxy-2,6-dimethylacetanilide

To a magnetically stirred 2-phase mixture of 2,6-dimethylphenylhydroxylamine (4.12 g, 0.03 mole) in 100 ml ether and sodium bicarbonate (2.80 g, 0.034 mole) in 12 ml water, cooled to -5° in an ice-salt bath, was added dropwise chloroacetyl chloride (Aldrich, 3.39 g, 0.03 mole) in 20 ml ether over a 30 min period. The reaction was stirred an additional 20 min at -5° and then poured into a separatory funnel and washed with two 50 ml portions of water. The pale yellow ether layer was filtered through anhydrous magnesium sulfate, evaporated

to a volume of 50 ml, and the product crystallized in the cold to yield 3.52 g white rhombic crystals, mp 138.5-140°; ir (KBr) 3200 cm^{-1} (OH), 1650 cm^{-1} (C=O); nmr δ ($CDCl_3$, TMS) 2.1 and 2.32 (s, aryl Me's), 3.76 and 4.41 (s, $O{=}CCH_2Cl$), 7.05 and 7.25 (m, aromatics) and 1.15 ppm (broad s, D_2O exchangeable OH). Integration indicated two isomers existing in a 1:2 ratio, presumably imino-enol: N-hydroxyamide.

Anal for $C_{10}H_{12}NO_2Cl$, calcd: C 56.21, H 5.66, N 6.56; found: C 56.33, H 5.60, N 6.51.

d. ω-Diethylamino-2,6-dimethylphenylacetohydroxamic acid or ω-Diethylamino-N-hydroxy-2,6-dimethylacetanilide or N-Hydroxylidocaine

A solution of ω-chloro-N-hydroxy-2,6-dimethylacetanilide (2.13 g, 0.01 mole) and diethylamine (Baker, 2.8 ml = 2.2 g, 0.03 mole) in 30 ml benzene was magnetically stirred for 4 hours at 65-70°. The orange-colored solution was cooled in ice and suction filtered to remove precipitated diethylamine hydrochloride, then extracted with three 5 ml portions of 10% HCl. The acid extracts were combined and carefully adjusted to pH 8.5 with 5N NaOH. The opaque mixture was back extracted with three 20 ml portions of purified chloroform, which were combined and washed with 10 ml of water and dried over anhydrous sodium sulfate. Rotary evaporation of solvent at room temperature yielded a pale yellow liquid which was recrystallized three times from purified hexane to yield 1.6 g small white needles, mp 81-82°; ir (CCl_4) 3250 cm^{-1} (OH), 1650

cm^{-1} (C=O); nmr δ ($CDCl_3$, TMS) 0.85 and 1.15 [t, J = 7 Hz, $N(CH_2\underline{CH_3})_2$], 2.30 and 2.35 (s, aryl Me's), 2.50-3.00 [overlapped q, J = 7 Hz, $N(\underline{CH_2}CH_3)_2$], 3.55 and 4.60 (s, $OCCH_2N$), and 7.10-7.20 ppm (m, aromatics plus OH exchangeable with D_2O). Integration indicated two isomers in a ratio of 1:1. High resolution EIMS calcd for $C_{14}H_{22}N_2O_2$, 250.16812; found 250.16873.

Anal for $C_{14}H_{22}N_2O_2$, calcd: C 67.17, H 8.86, N 11.19; found: C 67.40, H 8.70, N 11.30

The hydrochloride was made by adding the calculated amount of concentrated HCl to a solution of N-hydroxylidocaine in isopropanol and cooling the solution in a freezer to give small white crystals, mp 182-184°; ir (KBr) 3450 cm^{-1} (OH), 2700 cm^{-1} ($-N^+H$), 1665 cm^{-1} (C=O); nmr δ (Acetone-d_6, TMS) 1.45 [t, J = 7 Hz, $N(CH_2\underline{CH_3})_2$], 2.30 (s, aryl Me's), 3.55 [q, J = 7 Hz, $N(\underline{CH_2}CH_3)_2$], 4.09 (broad s, $-N^+H$ and OH), 4.72 (s, $O{=}CCH_2N$) and 7.23 ppm (s, aromatics).

e. ω-[α,α-d_4-Diethylamino]-N-hydroxy-2,6-dimethylacetanilide or N-Hydroxylidocaine-d_4

This deuterated analog was synthesized by the same procedure as the undeuterated compound using diethylamine-d_4 (Chapter I, p. 40) mp (HCl) 182-184°.

CIMS showed the following isotopic abundances: N-hydroxylidocaine-d_4 72.0% (m/e = 255), N-hydroxylidocaine-d_3 20.6%

(m/e = 254), N-hydroxylidocaine-d_2 5.9% (m/e = 253), N-hydroxylidocaine-d_1 1.5% (m/e = 252) and N-hydroxylidocaine 0% (m/e = 251).

f. ω-Ethylamino-2,6-dimethylphenylacetohydroxamic acid or ω-Ethylamino-N-hydroxy-2,6-dimethylacetanilide or N-HydroxyMEGX

A solution of ω-chloro-N-hydroxy-2,6-dimethylacetanilide (2.13 g, 0.01 mole) and anhydrous ethylamine (Eastman, 1.95 ml = 1.35 g, 0.03 mole) in 30 ml of purified p-dioxane was stirred for 4.5 hours at 50-55°. As the reaction proceeded a large amount of fine white precipitate formed. The slurry was cooled in ice and suction filtered to give a mixture of product and ethylamine hydrochloride (2.3 g, mp 70-102°). The precipitate was taken up in a 2:1 mixture of chloroform:pet ether and suction filtered to remove the ethylamine hydrochloride. The filtrate was then evaporated and the residue recrystallized twice from benzene to yield 1.4 g, mp 103-105°; ir (CCl_4) 3250 cm^{-1} (OH), 1650 cm^{-1} (C=O); nmr δ ($CDCl_3$, TMS) 1.05 and 1.17 (t, J = 7 Hz, $NCH_2\underline{CH_3}$), 2.15 and 2.20 (s, aryl Me's), 2.57 and 2.64 (q, J = 7 Hz, $N\underline{CH_2}CH_3$), 3.00 and 3.85 (s, $O{=}CCH_2N$), 5.95 (s, OH and NH exchangeable with D_2O) and 7.10 and 7.20 (s, aromatics). Integration indicated two isomers in an approximate ratio of 1:2 with the presumed N-hydroxy form predominant. High resolution EIMS calcd for $C_{12}H_{18}N_2O_2$, 222.13726; found, 222.13682.

Anal for $C_{12}H_{18}N_2O_2$, calcd: C 64.84, H 8.16, N 12.60; found C 64.75, H 8.41, N 12.62.

The hydrochloride was made by adding the calculated amount of concentrated HCl to a solution of the N-hydroxyMEGX in isopropanol and cooling the solution in a freezer to give small white crystals, mp 186-188°; ir (KBr) 3450 cm^{-1} (OH), 2700-2900 cm^{-1} ($-N^{+}H$), and 1665 cm^{-1} (C=O); nmr δ (Acetone-d_6, TMS) 1.40 (t, J = 7 Hz, $NCH_2\underline{CH_3}$), 2.35 (s, aryl Me's), 3.35 (q, J = 7 Hz, $N\underline{CH_2}CH_3$), 4.25 (broad s, $-N^{+}H$ and OH), 4.50 (s, $O{=}CCH_2N$), and 7.20 ppm (s, aromatics).

g. ω-[β-d_3-Ethylamine]-N-hydroxy-2,6-dimethylacetanilide or N-HydroxyMEGX-d_3

This deuterated analog was synthesized by the same procedure as the undeuterated compound using ethylamine-d_3 (Chapter I, p. 42), mp (HCl) 185-187°.

CIMS showed the following isotopic abundances: N-hydroxyMEGX-d_3 66.7% (m/e = 226), N-hydroxyMEGX-d_2 19.3% (m/e = 225), N-hydroxyMEGX-d_1 10.7% (m/e = 224) and N-hydroxyMEGX 3.3% (m/e = 223).

h. ω-[α-d_2-Ethylamino]-2,6-dimethylacetanilide or Lidocaine-d_2

This deuterated compound was synthesized in the same manner as lidocaine-d_4 using diethylamine-d_2 generated from N-ethylacetamide-d_2, mp 65-67°.

CIMS showed the following isotopic abundances: lidocaine-d_2 98.5% (m/e = 237), lidocaine-d_1 1.5% (m/e = 236) and lidocaine 0% (m/e = 235).

i. ω-Ethylamino-α-d_2-2,6-dimethylacetanilide or MEGX-d_2

This deuterated compound was synthesized by base catalyzed exchange of the methylene protons alpha to the amide carbonyl in D_2O. MEGX (206 mg, 1 mmole) was dissolved in 20 ml D_2O and heated at reflux for 12 hours with 50 mg anhydrous potassium carbonate. The mixture was then lyophilized and the exchange process repeated three times. After the final exchange the free base was extracted into 50 ml purified methylene chloride, washed with three 20 ml portions of distilled water and dried over anhydrous sodium sulfate. Rotary evaporation of solvent and recrystallization of the residue from hot hexane yielded 130 mg white needles, mp 50-51°; nmr showed the loss of the $O{=}CCH_2N$ resonance signal at 3.48 ppm when compared to the spectrum of MEGX.

CIMS showed the following isotopic abundances: MEGX-d_2 94.5% (m/e = 209), MEGX-d_1 5.5% (m/e = 208) and MEGX 0% (m/e = 207).

3. Studies with Lidocaine N-Oxide

a. Thermal Rearrangements

Solid lidocaine N-oxide (10 mg) was heated in a sealed tube for 15 minutes at 100° oil bath temperature. After cooling, the tube was broken and the residue subjected to tlc on Eastman #6060 Silica Gel GF plates using 70:20:10 cyclohexane:chloroform:methanol as the developing solvent. Short wave uv visualization indicated the presence of 6 distinct spots (rf A = .64, B = .53, C = .39, D = .25, E = .16 and F = .03);

4 reacted with ninhydrin spray to give pink colors (C, D, E, and F). Figure 1 shows that 3 of the spots co-chromatograph with the known compounds, lidocaine and/or cyclic metabonate, MEGX and lidocaine N-oxide.

Glc analysis of the mixture on 3% SE-30 at 150° showed 7 major peaks (Figure 1) with retention times of 1, 1.5, 2.5, 4, 5, 6.5, and 8.1 min. The peak with retention time of 4 min corresponds to MEGX and cyclic metabonate, while the peak at 5 min corresponds to lidocaine. Glc on 2% Carbowax at 160° shows 7 peaks with retention times of 1.5, 17, 18.5, 21.2, ~28, ~32, and 41 min. The peak at 17 min corresponds to cyclic metabonate, that at 21.2 min to lidocaine, and 41 min to MEGX.

b. Attempted Isolation of Lidocaine N-Oxide from Human and Rhesus Monkey Urine

To 100 ml of control human urine from a 0-24 hour urine collection, was added 1 ml of a solution containing 100 μg/ml of lidocaine N-oxide monohydrate. The sample was rotary evaporated at 20° (0.5 mm Hg) to a volume of 10 ml and extracted with 5 volumes of purified chloroform. Rotary evaporation of the chloroform yielded a residue which was chromatographed in 2 dimensions on a 20X20 cm silica gel GF plate, first using 70:20:10 cyclohexane:chloroform:methanol (rf of lidocaine N-oxide = 0.25), and secondly in 20:10:10:1 benzene:methanol:water: HOAc (rf of N-oxide = 0.82). A distinct uv absorbing spot corresponding to the rf value of the N-oxide could be detected in the urine extract.

This same procedure was followed using 100 ml of urine from a human subject who had received orally 500 mg of randomly tritiated lidocaine (50 uci),[27] and a Rhesus monkey after receiving a 100 mg i. v. dose of N-ethyl-^{14}-C-labeled lidocaine (6 uci).[28] No distinct uv spot could be seen after two-dimensional tlc of either extract, and no radioactivity above background could be determined by scintillation counting of an area from 0. 5 cm below to 0. 5 cm above the appropriate rf area. Repeating the experiment with the addition of 1 mg of cold lidocaine N-oxide to the urine revealed the lidocaine N-oxide under uv visualization of the developed tlc plates, but no radioactivity could be measured in the isolated spot.

4. Studies with 2, 6-Dimethylphenylhydroxylamine

a. Glc Analysis

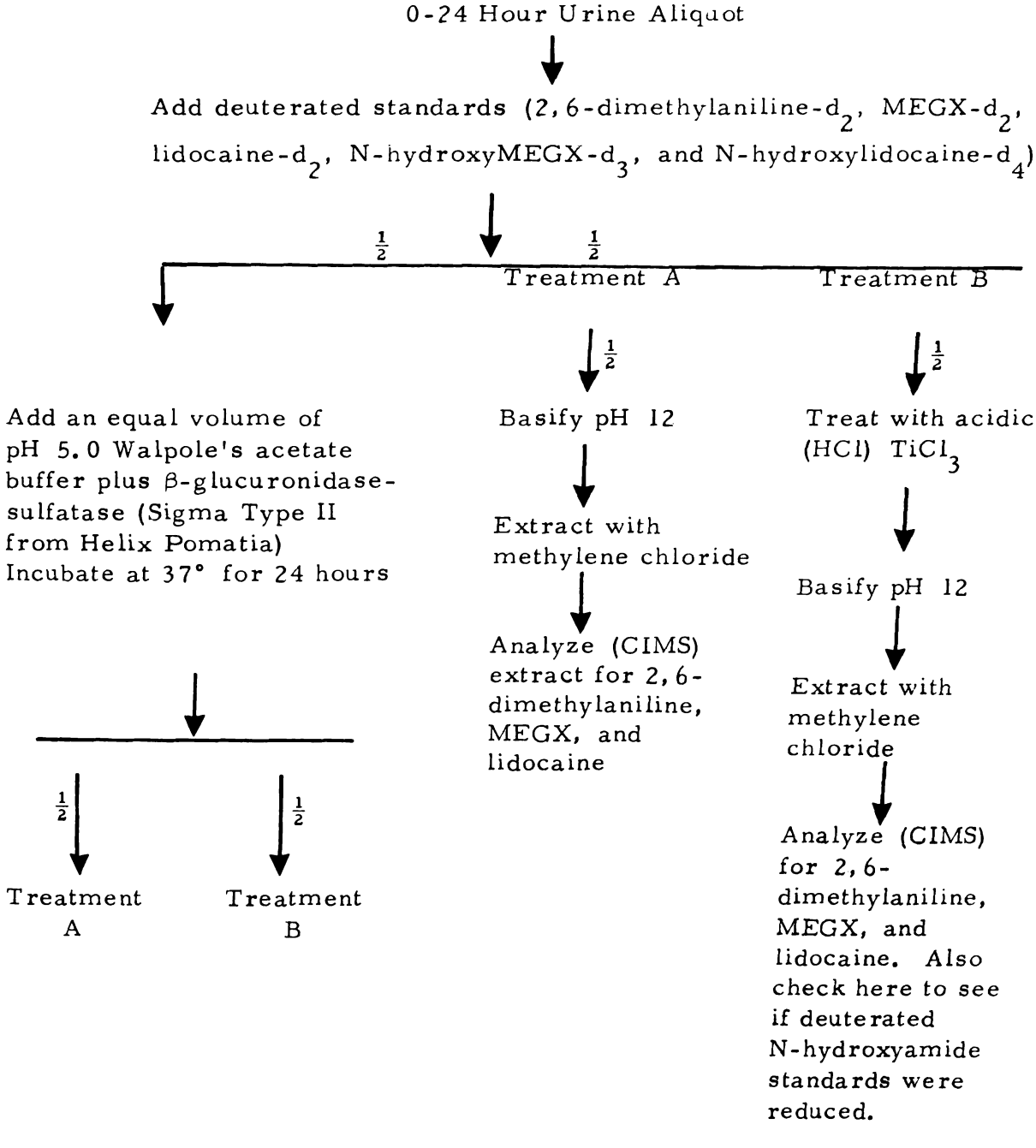
Glc analysis of the pure aromatic hydroxylamine on 3% OV-17 at 150° produced four peaks with retention times of 0. 8, 1. 2, 1. 6, and 4. 4 min. The major peak at 1. 2 min corresponds to 2, 6-dimethylaniline, and that at 4. 4 min corresponds to 2-amino-3-methyl-benzyl alcohol.

b. Analysis of 2, 6-Dimethylphenylhydroxylamine in Human Urine

(Refer to Scheme 5 for a flow diagram of the analysis procedure).

Fifty ml aliquots of the urine containing the deuterium standards from subjects A and B (0-24 hours after an oral dose of 250 mg of lidocaine) were analyzed indirectly for the hydroxylamine within 24 hours

Scheme II-5. Flow diagram for the indirect quantitative analysis of N-hydroxy compounds using a titanium trichloride ($TiCl_3$) reduction procedure combined with the CIMS-stable isotope dilution technique.



after collection. One half of each aliquot was extracted at pH 12.0 with an equal volume of ether and the ether dried over Drierite. The other half was treated in the dark, under nitrogen in a polyethylene glove bag (IRI, Cheltenham, Pa.) with a mixture of 2 ml of 20% $TiCl_3$ (Baker) plus 2 ml concentrated HCl in 6 ml water.[29] A sample of pure 2,6-dimethylphenylhydroxylamine (2.5 mg) in 25 ml of blank urine was treated in the same manner as a control.

All $TiCl_3$ treated samples were heated for 20 min at 50° in a water bath, then cooled and neutralized with the calculated amount of solid sodium hydroxide. The purple-black, semi-solid mixture was then basified to pH 12 with 5N NaOH and centrifuged in 50 ml pyrex culture tubes at 2500 rpm for 10 min. The supernatant was removed and shaken with an equal volume of ether and the extract dried over Drierite. Removal of solvent from each sample yielded a residue which was subjected to CIMS at 90° in methanolic HCl. Results showed no increase in the amounts of 2,6-dimethylaniline after $TiCl_3$ treatment.

5. Studies with N-Hydroxylidocaine and N-HydroxyMEGX

a. Reaction with $TiCl_3$ and Glc Analysis

To determine if the N-hydroxyamides were reducible with $TiCl_3$, separate solutions were made up of each of the N-hydroxyamides and their hydrochloride salts (2.5 mg in 10 ml water). Two ml of a second solution, consisting of 1 ml concentrated HCl, 5 ml water, and 6 ml 20% $TiCl_3$

solution, was added to each solution of N-hydroxyamide. The reaction sequence was carried out in a darkened room in a nitrogen-filled glove bag. After heating each solution for 10 minutes at 50°, the dark purple mixture was basified to pH 12 with 5N NaOH and extracted with 30 ml of ether. After drying (Drierite), rotary evaporation yielded a residue which was dissolved in 1 ml of methanol. One half of the residue was chromatographed on silica gel GF plates (Eastman #6060) using 70:20:10 cyclohexane:chloroform:methanol, and the other half was subjected to glc analysis on 3% OV-17. Both procedures showed that N-hydroxylidocaine and N-hydroxyMEGX had been quantitatively reduced to lidocaine and MEGX, respectively.

Glc analysis of pure N-hydroxylidocaine on 3% OV-17 at 185° showed 5 peaks with retention times of 1, 1.5, 2, 8.5, and 9.5 min. The major peak at 9.5 min corresponded to lidocaine. Glc analysis of N-hydroxyMEGX under identical conditions gave peaks at 1, 1.5, 2, 4.5, 7 and 8 min with the major peak at 8 min corresponding to MEGX.

b. Analysis of N-Hydroxyamides in Human Urine

(Refer to Scheme 5 for a flow diagram of the analysis procedure)

1) $TiCl_3$ Treatment and CIMS-Stable Isotope Dilution Analysis

Urine samples were collected from subjects A and B after receiving an oral dose of lidocaine (250 mg) as described in Chapter I, p. 57.. Immediately after collection, 1/20 of each sample was transferred to a bottle containing 0.2 mg lidocaine-d_2, 0.4 mg MEGX-d_2, 0.3 mg

N-hydroxylidocaine-d_4, and 0.3 mg N-hydroxyMEGX-d_3. Another subject (C) received ammonium chloride (5 g) in divided doses for 24 hours prior to ingesting a 250 mg dose of lidocaine, and throughout the 24-hour urine collection period after lidocaine dosage. Urine samples were collected 0-2, 2-4, 4-8, and 8-24 hours after dosage, with the pH ranging from 5.0-5.3 during this period. To 1/10 aliquots of this subject's urine was added 1.0 mg lidocaine-d_2, 1.6 mg MEGX-d_2, 1.2 mg N-hydroxylidocaine-d_4 and 1.6 mg N-hydroxyMEGX-d_3. One half of this aliquot was used for the $TiCl_3$ assay and the other half used for tlc separation as described in Part 2. All urine samples were stored under nitrogen in a freezer (-15°) and worked-up on the day of the final urine collection.

One fourth ($\frac{1}{4}$) of each aliquot was extracted with two volumes of methylene chloride after basifying to pH 12 with 5N NaOH. The extract was filtered through anhydrous sodium sulfate and rotary evaporated at 20° to yield a residue which was dissolved in 0.05 ml of ethanol and the CI mass spectrum determined at 120°. Another fourth of each aliquot was reacted with $TiCl_3$ under acidic conditions as described previously for 2,6-dimethylphenylhydroxylamine, and the supernatant from centrifugation extracted with two volumes of methylene chloride. Filtration of the extract through anhydrous sodium sulfate and rotary evaporation yielded a residue which was subjected to CIMS as described.

The remaining half of each aliquot was treated for 24 hours with 0.5 ml of β-glucuronidase-sulfatase in an equal volume of pH 5.0

Walpole's acetate buffer on a shaker-incubator (37°, 100 oscillations/min). After 12 hours an additional 0.5 ml of the enzyme preparation was added. Each hydrolyzed aliquot was then divided into two portions and treated exactly as described for the unhydrolyzed urine samples.

Ratios of the MH^+ ions for metabolites vs. deuterated standards were determined as previously described in Chapter I, p. 60. Results showed no increase in lidocaine or MEGX levels in subject B, while approximately a 5% increase could be detected in the samples from subjects A and C, although this approaches the limits of accuracy for this technique.

2) Tlc Separation of the N-Hydroxyamides from Human Urine

The other half of the 1/10 aliquot of the human urine collected under constant acidic urinary pH, containing deuterated standards, was divided into two portions and one portion treated as before with β-glucuronidase-sulfatase. Both portions were then extracted with 3 volumes of purified methylene chloride, and each of the combined extracts filtered through anhydrous sodium sulfate and rotary evaporated. The residues were taken up in 200 ul of purified methanol and chromatographed on 20X20 cm silica gel GF plates using 85:15 methylene chloride:acetone as developing system. The N-hydroxyamides are visible as pale blue spots under short wave uv. This system separates N-hydroxylidocaine (rf = 0.15) from m-hydroxylidocaine (rf = 0.35), although it does not separate N-hydroxyMEGX (rf = 0.08) from m-hydroxyMEGX (rf = 0.10). The

area containing the N-hydroxyamides was scraped off, eluted with purified methanol, and the methanol extract re-chromatographed on silica gel GF in 90:5:10 benzene:cyclohexane:methanol. In this system, N-hydroxylidocaine has an rf = 0.35, N-hydroxyMEGX an rf = 0.27, and m-hydroxyMEGX an rf = 0.16. The area corresponding to the N-hydroxyamides was scraped off and eluted with purified methanol. The residues from both extracts, before and after β-glucuronidase-sulfatase, were subjected to CIMS at 200°. The presence of the deuterated standards was confirmed by MH^+ ions at m/e = 255 (N-hydroxylidocaine-d_4) and m/e = 226 (N-hydroxyMEGX-d_3). No peaks corresponding to the MH^+ ions for possible N-hydroxyamide metabolites at m/e = 223 or m/e = 251 were found.

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CHAPTER III

A NEW METABOLITE OF LIDOCAINE; ITS POSSIBLE SIGNIFICANCE

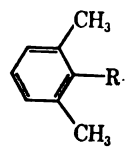
A. INTRODUCTION

In the course of a study concerning the biotransformation of lidocaine (1, Figure 1), Beck and Trager[1] discovered a new metabolite, N^1-ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (2=cyclic metabonate). Further investigation revealed the presence of a second new metabolite which was shown to be, N^1-ethyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (3).

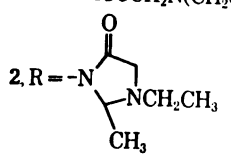
In a series of papers in 1960, Hollunger[2] demonstrated that in rabbit, liver enzymes were largely responsible for the biotransformation of lidocaine and that a key metabolite was MEGX (4). Later Beckett and Boyes[3] demonstrated that MEGX was a major biotransformation product of lidocaine in man and their report has been subsequently confirmed by work reported in this thesis and previously by others.[4]

Since MEGX is known to be a major metabolic product in man, it seemed possible that cyclic metabonate might be arising via an *in vivo* intermolecular condensation between MEGX and acetaldehyde, and 3 from condensation of MEGX with some one carbon fragment at the oxidation level of formaldehyde. The formation of cyclic metabonate might also be

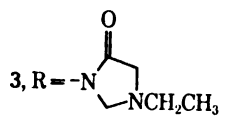
Figure III-1. Structures of compounds discussed in Chapter III.



1, R = NHCOCH$_2$N(CH$_2$CH$_3$)$_2$



2, R = -N(O)(CH$_2$CH$_3$)(CH$_3$)

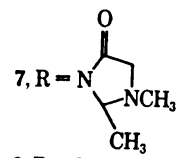


3, R = -N(O)(CH$_2$CH$_3$)

4, R = NHCOCH$_2$NHCH$_2$CH$_3$

5, R = NH$_2$

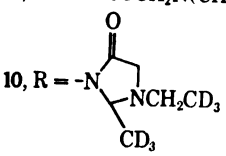
6, R = NHCOCH$_2$N(CH$_2$)$_4$



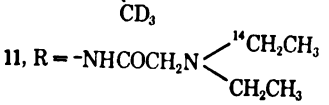
7, R = -N(O)(NCH$_3$)(CH$_3$)

8, R = NHCOCH$_2$NCH$_3$

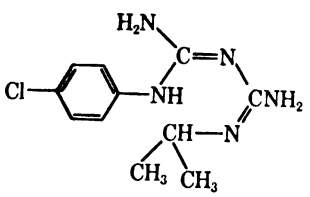
9, R = NHCOCH$_2$N(CH$_2$CD$_3$)$_2$



10, R = -N(O)(NCH$_2$CD$_3$)(CD$_3$)



11, R = -NHCOCH$_2$N(*CH$_2$CH$_3$)(CH$_2$CH$_3$)



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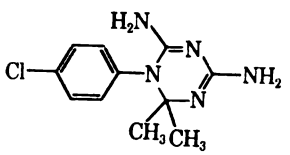

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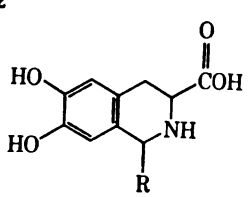


14, R = CH$_3$

15, R = H

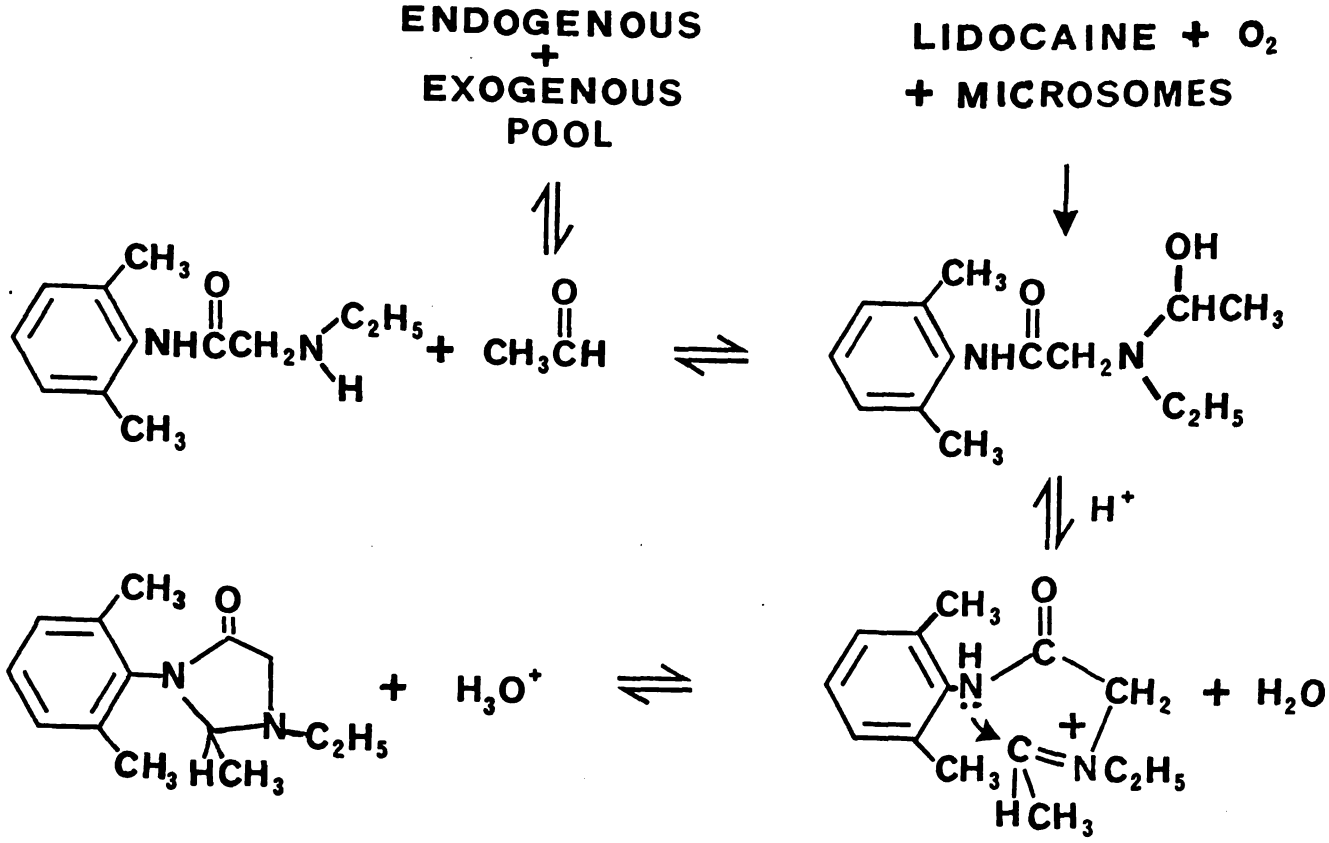
rationalized as proceeding via an intramolecular cyclization following initial oxidation.

The pathways available for the formation of cyclic metabonate are shown in Scheme 1. There is considerable evidence that enzymatic N-dealkylations proceed by way of a carbinolamine intermediate such as that shown, which then either spontaneously or enzymatically dissociates to an amine and an aldehyde.[5] Another possibility is acid catalyzed dehydration of the carbinolamine to an electrophilic imminium ion which, in the peculiar case of lidocaine, can react with the non-bonded lone pair on the amide nitrogen in a Mannich reaction[6] to produce the cyclic metabonate. Of major significance is that both pathways from carbinolamine intermediates are known to be operative chemically,[7] therefore there is every reason to believe that they are operative in a physiological situation. Thus, the generation of cyclic metabonate in vivo seemed a real phenomenon, one that might provide insight into mechanisms of oxidative amine metabolism.

To investigate the origin of this metabolite several experiments were conducted:

1. Since formation of cyclic metabonate may have occurred on an enzyme surface, an nmr investigation using an asymmetric pseudo-contact shift reagent was carried out on metabonate isolated from human urine to determine the possibility of chirality at the C-2 carbon atom.
2. A series of controls and labeling experiments was undertaken.

Scheme III-1. Proposed mechanism for the formation of cyclic metabonate (2).


ENDOGENOUS
+
EXOGENOUS
POOL
LIDOCAINE + O_2
+ MICROSOMES
CH_3
O
$NHCCH_2N$
C_2H_5
H
CH_3
+ CH_3CH
O
OH
$CHCH_3$
C_2H_5
H^+
N
$N-C_2H_5$
CH_3 HCH_3
+ H_3O^+
H
CH_2
+ H_2O
CH_3
NC_2H_5
HCH_3

3. Kinetic and equilibrium experiments were performed on the reaction of acetaldehyde with MEGX and exchange of acetaldehyde with the C-2 unit in cyclic metabonate.
4. In vitro experiments were carried out investigating the microsomal formation of cyclic metabonate.

As will be shown, the results of these experiments clearly demonstrate the ease of condensation reactions between MEGX and aldehydes, the complex nature of the reactions involved, and the care that must be exercised in interpreting the results of apparently simple processes.

B. INITIAL STUDIES

1. Isolation of Human Metabolites

After oral administration of 500 mg of randomly tritiated lidocaine to three normal human volunteers, urine and feces were collected, frozen and stored over a period of 72 hours.[1] No attempt was made to control urinary pH during these studies. Of the radioactivity present in the urine, 5-10% could be accounted for as extractable organic bases while the total radioactivity present represented 50% of the administered dose. Homogenization of the feces followed by oxidation of an aliquot and scintillation counting indicated that this route of elimination is of minor importance for lidocaine and its metabolites. Hence, at this point the feces were discarded. Since most of the radioactivity appeared in the urine 8 hours after administration, this sample was selected for initial investigation and isolation of the organic bases present. Glc separated the base isolate

into five major components. Each component was collected from glc via a stream splitter and saved for analysis.

2. Mass Spectroscopy

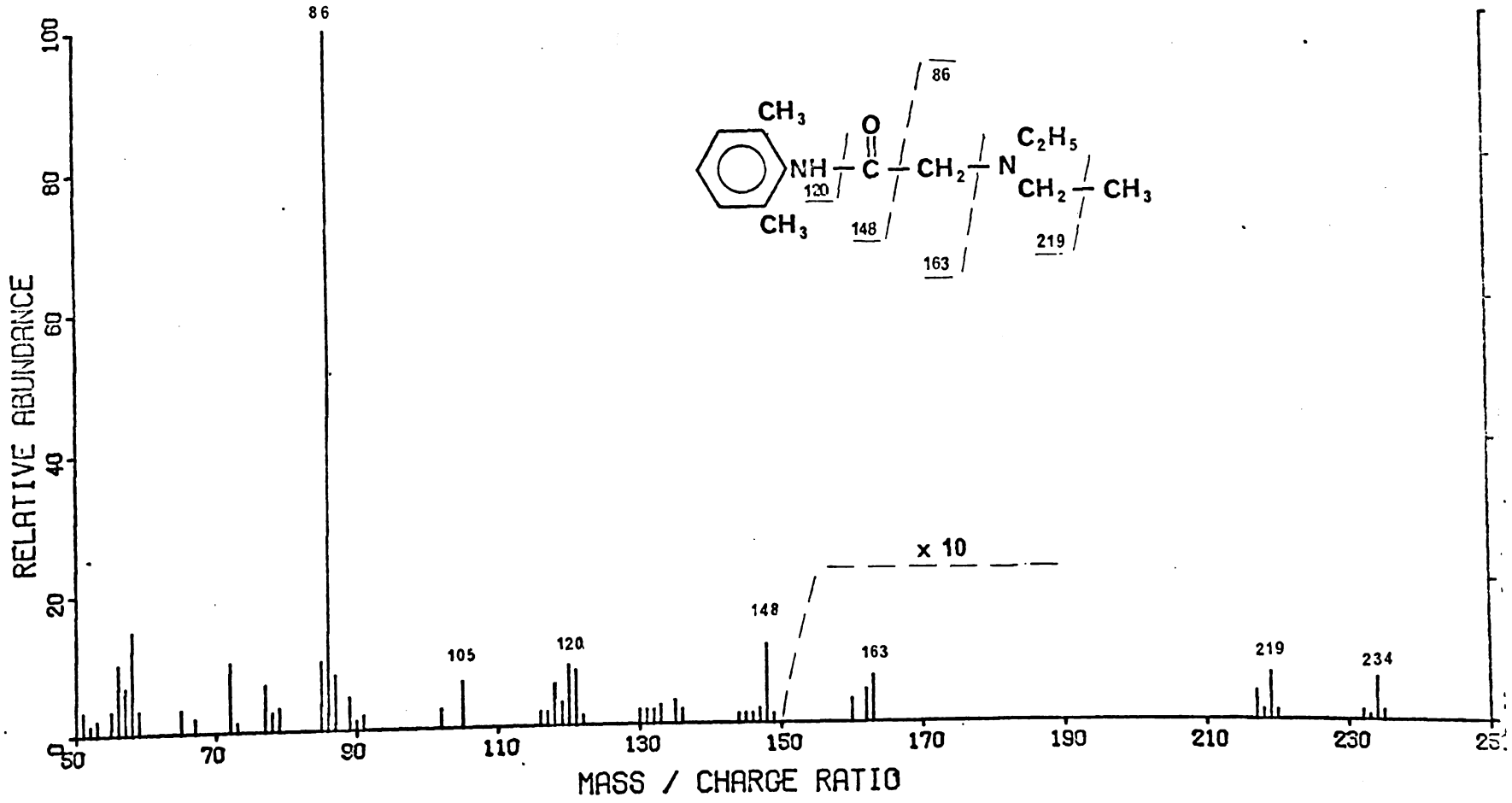
To establish a capability for elucidating the structure of any unknown metabolite, a mass spectral study was undertaken of the behavior of lidocaine and one of its known metabolites, MEGX. The EI mass spectrum of lidocaine is reproduced in Figure 2. The major fragmentation routes are depicted in Scheme 2 and are supported by exact mass measurement and metastable scanning. As expected, both compounds fragmented primarily by homolytic cleavage of the carbon-carbon bond between the carbonyl and methylene groups to generate imminium ions as the base peak ions[8a] at m/e = 86 ($C_5H_{12}N$) and m/e = 58 (C_3H_8N) for lidocaine and MEGX, respectively. Exact mass measurement of the ions at m/e = 148, 120 and 105 are consistent with the structures depicted in Scheme 2, and as such represent the other half of the molecule. Therefore it appeared that metabolic transformations could be detected by changes in the atomic composition of these ions.

3. Identification of Metabolites and Proof of Structure

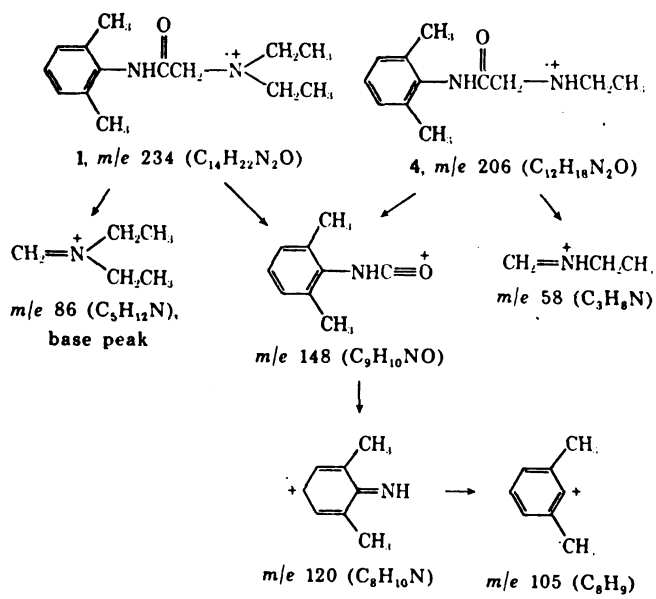
Of the five major radioactive bases isolated, three proved to be known metabolites. These were lidocaine, MEGX, and 2,6-dimethylaniline (5). These structures were confirmed by retention times on two different glc systems, isolation of small quantities of each by glc using a stream splitter, scintillation counting and high resolution mass spectrometry.

Figure III-2. EI mass spectrum of lidocaine.

LIDOCAINE EI



Scheme III-2. Mass spectral fragmentation patterns for lidocaine and MEGX.

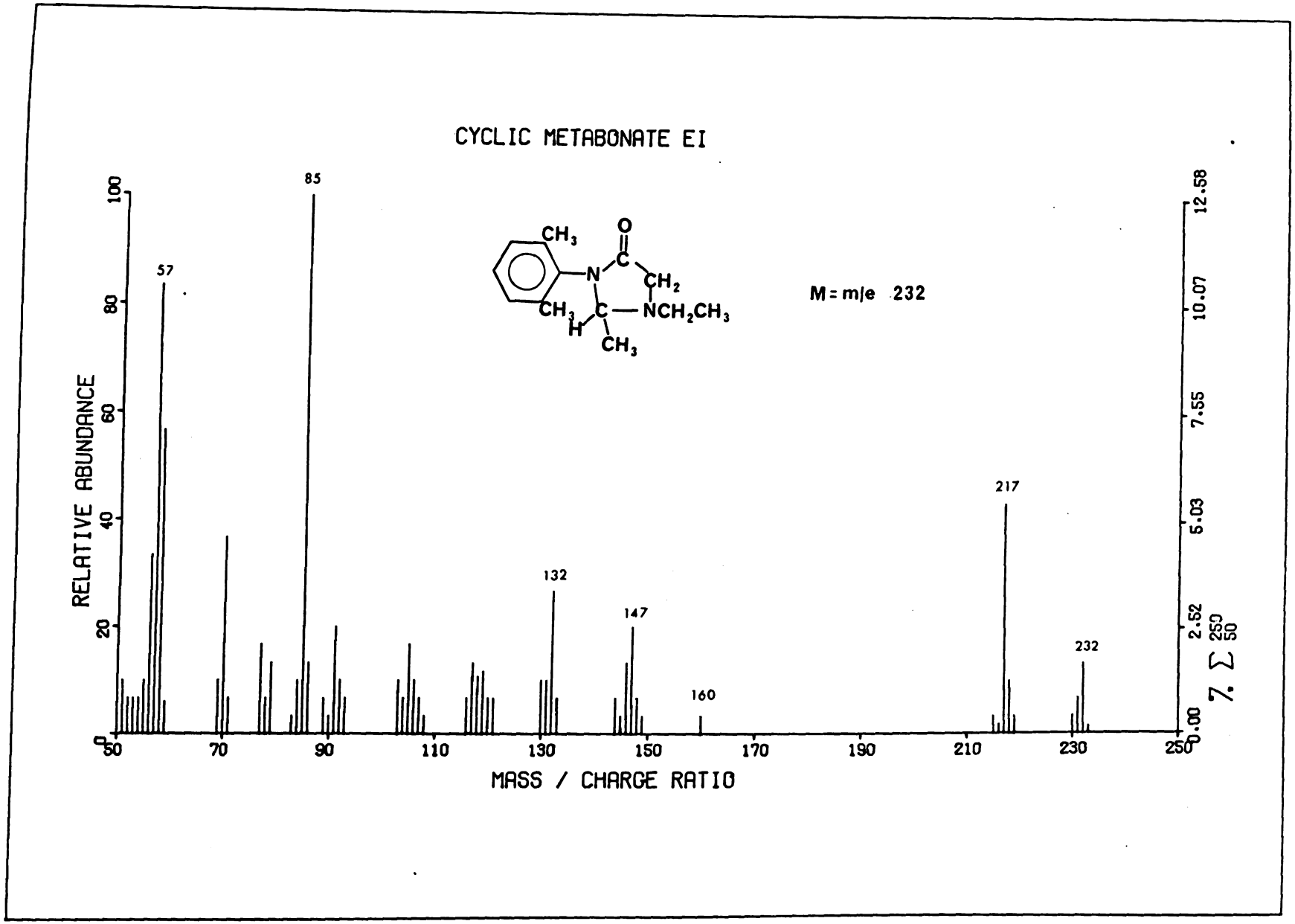

1, m/e 234 (C14H22N2O)
4, m/e 206 (C12H18N2O)
m/e 86 (C5H12N), base peak
m/e 148 (C9H10NO)
m/e 58 (C3H8N)
m/e 120 (C8H10N)
m/e 105 (C8H9)

The remaining two peaks were unknown, although collection and scintillation counting confirmed that they were lidocaine derived. One of these, the largest of all five peaks, was subjected to mass spectrometry and was found to have a parent ion at m/e = 232 as shown in the normalized EI spectrum, Figure 3. This suggested that the unknown contained one additional unit of unsaturation. Somewhat surprisingly, the base peak ion in the spectrum of this material occurred at m/e = 85. If the diethylaminomethylene portion of the molecule were intact, the compound should still give a base peak at m/e = 86. However, if cyclization to a substituted pyrrolidine such as 6 (Figure 1) or some isomeric substance had occurred, the base peak ion should be at m/e = 84.

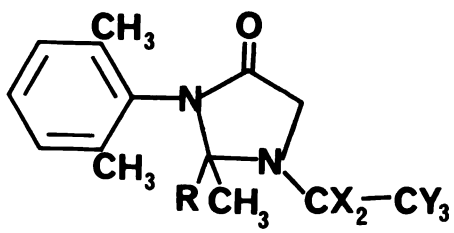
On the basis of chemical stability and the mass spectrum, the imidazolidinone 2 was considered a likely possibility. The base peak at m/e = 85 can be rationalized mechanistically as shown in Scheme 3. This and the other possible fragmentation routes are supported by the EI mass spectra of various specifically deuterated analogs of the cyclic metabonate as illustrated. The driving force for the fragmentation is presumably the generation of a neutral isocyanate and allylic radical cation.[8b] Certainly, this is a more reasonable process than generating a base peak ion at m/e = 86 or m/e = 84 from such a molecule. Further, cyclic metabonate (2) was an attractive possibility because its formation could be rationalized as proceeding through a well established metabolic pathway, that of oxidative N-dealkylation.

Compounds similar to the cyclic metabonate have been prepared by the condensation of an aldehyde with a suitable secondary amine.[9]

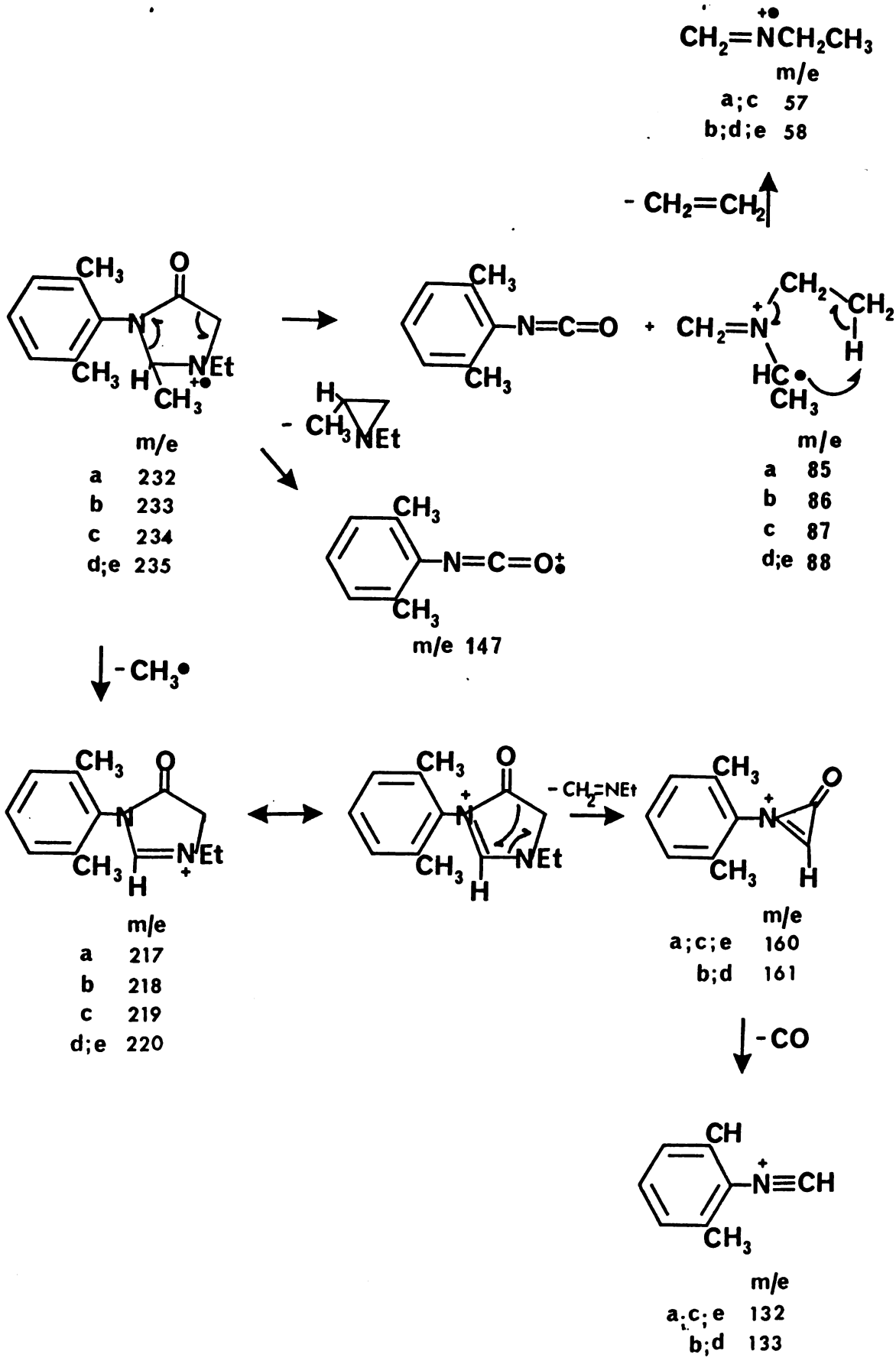
Figure III-3. EI mass spectrum of the cyclic metabonate (2).


CYCLIC METABONATE EI
M = m/e 232
RELATIVE ABUNDANCE
MASS / CHARGE RATIO
57
85
132
147
160
217
232
0
20
40
60
80
100
50
70
90
110
130
150
170
190
210
230
250
0.00
2.52
5.03
7.55
10.07
12.58
% Σ 250 50

Scheme III-3. Possible EI mass spectral fragmentation routes for cyclic metabonate (2).



	R	X	Y
a	H	H	H
b	D	H	H
c	H	D	H
d	D	D	H
e	H	H	D


CH2=N+•CH2CH3
m/e
a;c 57
b;d;e 58
- CH2=CH2
m/e
a 232
b 233
c 234
d;e 235
- CH3•
m/e 147
m/e
a 85
b 86
c 87
d;e 88
m/e
a 217
b 218
c 219
d;e 220
- CH2=NEt
m/e
a;c;e 160
b;d 161
- CO
m/e
a;c;e 132
b;d 133

Hence, acetaldehyde was condensed with MEGX. The reaction mixture was separated by glc and found to contain two peaks distinct from starting material. The major peak had a retention time identical to the unknown and was purified by further recrystallization. Admixture of the purified synthetic material to the metabolite and recrystallization to constant specific activity confirmed the assigned structure.

The nmr of the cyclic metabonate and one of its deuterated analogs is shown in Figure 4. The stereo-picture shows a proposed preferred conformation of one enantiomer of the cyclic metabonate based on an analysis of its nmr spectrum and the spectra of its deuterated analogs (a, b and d as shown in Scheme 3). Some interesting conclusions of the study include the following:

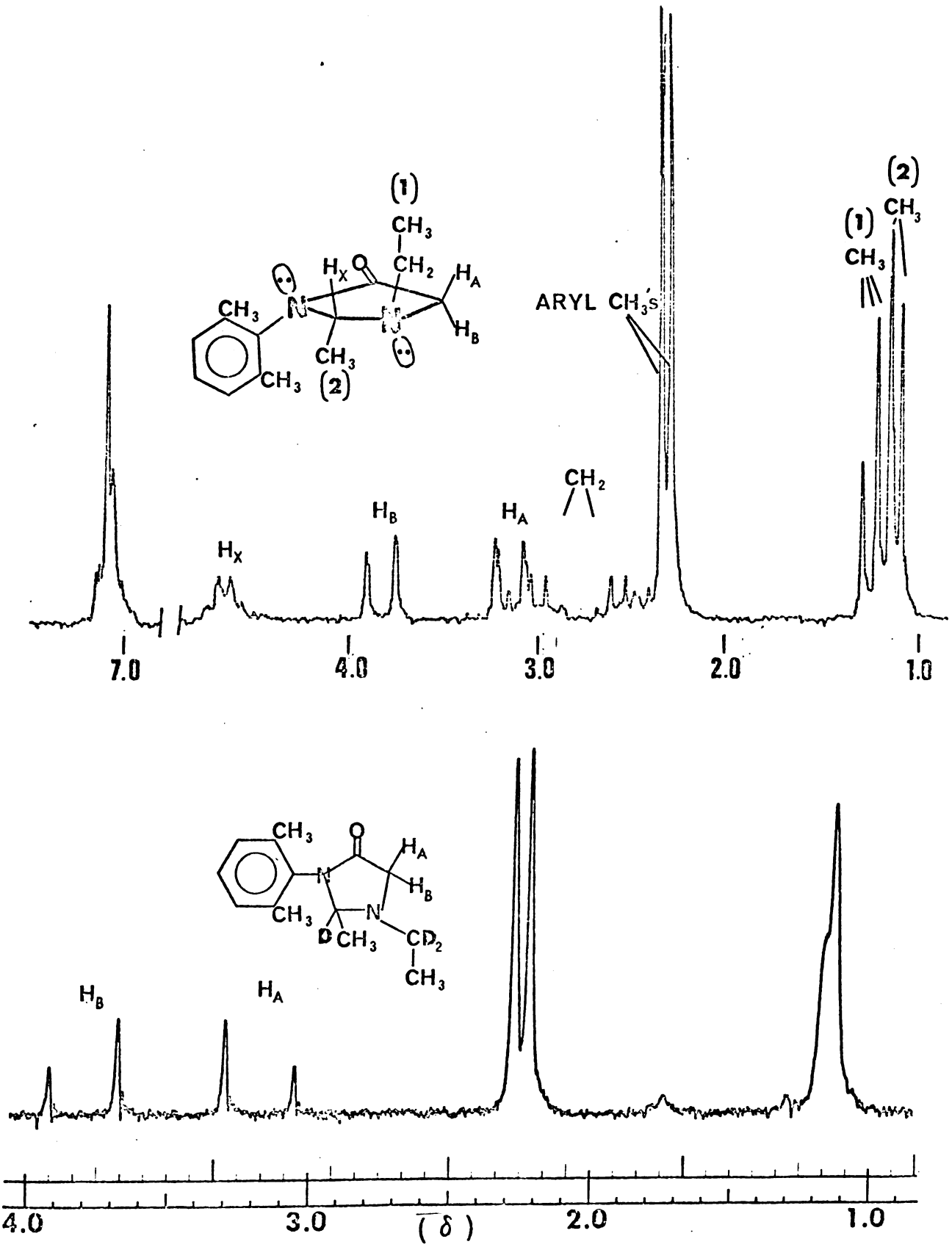
1) There are different average chemical shifts for the methylene protons of the N-ethyl side chain (2.47 and 2.97 ppm) leading to octuplets with J_{gem} = 14.0 Hz and J_{Me} = 5.5 Hz. This indicates that these two hydrogens never have the same average magnetic environment.

2) There are different average chemical shifts for the C-2 hydrogens of the ring methylene group (3.20 and 3.70 ppm). The downfield shift of one proton (H_b) is probably caused by deshielding from the N^1-amino group lone pair.[10a]

3) The two ring methylene hydrogens show interesting secondary splitting besides the obvious geminal coupling. This splitting is lost when deuterium is introduced into the C-2 ring

Figure III-4a. Nmr spectrum of cyclic metabonate (2) (100 MHz, $CDCl_3$, TMS).

Figure III-4b. Nmr spectrum of cyclic metabonate-d_3 (see structure 2d in Scheme 3) (60 MHz, $CDCl_3$, TMS).


(1)
CH3
CH2
HX
HA
HB
CH3
CH3
CH3
(2)
ARYL CH3's
(2)
CH3
(1)
CH3
CH2
HB
HA
HX
7.0
4.0
3.0
2.0
1.0
CH3
O
HA
HB
CH3
N
CH3
CD2
CH3
HB
HA
4.0
3.0
(δ)
2.0
1.0

methine carbon. This indicates that the secondary splitting in ring methylene hydrogens is due to long-range coupling from the ring methine hydrogen. It is noteworthy that the coupling is greater (1.5 Hz compared to 0.5 Hz) for that methylene proton (H_a) cis to the methine proton (H_x) which thus obeys the W-rule.[10b] This assignment also agrees with the proposed trans relationship between the C-2 methyl group and N^1-ethyl group in the favored conformation, since it is the downfield ring methylene hydrogen (H_b) which has the smallest long range coupling constant. This proton has a cis relationship to the nitrogen lone pair leading to the downfield shift, and trans relationship to the ring methine hydrogen (H_x) leading to the small long range coupling.

These nmr characteristics combined with the mass spectral and glc properties are consistent with the assigned structure 2 for the cyclic metabonate.

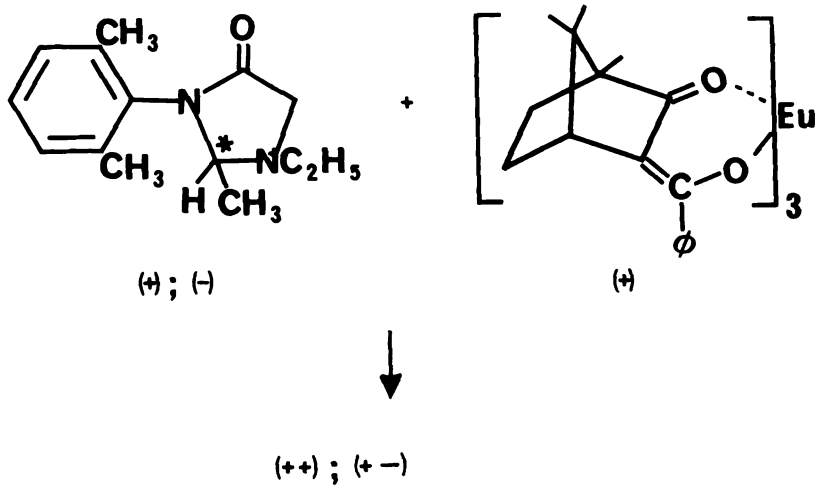
The last of the five major peaks that was still unidentified was subjected to mass spectroscopy. The molecular ion occurred at m/e = 218, while the base peak ion occurred at m/e = 71. The similarity in behavior of this material upon electron impact to that displayed by the cyclic metabonate immediately suggested either 3 or 7 as possible structures for the metabolite. Both were synthesized, 3 by the condensation of MEGX with formaldehyde, and 7 by a similar condensation of ω-methylamino-2,6-dimethylacetanilide (8) with acetaldehyde. Mass

fragmentation patterns and glc retention times indicated that the behavior of the metabolite was identical to 3, but different from 7. Admixture of the synthetic material to the urinary metabolite and recrystallization to constant specific activity established the structure as 3.

C. THE USE OF AN ASYMMETRIC EUROPIUM SHIFT REAGENT TO DETERMINE POSSIBLE CHIRALITY IN A METABOLITE OF LIDOCAINE

Since their development just a few years ago, lanthanide shift reagents have been extensively used for nmr structural investigations of organic molecules. More recently, the asymmetric shift reagents have been used to determine enantiomeric purity,[11] and we have extended this use to include the determination of possible chirality in a metabolite of lidocaine isolated from human urine.

Although lidocaine itself possesses no asymmetric centers, the cyclic metabonate contains an asymmetric carbon atom (C-2) as shown (*). Reaction of the synthetic racemic metabolite with the pseudo-contact shift reagent, tris-[3-(benzylhydroxymethylene)-d-camphorato] europium (III),[12] should produce two diastereomeric complexes in equal proportions as illustrated. Hopefully these complexes have different magnetic environments.

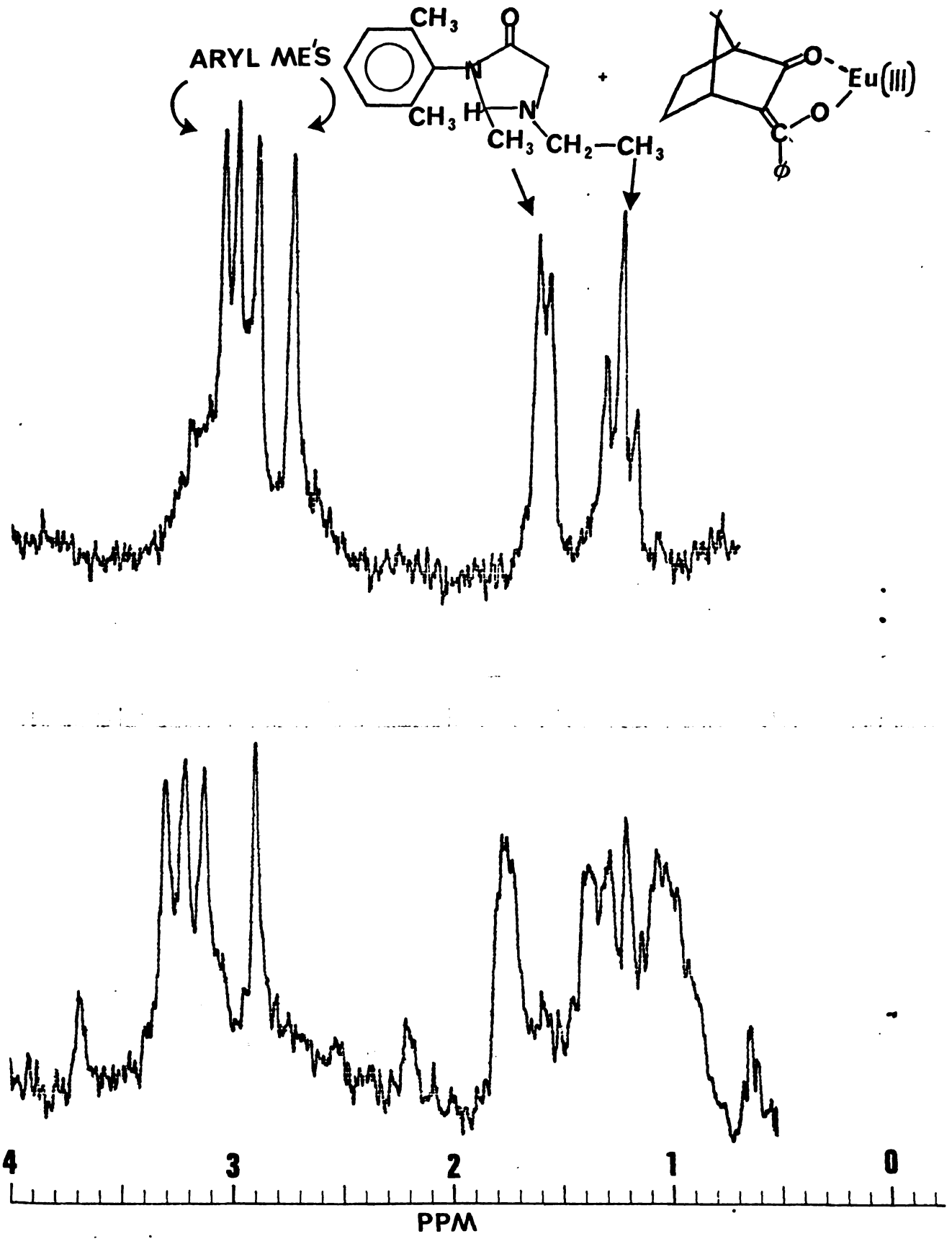


The interaction of a pseudo-contact shift reagent with a substrate has been shown to alter chemical shifts by formation of complexes in which there is a perturbation of electron spin density via magnetic interaction.[11] Since this formation is an equilibrium phenomena, common practice is to successively add known amounts of the reagent to a solution of the compound and record the nmr spectrum after each addition. Spectra were run (100 MHz) of synthetic cyclic metabonate (12 mg in 0.5 ml CCl_4, ~0.1 M) using from 0-50 mg of the shift reagent in 10 mg increments. The most distinct observable change in the spectrum (Figure 5a) was the downfield shift of the aryl methyl groups originally appearing as two singlets at 2.21 and 2.26 ppm. After the addition of 30 mg (~0.06 M) of the asymmetric shift reagent, two pairs of singlets could be seen due to the two diastereoisomers formed from the cyclic metabonate enantiomers and the chiral shift reagent. Resonance absorption occurred at 2.72, 2.87, 2.97 and 3.02 ppm as can be seen. Another noticeable change in the spectrum is the downfield shift of the C-2 methyl group doublet with respect to the N^1-$CH_2\underline{CH}_3$ triplet.

Approximately 5-7 mg of reasonably pure cyclic metabonate was isolated by ether extraction (pH 12) and tlc from 5 litres of urine. The urine represented aliquots of the combined output from three human subjects orally dosed with 500 mg randomly tritiated lidocaine.[1] The 100 MHz nmr spectrum of the scan containing 20 mg of the shift reagent is almost identical with respect to the aryl methyl group resonances (Figure

Figure III-5a. Nmr spectrum of synthetic cyclic metabonate (12 mg) + asymmetric shift reagent (30 mg) taken in 0.5 ml CCl_4 (100 MHz, TMS).

Figure III-5b. Nmr spectrum of metabolite 2 (5-7 mg) isolated from human urine. The spectrum was taken in 0.5 ml CCl_4 + asymmetric shift reagent (20 mg) (100 MHz, TMS).


ARYL ME'S
CH3
O
N
CH3 H N
CH3 CH2—CH3
O
Eu(III)
O
C
ϕ
4
3
2
1
0
PPM

5b) as that of the synthetic compound, showing that the isolated metabonate was a racemate.

D. CONTROLS AND LABELING EXPERIMENTS

1. Initial Monkey Studies

To investigate the genesis of the cyclic metabolites by utilization of isotopically labeled materials it was necessary to have an animal model that would emulate the fate of the drug in man. To this end the experiments were repeated in the Rhesus monkey. The monkey was administered an isotonic solution of lidocaine and the urine collected and worked up in a fashion identical to that which had been used previously.[1] Under these conditions both 2 and 3 were isolated and identified.

Since the experiments were reproducible in the monkey, attempts were made to determine whether these apparent metabolites were in fact artifacts. The solvents, either ether and/or methylene chloride, that had been used for extractions, were spectral grade and reputedly contained less than 0.001% aldehyde impurities. Both solvents were meticulously purified and freed of aldehydes, using the method outlined by Schwartz and Parks.[13]

2. Control Experiments

Extraction of samples of both human and monkey urine after treatment with lidocaine was accomplished using "unpurified" and purified spectral solvents for extraction. The results of these experiments indicated

that most, if not all, of 2 and 3 was due to reaction between MEGX and the aldehydes contaminating the solvent (Figure 6a). However, glc peaks corresponding to both 2 and 3 could consistently be detected (Figure 6b) from the control experiment. Conversely, if lidocaine was added to the urine and extracted with ether these peaks could not be detected (Figure 6c).

These data imply that 2 and 3 result from either residual contamination in the purified solvents or that they arise from reaction with minute quantities of aldehydes present in urine. To investigate these possibilities, MEGX was allowed to sit in purified ether for 24 hours. The ether was evaporated and the residue analyzed by glc. No cyclic metabolites could be detected. The control experiments were repeated by adding MEGX to both monkey and human urine. The urines were allowed to sit for 48 hours, each was divided into four aliquots, and each aliquot was separately extracted with purified ether, methylene chloride, chloroform, and carbon tetrachloride. In all cases, both cyclic metabolites could be detected on analysis and the results from all four experiments were identical within experimental error.

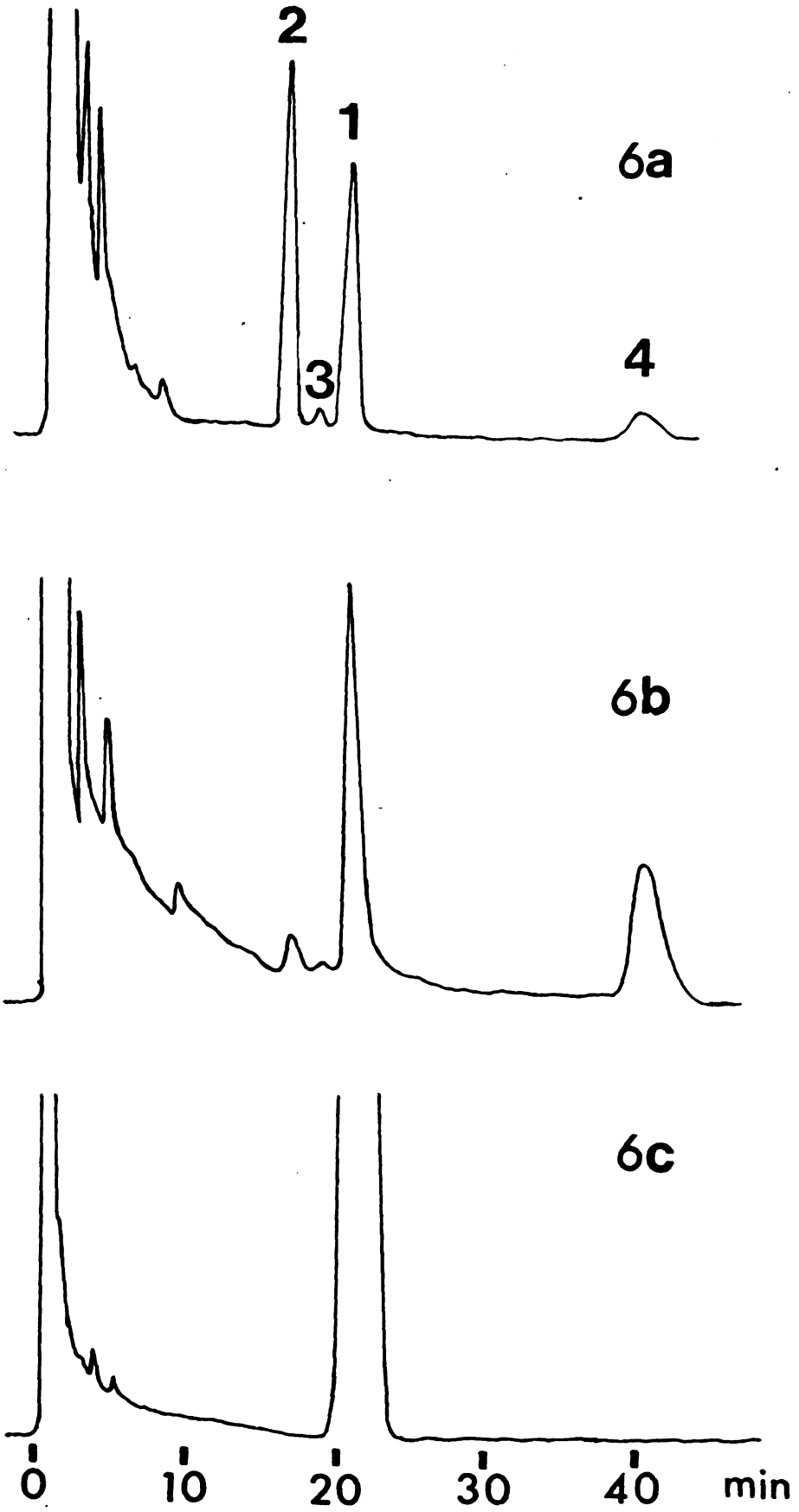
Since both metabolites could be detected in urine after using purified solvents, it seemed possible that at least some small portion of these materials could be arising *in vivo* even though there were no readily apparent differences in concentration between the results of these experiments and the controls.

Figure III-6. Glc trace of the basic metabolites isolated from

a) human urine extracted with unpurified ether

b) human urine extracted with purified ether

c) lidocaine added to urine and extracted with purified ether.


2
1
6a
4
3
6b
6c
0
10
20
30
40
min

3. Labeling Experiments Under Normal Physiological Conditions

In order to determine if some small quantity of cyclic metabonate was arising via an in vivo intramolecular process, we synthesized specifically labeled lidocaine-d_6 (9) and administered it to the monkey. The assumption was made that if any cyclic metabonate was being generated by an intramolecular process, then a parent ion at m/e = 238 should be detectable in its mass spectrum even if 10 (refer to Figure 1) was only a small fraction of the isolated material. To ease the isolation problems, unpurified methylene chloride was used for extraction, the metabolites isolated, and the mass spectrum determined. No ion at m/e = 238 could be detected, although a large parent ion was found at m/e = 235.

In a similar experiment, lidocaine containing ^{14}C in the methylene group of one of the N-ethyl groups (11) was synthesized. After determining the specific activity, it was administered to a Rhesus monkey and the experimental protocol described above was repeated. The cyclic metabolites 2 and 3 were isolated and their specific activities were determined. Within experimental error, the specific activity had fallen to 50% of the administered lidocaine. Clearly, this result is consistent with the deuterium experiment. Unfortunately, these experiments do not exclude the possibility of the labels being lost via equilibrium with aldehydes present in plasma, urine, and/or solvent. In fact, the following experiment, in which purified solvents were used, demonstrates that the aldehydes present in urine are sufficient to equilibrate with cyclic metabonate and MEGX.

Doubly-labeled MEGX, containing ^{14}C in the carbonyl group and tritium in the aromatic ring, of known specific activity was added to an 8 hour urine collection from a human volunteer who had received lidocaine, and the urine was allowed to stand for 12 hours. The urine was processed as previously described, and then extracted with purified solvents. The isolated cyclic metabonate (2) as well as labeled MEGX now had the same specific activity, although it was different from and less than the starting specific activity. This result implies that cyclic metabonate and MEGX must be in equilibrium, and the rate of equilibrium probably limits the possibility of detecting an intramolecular process.

The reaction appeared efficient enough to scavenge minute quantities of aldehydes under very mild conditions. To investigate just how efficient the reaction might be, 1 μg/ml of MEGX was allowed to react with 1μg/ml of acetaldehyde in a phosphate buffer at room temperature. The reaction mixture was concentrated after removing excess aldehyde and injected directly into a gas chromatograph. The resultant glc trace indicated a 20% conversion of MEGX to cyclic metabonate under these conditions. This suggested that if the in vivo concentration of acetaldehyde could be increased the formation of the metabolite might be induced.

4. Labeling Experiments With Concomitant Administration of Ethanol

To determine if the formation of cyclic metabonate could be induced, ethanol-d_5 was infused into the monkey along with lidocaine in phosphate buffer. The urine was collected and processed with purified solvents as

described previously. A glc trace indicated that the peak attributable to cyclic metabonate from the alcohol experiment was approximately 25 times as great as that obtained from a simultaneously run control. The metabolite was collected and subjected to mass spectroscopy, and was found to have 39% containing one or more atoms of deuterium. This result is based on the abundance of the M+1, M+2 and M+3 ions minus the contribution of the M+1 due to the natural abundance of ^{13}C. No ion at M+4 could be detected. This result indicated that the acetaldehyde produced *in vivo* from the alcohol was losing some of its deuterium via enolization prior to condensation, as has been previously noted by Truitt,[14] and the acetaldehyde was exchanging via equilibrium in the normal aldehyde pool.

To gain confirmation for this result and unambiguously determine whether the cyclic metabonate was being produced from an *in vivo* source of acetaldehyde, the experiment was repeated using ethanol-1-^{14}C. The metabolite was isolated, subjected to scintillation counting, and found to be radioactive. Mass spectroscopy confirmed its structure. Determination of its specific activity revealed that approximately 35% dilution had occurred. Several explanations seem possible for this observation. Either the labeled acetaldehyde is being diluted in the normal metabolic pool prior to condensation, and/or the presence of excess acetaldehyde is enhancing formation of cyclic metabonate via an intramolecular reaction. The difference in the amount of label incorporated in the two experiments,

39% in the deuterium experiment and 65% in the ^{14}C experiment, is intriguing. It may simply be biological variability, and/or it may mean that deuterium is exchanging with some hydrogen source, and/or it may be due to different rates of equilibration in the two experiments.

These studies indicate that the cyclic metabolites can be generated in vivo after lidocaine administration when the plasma levels of appropriate aldehydes are artifically increased, but that their formation under normal physiological conditions cannot be unambiguously determined in urine. Results of the CIMS-stable isotope labeling study reported in Chapter I, did however indicate that small amounts of cyclic metabonate (10-100 ng/ml) were present in human plasma after an oral dose of 250 mg lidocaine (Table I-2).

E. KINETIC AND EQUILIBRIUM EXPERIMENTS

Referring back to Scheme 1, all indications are that the cyclic metabonate is arising from the interaction of MEGX with exogenous and endogenous pools of acetaldehyde. However, if equilibrium is rapidly established between products and intermediates under physiological

conditions, the direct intramolecular cyclization pathway from carbinolamine to cyclic metabonate may not be detected by looked at blood or urine samples. Results of the labeling experiments suggested that the C-2 unit of the cyclic metabonate, which is at the oxidation state of acetaldehyde, does exchange with acetaldehyde in the urine. Subsequent work presented in this section confirms the fact that a definite pH dependent equilibrium is established between MEGX and cyclic metabonate, and that there is a rapid rate of exchange of the C-2 unit in the cyclic metabonate with acetaldehyde in the medium.

1. Equilibrium Studies

The equilibrium concentration ratios of cyclic metabonate vs. MEGX at four pH values considered within the range of urinary pH[15] were determined by reacting equi-molar quantities of ^{14}C-labeled acetaldehyde and MEGX (1.4 mM in 0.1 M phosphate buffer of appropriate pH). After 24 hours at 26° in sealed culture tubes the reactions were stopped with the addition of saturated sodium bisulfite solution and extracted with purified benzene. The extracts were analyzed by scintillation counting. The averages (±3%) of 3 runs using duplicate samples are given in Table 1. The results show that equilibrium between cyclic metabonate and MEGX + acetaldehyde is pH dependent and that the ratio of metabonate to MEGX decreases remarkably under more acidic conditions. Therefore, although the metabonate may be formed and present in body tissue and blood, once in the urine (human urine

average pH = 6.25)[15] most will break down to MEGX and acetaldehyde. During any acidic collection or work-up of urine the metabonate would be virtually non-existent.

The equilibrium position described above in buffer solution is undoubtedly shifted even more towards MEGX in biological fluids since many nucleophilic groups (amino groups, sulfhydryl groups) are present to scavange the released acetaldehyde. When radioactive cyclic metabonate was incubated at 37° with samples of fresh whole human blood (pH 7.4), equilibrium was reached in approximately 60 minutes with the ratio of metabonate:MEGX = 0.95, compared to 1.46 in 0.1 M phosphate buffer at the same pH.

TABLE III-1

Equilibrium Ratios of Cyclic Metabonate vs. MEGX at Various pH Values

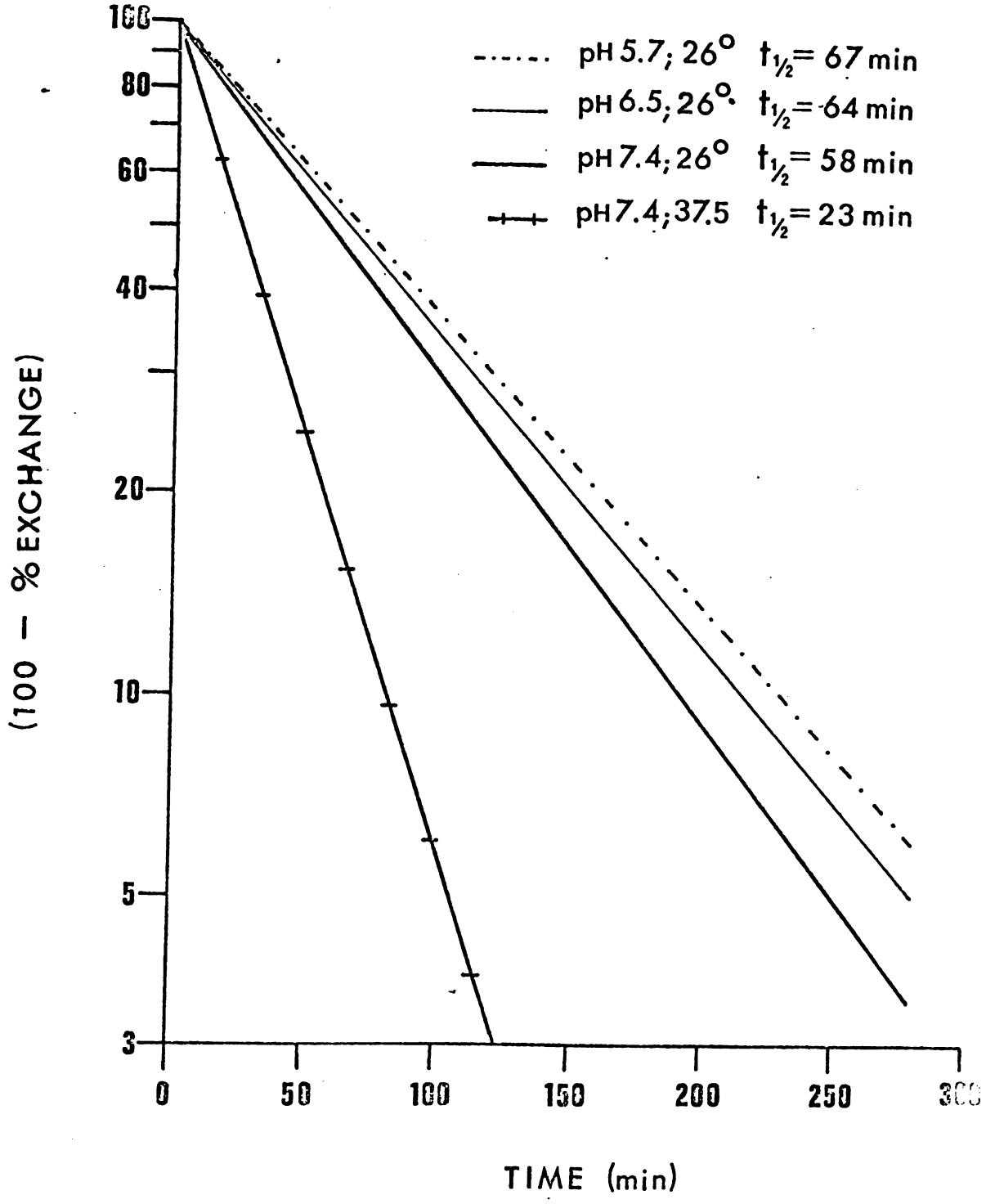
pH	Cyclic Metabonate/MEGX
5.7	.024
6.5	.159
7.4	1.46
8.0	2.09

2. Acetaldehyde Exchange with Cyclic Metabonate

Since a true equilibrium exists between cyclic metabonate and MEGX + acetaldehyde as shown in Scheme 1, a series of experiments was carried out to determine the rate of exchange of the C-2 unit in the cyclic metabonate with radioactive acetaldehyde present in the solution. To a series of culture tubes containing 1 ml of a freshly prepared solution of cyclic metabonate (100 μg/ml) in 0.1 M phosphate buffer pH 7.4, was added 0.1 ml of a solution containing ^{14}C-labeled acetaldehyde (61.5 μg, 10^4 dpm). The tubes were shaken on an incubator-shaker and samples removed and extracted at appropriate time intervals through at least 10 half-lives to obtain a good end point. The rate of exchange was monitored by scintillation counting of the extract. To insure that the radioactivity recorded was due to the formation of labeled cyclic metabonate, a portion of each extract was analyzed by glc on 3% OV-17. The presence of a single peak with a retention time of 17 min indicated that only cyclic metabonate was present. To insure this conclusion, extracts from duplicate zero-time samples, an intermediate sample, and end-point samples from a run at pH 7.4 were evaporated under a gentle stream of nitrogen and the residue recrystallized to constant specific activity with added cold cyclic metabonate. The activity of the recrystallized cyclic metabonate from each sample period accounted for at least 97% of the activity recorded in the original extracts.

Based on the analysis of Duffield and Calvin,[16] regardless of the actual kinetics of an exchange process, the rate of appearance of radiolabel in the compound will be a first-order process. Semi-logarithmic plots of the exchange reaction of acetaldehyde-^{14}C with the C-2 unit of cyclic metabonate in 0.1 M phosphate buffer under various conditions of pH and temperature are shown in Figure 7. All lines are drawn from a linear regression analysis of the data with correlation coefficients equal to or greater than 0.99 ($r \geq 0.990$). The first thing to note is that exchange does take place, and secondly that at 37.5°, pH 7.4, the half-life ($t_{\frac{1}{2}}$) for exchange is only 23 minutes. Thus, detection of a direct intramolecular reaction from carbinolamine to cyclic metabonate would be very difficult under the in vivo conditions used.

Figure III-7. Semi-logarithmic plots of the exchange reaction of acetaldehyde-^{14}C with the C-2 unit of cyclic metabonate as observed in 0.1 M sodium phosphate buffer under various conditions of pH and temperature.



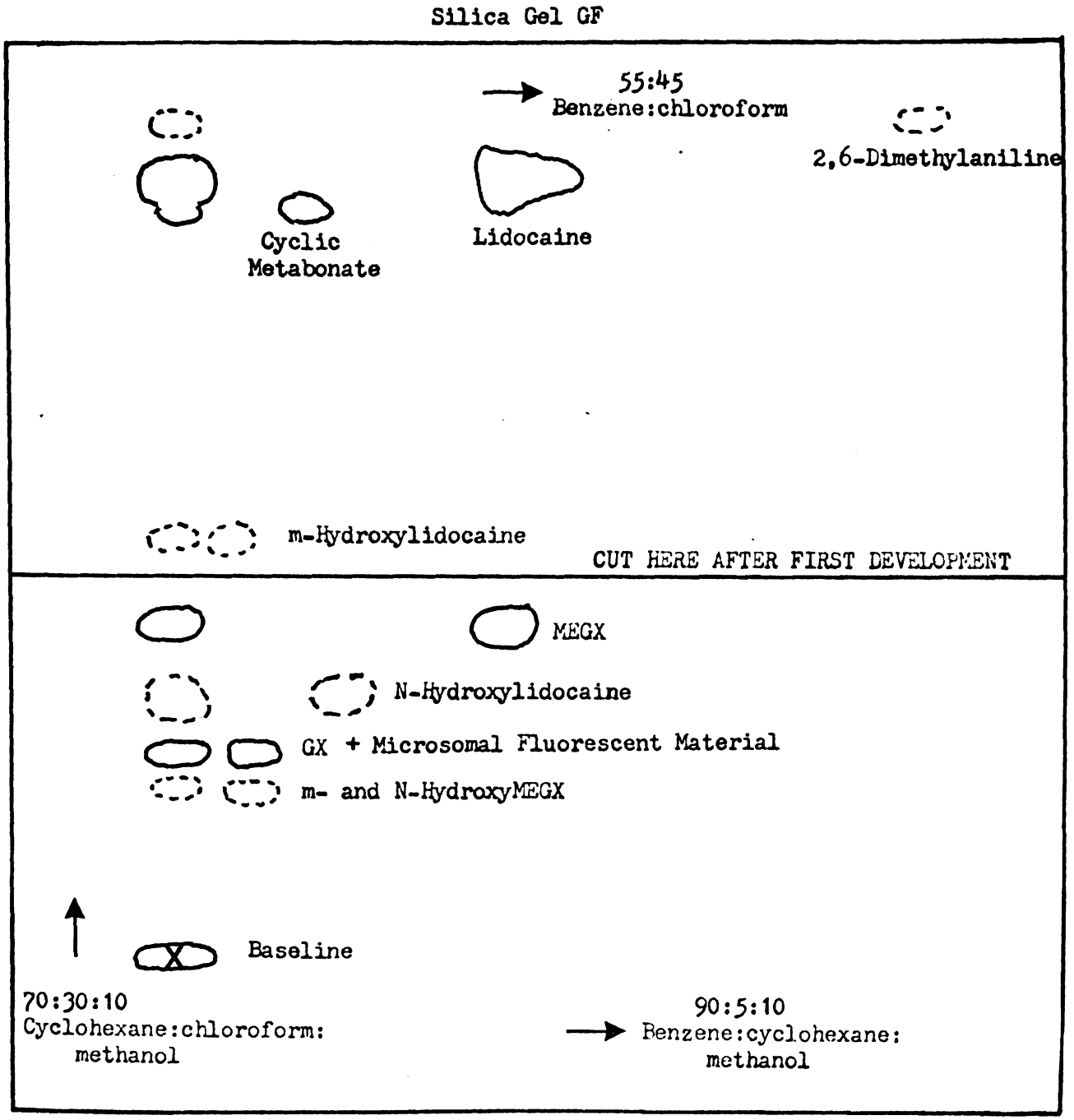
Semi-logarithmic plot of the exchange reaction of Acetaldehyde-c-14 with Cyclic Metabonate in 0.1 M Phosphate Buffer

F. MICROSOMAL EXPERIMENTS

Since detection of a direct intramolecular pathway by analyzing urine or blood samples is impeded by equilibration and exchange reactions, in vitro experiments with rat liver microsomes were carried out. Incubations were conducted with radio-labeled (^{3}H and ^{14}C) lidocaine and metabolites were isolated by two-dimensional tlc as shown in Figure 8. Quantification was accomplished by scintillation counting. Controls were run with radio-labeled MEGX to insure that no cyclic metabonate was arising as an artifact of the isolation and analysis procedures. Results of the experiments and controls indicated that 78-80% of the intial activity was recoverable in the solvent extracts. Table 2 shows the results of the extractable metabolites from three separate incubations and tlc separations.

Glc analysis showed the presence of lidocaine (retn time 9.5 min), MEGX (retn time 9.8 min), and cyclic metabonate (retn time 8.0 min). Other metabolites, except possibly GX, could not be detected by glc. Greater than 99% of the radioactivity applied to the lidocaine control plate was concentrated in the lidocaine rf area. A small amount of activity (~0.3% of the total) had to be subtracted from the cyclic metabonate separated area to account for some tailing of the radioactivity from lidocaine into this region. In the control of radioactive MEGX, no radioactivity was detected in the tlc separated cyclic metabonate region showing that no artifact was formed in the incubation and work-up procedures.

Figure III-8. Two-dimensional tlc separation (Eastman #6060 silica gel GF plates, 20 x 20 cm) of lidocaine metabolites extracted from a microsomal incubation with methylene chloride.



Areas that fluoresced without added standards.

Areas determined with the addition of standards and radioactive counting.

TABLE III-2

Quantification of Lidocaine and Metabolites from Microsomal Metabolism

Data were derived from the average radioactivity (dpm) of duplicate samples from three separate incubations, each incubation using the microsomes isolated from the pooled livers of three male Sprague-Dawley rats.

Metabolite Separated by tlc	Percent of Metabolite Found in Extract		
	Incubation 1	Incubation 2	Incubation 3
Lidocaine	79.0	74.5	82.0
MEGX	14.0	17.3	14.6
Cyclic Metabonate[a]	3.0	1.6	1.2
GX[b]	1.5	0.6	0.5
N-Hydroxylidocaine[b]	0.5	0.5	0.6
m-Hydroxylidocaine[b]	0.7	0.7	0.3
N-and/or m-Hydroxy MEGX[b]	0.5	0.3	0.2
2,6-Dimethylaniline[b]	0	0.6	0.3
Other	0.8	3.9	0.3

[a]Corrected for small amount of activity tailing from lidocaine.

[b]These metabolites have only been tentatively idenfified by the addition of "cold" synthetic metabolites to the extract and subsequent tlc separation and scintillation counting.

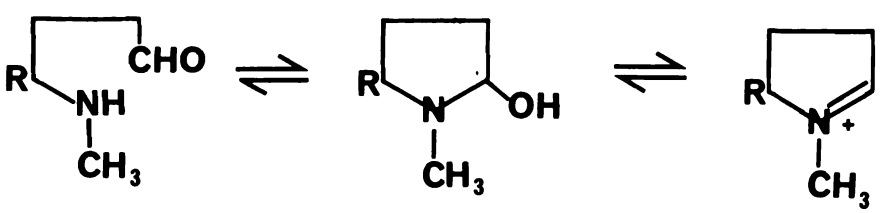
The results indicate that lidocaine is primarily metabolized by N-dealkylation, although a small amount of cyclic metabonate is formed. However, since acetaldehyde is a product of the N-dealkylation process (refer to Scheme 1 reproduced on p. 156), this metabonate may still be arising from condensation of MEGX and acetaldehyde. If this is the case, its concentration should increase over time. Therefore, an incubation was performed with extraction of duplicate samples at 0, 5, 10 and 15 minutes. The 0-time sample was used to determine the amount of radioactivity tailing from the lidocaine tlc region into the cyclic metabonate area. The results show ratios of cyclic metabonate vs. MEGX as follows:

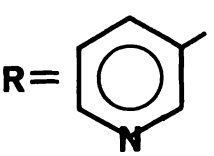
Time	Cyclic Metabonate/MEGX
5 min	0.099
10 min	0.088
15 min	0.069

These results suggest that at least some of the cyclic metabonate analyzed is formed as a result of the direct imminium ion pathway shown in Scheme 1. After formation, equilibrium is established with MEGX + acetaldehyde. Based on a product analysis of the microsomal oxidation, assuming that this reflects the kinetics of the enzyme reaction during the time period analyzed, the dehydration pathway from the intermediate carbinolamine to imminium ion proceeds at a slower rate than the fragmentation pathway to MEGX and acetaldehyde. This agrees with the

kinetics observed in the solution chemistry of imine formation, where it has been shown that above pH 5 aliphatic amines react with aldehydes or ketones with rapid equilibration between aldehyde + amine $\rightleftharpoons$ carbinolamine, and rate-determining loss of water from the carbinolamine.[17]

Recently, Murphy[18] has reported trapping nicotine $\Delta^{1'(5')}$ imminium ion as an intermediate in the enzymatic oxidation of nicotine. As shown below, the proposed sequence involves formation of the carbinolamine which then can break down to an aldehyde and amine contained in the same cyclic structure which entropically favors the formation of the imminium ion compared to the case of lidocaine where acetaldehyde is generated. The author pointed out the possibility of a rapid equilibrium being established between the three structures although the equilibrium positions were not determined.

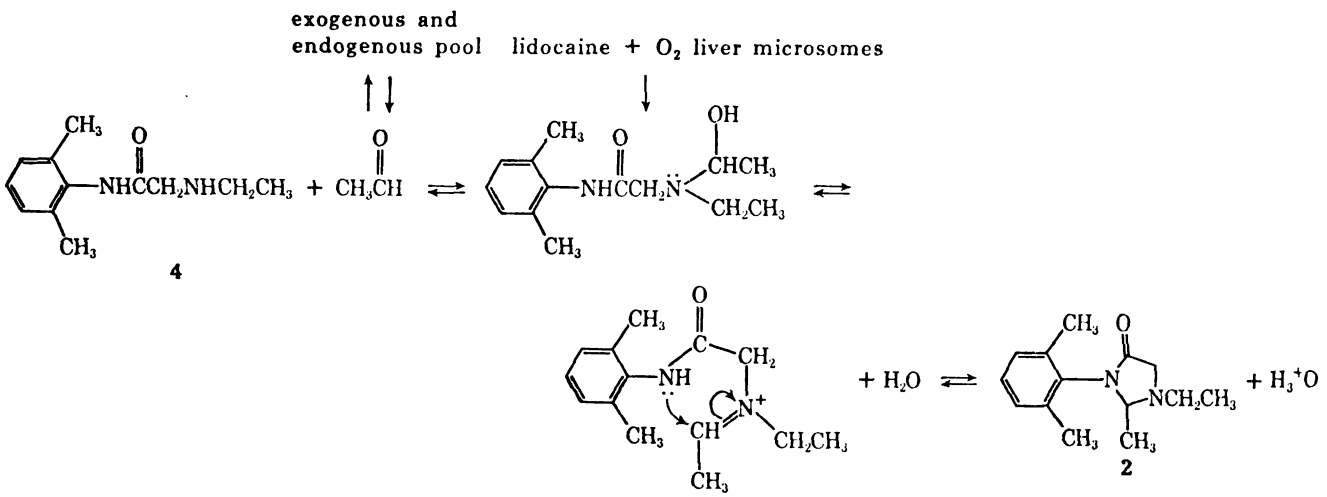




G. DISCUSSION

The generalized mechanism for the formation of cyclic metabonate which is consistent with all our results has been depicted in Scheme 1 which is reproduced below.

Scheme III-1 (Reproduced from p. 124)



There is considerable evidence that enzymatic N-dealkylations proceed by way of a carbinolamine intermediate such as that shown, which then either spontaneously or enzymatically disassociates to an amine and an aldehyde.[5] In the case of lidocaine, because of the availability of a nonbonded lone pair of electrons five atoms away from the electrophilic

site, a second reaction becomes theoretically possible, that of an intramolecular nucleophilic attack by the amide nitrogen on the imminium cation carbon, resulting in ring closure and the generation of a stable non-charged molecule. Moreover, there is precedent[19] for a similar intramolecular reaction occurring in vivo in the course of the metabolism of the antimalarial agent proguanil (12, Figure 1) to generate the biologically active triazine, 13. From a strictly chemical viewpoint, this latter reaction can be viewed as a Mannich reaction.[6]

Thus, MEGX and cyclic metabonate are related through a common intermediate, the carbinolamine, obtained from reaction with acetaldehyde. It could, therefore be anticipated that the three substances might be in measurable equilibrium depending on their relative stabilities and reaction conditions. That this is the case, and that equilibrium is extremely fascile under very mild conditions, is apparent from the results obtained. Indeed, the fascile nature of the reaction precludes the possibility in this thesis of determining just how much of the cyclic metabonate might arise in man from the originally administered lidocaine via an initial intramolecular cyclization, although measurable amounts were found in the in vitro rat liver microsomal experiments. Even presupposing significant amounts of cyclic metabonate were generated in this fashion, this would not be detectable on urine analysis because equilibrium between cyclic metabonate, MEGX, and the endogenous or exogenous (urine, solvents) aldehyde pool would have already occurred. In fact, the nmr study revealing the

metabonate as a racemate, and the in vivo labeling studies indicated that all metabonate found in the urine probably arises from this mechanism.

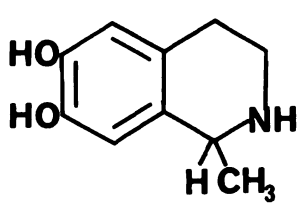
The results reported in this chapter appear to have bearing on four areas of current research.

1) Recently, Freund[20] has reported that lidocaine is an effective agent in preventing ethanol withdrawal seizures in addicted mice. It may be that the drug is exerting its pharmacological action via its membrane stabilizing properties. However, this effect may in some way be associated with the propensity of the lidocaine metabolite, MEGX, to react with aldehydes.

2) Beckett[21] has coined the term "metabonate" for a substance which appears to be a metabolite but which in reality is formed during isolation and/or analysis. The ease in which the cyclic metabolites 2 and 3 can be artifactually formed certainly illustrates the problem and opens to question the genesis of natural products formed by similar reactions. For example, while isolating L-Dopa from velvet beans, one group of workers[22] reported finding 14 (Figure 1) as a new naturally occurring amino acid. Similarly, a second group[23] reported finding 15 as a naturally occurring amino acid from the seeds of Mucuna mutisiana. Since both compounds could result chemically[24] by condensation of acetaldyhyde with L-Dopa in the case of 14, and from formaldehyde in the case of 15, the validity of these compounds as natural products is open to some question.

3) Much attention has been focused recently on the in vivo and in vitro generation of substituted tetrahydroisoquinolines from the condensation of exogenous or endogenously induced levels of aldehydes and the hormonal amines.[24-30] These substances are known to be pharmacologically active in their own right and several groups of workers have speculated as to their possible involvement in the dependence and withdrawal phenomena of morphine addiction[25,28,29] and alcoholism.[30] Our results would indicate that quantification of levels of certain of these condensation products might be extremely difficult. For example, Sandler, et al.,[31] have recently reported the in vivo formation of aldehyde condensation products in patients being treated for Parkinsonism with L-Dopa. As pointed out in their paper, they could not meaningfully interpret their quantitative data on urine levels because of great variability from determination to determination.

We have carried out preliminary work which indicates that salsolinol, the condensation product of dopamine and acetaldehyde shown below, is formed slowly at pH 7.4. Under pseudo-first order conditions of excess dopamine $t_{\frac{1}{2}}$ = 40 min, whereas under the same conditions with MEGX and acetaldehyde $t_{\frac{1}{2}}$ = 8 min. However, as might expected because of the C-C bond formed in salsolinol vs. a C-N bond in cyclic metabonate, acetaldehyde exchange in salsolinol appears to be negligible.



4) The generality of the binding of low molecular weight drugs or their degradation products to tissue protein components is becoming increasingly recognized.[32-34] In the case of lidocaine, its metabolite, MEGX readily condenses with acetaldehyde to form cyclic metabonate (2) even though a sterically hindered amide nitrogen must function as a nucleophile. This implies that good nucleophiles such as the sulfhydryl group of cysteine, or the ring nitrogen of histidine, could effectively compete for reaction with a reactive intermediate formed in the process of dealkylation, or in the reverse direction with aldehyde. Murphy,[18] has recently detected the formation of an analogous imminium ion in the oxidative metabolism of nicotine, and he hypothesized that this electrophilic intermediate might bind at certain nucleophilic receptor sites. If such reactive electrophilic intermediates are a general phenomena in N-dealkylation processes or in the presence of alcohol ingestion, then perhaps the biological responses, either efficacious or toxicological, of various amine drugs may partially be explained in terms of reactions of such intermediates with a nucleophile of a critical macromolecule.

H. EXPERIMENTAL SECTION

1. Synthesis and Labeling Studies

a. Materials

Melting points were taken by capillary on a Thomas-Hoover Uni-Melt instrument and are uncorrected. Uv spectra were recorded on a Cary 16 spectrophotomenter; ir on a Perkin-Elmer 337 spectrophotometer; nmr on a Varian A-60A (δ) or a JEOL 100 MHz (δ^{100}); mass spectra were taken on an AEI MS902 (direct inlet, 70 eV). Vpc analyses were obtained on a Varian 2100 gas chromatograph equipped with flame ionization detectors using a 5.5 ft X 0.25 in X 2 mm i.d. glass U column freshly packed with 2% Carbowax 20M on 80-100 mesh KOH-washed Chromosorb W. Conditions employed were: column temperature 160°; injector 250°; detector 250°; N_2 carrier gas flow 40 ml/min; hydrogen and air adjusted to give maximum flame response; chart speed (Varian A-25 recorder) 0.1 in/min. The column was equipped with a 10:1 splitter for collection of samples in capillary tubes for mass spectral analysis or scintillation counting. A Tri-Carb Model 3375 scintillation counter was used for radioactivity determinations. All determinations were made in 10 ml Aquasol (NEN) and corrected for quenching using internal standardization with either toluene-^{14}C standard (NEN) or toluene-^{3}H (NEN).

b. Isolation of Basic Metabolites from Human and Monkey Urine

Administration of lidocaine to human volunteers was accomplished via the oral route with 500 mg encapsulated doses.[1] Male Rhesus monkeys, restrained in metabolism chairs, were administered by i.v. infusion 100 mg of lidocaine dissolved in sterile water for injection made acidic with a small amount of HCl, neutralized with NaOH, made isotonic with NaCl, made to a volume of 40 ml, and filtered through a millipore filter. Infusion was maintained for 20 hours at a rate of 2 ml/hr. Aliquots of the total urine collected for 36 hours were extracted (pH 11) with equal volumes of solvent[35] by shaking for 30 min on an automatic shaker in centrifuge tubes. The samples were then spun down for 20 min at 1000 xg to help break emulsions. Combined extracts were extracted with 2.5 volumes of 10% HCl and this acid extract basified to pH 11 with 5N NaOH followed by back extraction with 2 volumes of solvent. After drying over anhydrous sodium sulfate, solvent was removed at room temperature on a rotary evaporator, the residue taken up in 1 ml of solvent and subjected to glc. Retention times on a freshly packed column are: 5 1.2 min; 2, 17 min; 3, 19 min; 1, 21.2 min; 4, 41 min. Retention times on a column packed with 3% OV-17 on A/W Chromosorb W 80-100 mesh are: 5, 1 min; 2, 8 min; 3, 7.9 min; 1, 9.5 min; 4, 8.8 min (185°).

c. ω-Ethylamino-2,6-dimethylacetanilide or MEGX (4)

This compound was synthesized as described in Chapter I, Experimental.

d. N^1-Ethyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone or Cyclic Metabonate (2)

To an ice-bath cooled, magnetically stirred solution of MEGX, (20.6 g, o.1 mole) in 2 l of ether, was added two 10 ml portions of acetaldehyde. The reaction was warmed slowly to room temperature and allowed to stir for 5 hours after which period solvent was removed on a rotary evaporator to give an orange liquid. Recrystallization from pet ether (30-60°) yielded cyclic metabonate, 9.8 g (48%), mp 62-63°; ir (KBr) 1715 cm^{-1} (lactam C=O); nmr δ^{100} (benzene-d_6, TMS)[36] 0.86 (t, J = 7.3 Hz, $NCH_2\underline{CH_3}$), 0.87 (d, J = 5.5 Hz, C-2 methyl), 2.05 and 2.56 (non-equivalent s, aryl Me's), 1.95 and 2.49 (non-equivalent octets, J_{gem} = 14.5 Hz, J_{Me} = 7.3 Hz, $N\underline{CH_2}CH_3$), 2.87 and 3.66 (non-equivalent doublets, J_{gem} = 14.0 Hz, C-5 ring methylenes), 4.12 (q, J_{Me} = 5.5 Hz, C-2 ring methine) and 7.12 ppm (m, aromatics). High resolution mass spectrum, calcd for $C_{14}H_{20}N_2O$, 232.15782; found, 232.15756.

Anal for $C_{14}H_{20}N_2O$, calcd: C 72.30, H 8.68, N 12.06; found: C 72.22, H 8.87, N 11.88.

e. N^1-Ethyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (3)

To a solution of MEGX (1.0 g, 4.85 mmoles) in 100 ml benzene, was added a two-fold excess of 35% aqueous formaldehyde. The reaction was refluxed for 3 hours, cooled, washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of benzene with a stream of nitrogen gas yielded a white crystalline mass which was recrystallized from purified pet ether to give 0.6 g of white needles, mp 100-101°; ir (KBr) 1715 cm^{-1} (lactam C=O); nmr δ ($CDCl_3$, TMS), 1.16 (t, J = 7 Hz, $NCH_2\underline{CH_3}$), 2.27 (s, aryl Me's), 2.74 (q, J = 7 Hz, $N\underline{CH_2}CH_3$), 3.47 (m, C-5 methylenes), 4.20 (m, C-2 methylenes) and 7.13 ppm (s, aromatics). High resolution mass spectrum, calcd for $C_{13}H_{18}N_2O$; 218.14191, found: 218.14127.

Anal for $C_{13}H_{18}N_2O$, calcd: C 71.53, H 8.31, N 12.83; found: C 71.43, H 8.39, N 12.75.

f. N^1-Methyl-2-methyl-N^3-(2,6-dimethylphenyl)-4-imidazolidinone (7)

1) ω-Chloro-2,6-dimethylacetanilide (10 g, 0.05 mole) in 50 ml dry benzene was added to a four-fold excess of methylamine in a stainless steel bomb cooled in dry-ice/acetone. The bomb was sealed and heated for 18 hours at 100°. After cooling, the bomb was unsealed and warmed to room temperature. The reaction mixture was extracted twice with 5% HCl, the combined acid extracts backwashed with ether, basified with KOH pellets, and extracted with methylene chloride to give 2.7 g of ω-N-methyl-2,6-dimethylacetanilide, mp 41°.

2) A solution of ω-N-methyl-2,6-dimethylacetanilide, (1 g, 4.9 mmoles), in 100 ml benzene was refluxed with excess acetaldehyde for one hour. After cooling, the reaction mixture was washed with saturated sodium chloride solution and dried over anhydrous sodium sulfate. Removal of solvent under nitrogen gave an oil which was crystallized from purified pet ether to yield white crystals, mp 139-142°; nmr δ ($CDCl_3$, TMS) 1.3 (m, C-2 methyl), 2.20 (s, aryl Me's), 2.45 (s, NCH_3), 3.25, 3.70 and 3.80 (m, C-2 and C-5 hydrogens) and 7.07 (s, aromatics); EI mass fragments at m/e 218, 203, 147, 132, 117, 105, 91, 71 and 57 (base peak).

g. ω-[β,β-d_6-Diethylamino]-2,6-dimethylacetanilide or lidocaine-d_6(9)

1) A mixture of acetic anhydride-d_6 (Stohler, 5.0 g, 46 mmoles) and acetonitrile-d_3 (Stohler, 1.5 g, 34 mmoles) was hydrogenated (30 psi, H_2) on a Paar shaker over 60 mg Adam's catalyst for 4 hours. An additional 30 mg Adam's catalyst was then added and the hydrogenation continued an additional 2 hours. After removal of reduced Pt by suction filtration, the filtrate was distilled through a short-path distillation head to yield N-ethylacetamide-d_6, 2.2 g (71%).

2) To an ice-bath cooled solution of AlH_3 (~31 mmoles) in THF, was added dropwise a THF solution of N-ethylacetamide-d_6 (1.5 g, 16.5 mmoles) over 45 min. After 18 hours excess AlH_3 was destroyed with 2 ml of 1:1 THF:water followed by hydrolysis of aluminate complexes with 8 ml of 15% NaOH. The liberated diethylamine-d_6 was co-distilled with THF at 59-64°.

3) To the 60 ml of THF solution containing diethylamine-d_6, was added ω-chloro-2,6-dimethylacetanilide (1.20 g, 6 mmoles) and the solution stirred at reflux for 2 hours. To the solution was added 300 mg NaOH in 1.5 ml of water and the reaction refluxed an additional 4 hours. Rotary evaporation of solvent yielded a pale yellow liquid which was taken up in ether and washed with water followed by extraction with 10% HCl. The acid extract was basified with 5N NaOH and back-extracted into ether. The ether extract was washed with water, filtered through anhydrous sulfate, and dried over Drierite. Removal of solvent and recrystallization from petroleum ether yielded 780 mg white crystalline solid, mp 65-67°; nmr δ ($CDCl_3$, TMS), 2.23 (s, aryl Me's), 2.66 [broad m, $N(\underline{CH_2}CD_3)_2$], 3.20 (s, $O{=}C\underline{CH_2}N$), 7.07 (s, aromatics), and 6.90 ppm (broad s, HNC=O). High resolution mass spectrum calcd for $C_{14}H_{16}N_2O(-D_6)$: 228.12625, found: 228.12703; overall deuterium incorporation, 94%.

h. Metabolic Studies with Lidocaine-d_6

A 4.8 Kg Rhesus monkey was administered i.v. a total of 100 mg of lidocaine-d_6 over 20 hours and aliquots of the 36-hour urine were extracted as previously described using reagent grade ether. Mass spectral analysis of the cyclic metabonate collected from glc showed that only three deuteriums were retained in the metabolite on the N-ethyl side chain; mass fragments, m/e = 235, 220, 160, 147, 132, 117, 105, 91, 88 (base peak), and 70.

i. ω-[α-^{14}C-Diethylamino]-2,6-dimethylacetanilide (11)

This compound was synthesized by the procedure described for lidocaine-d_6 from acetonitrile-^{14}C (Amersham-Searle) and redistilled acetic anhydride. The product was recrystallized five times from purified pet ether to constant specific activity and the radiochemical purity determined by glc and tlc (cyclohexane:chloroform:methanol 70:20:10) with the aid of a radiochromatographic scanner. A final specific activity (sp. act.) of 0.060 μCi/mg was obtained.

j. Metabolic Studies with Radio-labeled Lidocaine (11)

A 4.8 Kg Rhesus monkey was administered i.v. a total of 100 mg of 11, sp. act. 0.060 μCi/mg, over 20 hours. The cyclic metabolites 2 and 3 were isolated from an aliquot of the 36-hour urine and collected from glc. Reinjection was used to verify their purity. Two independent methods were then used to determine the sp. act. of the isolated metabolites. In the first method, 2 was taken up in 300 μl of pure ethanol and its uv spectrum determined at 264 nm. The absorbance obtained was compared to that obtained from the spectrum of a known concentration of an authentic sample. Having determined the concentration, a 100 μl aliquot was used for scintillation counting. The sp. act. was determined and found to be 0.029 μCi/mg or ~49% of that present in the originally administered lidocaine. In the second method, standard curves of known concentrations of cyclic metabolites 2 and 3 vs. lidocaine were

determined by glc. A known amount of lidocaine was added to 100 μl aliquots of each of the metabolites in pure ethanol, the samples subjected to glc, and the concentration of each determined from the standard curves. Another 100 μl aliquot was counted and again the sp. act. in both cases was found to be 49% of that of the administered lidocaine.

k. The Reaction of MEGX with Acetaldehyde Under Conditions of "Physiological" Concentration and pH

To 1 l of sodium phosphate buffer (0.1 M, pH 7.4) was added with stirring 1 ml of a 1 mg/ml solution of acetaldehyde in distilled water followed 15 min later by 1 ml of a 1 mg/ml solution of MEGX in phosphate buffer. The solution was stirred for 4 hours at room temperature followed by a four-hour period of bubbling nitrogen gas through the solution in order toremove as much of the remaining acetaldehyde as possible. The buffered reaction mixture was rotary evaporated (25°, 1-5mm Hg) to a volume of 50 ml and a 10 ul sample was injected onto a glc column. Glc analysis indicated 20% conversion of MEGX to cyclic metabonate based on standard curves of the two compounds on the Carbowax column used.

l. Study with Ethanol-d_6

A 5.2 Kg Rhesus monkey was infused i.v. with a 40 ml solution containing 10 ml Xylocaine (Astra), 10 mg/ml, plus 10 ml of 95% ethanol-d_6 (Stohler Isotope), plus 20 ml of normal saline for injection.

Infusion was maintained at 2 ml/hour for 20 hours. The total 36-hour urine was combined and extracted with aldehyde-free methylene chloride as previously described. Cyclic metabonate was collected from glc and subjected to EI mass spectral analysis which showed peaks at m/e = 232 (61%), 233 (23%), 234 (11.5%), 235 (4.5%) and 236 (0%).

Controls:

1) Two weeks before co-administration of ethanol-d_6 and lidocaine, the same Rhesus monkey was administered 100 mg lidocaine by i. v. infusion in the usual manner. The total 36-hour urine was combined and extracted with aldehyde-free methylene chloride. Comparative glc analysis of the basic metabolites from ethanol-d_6 vs. control showed enhanced levels (~25X) of cyclic metabonate in the ethanol-d_6 study.

2) One week prior to infusion with ethanol-d_6, the same monkey was infused with 10 ml unlabeled 95% ethanol in 30 ml of normal saline and 3 mg of MEGX was added to the urine collection pail. Urine was collected for 36 hours and extracted with aldehyde-free methylene choride in the same manner. Comparative glc analysis of the basic metabolites from the ethanol-d_6 study vs. control extract showed enhanced levels (~20X) of cyclic metabonate in the labeled ethanol study, based on relative peak areas of cyclic metabonate to MEGX. This indicates that the enhanced levels of cyclic metabonate found under conditions of ethanol administration do not arise from the collected urine.

m. Study with Ethanol-1-^{14}C

A 4.7 Kg Rhesus monkey was infused i.v. with a 40 ml solution containing 10 ml Xylocaine (Astra), 10 mg/ml, plus 10 ml 95% ethanol containing 500 μCi ethanol-1-^{14}C (NEN), and 20 ml of normal saline. The experimental and isolation conditions were the same as those used in the ethanol-d_6 study. The collected sample of cyclic metabonate was taken up in 200 μl of pure ethanol and a 100 μl aliquot counted. A 10 μl aliquot was reinjected to indicate the chromatographic purity of the collected metabolite. Another 2x10 μl aliquots were co-chromatographed on the Carbowax system with known concentrations of lidocaine to determine the concentration of cyclic metabonate using a standard curve of peak area ratios. The sp. act. of the administered ethanol was 2.96 μCi/mmole. The specific activity of the extracted metabolite was 1.81 μCi/mmole. This indicates a 35-40% dilution of radioactive label in the cyclic metabonate. The remaining sample was evaporated under a stream of nitrogen and subjected to mass spectral analysis where the fragmentation was found to be identical with synthetic cyclic metabonate.

n. Doubly-labeled Lidocaine and MEGX

Both lidocaine and MEGX were synthesized with tritium (random) in the aromatic ring and specifically with ^{14}C in the carbonyl group.

1) Tritiated water (Bio-Rad, 2 ml, ~500 μCi), was distilled twice in vacuo using a high vacuum manifold. To this was added freshly prepared platinum black (50 mg) and freshly distilled 2,6-dimethylaniline (1.0 g,

8.2 mmoles). Concentrated sulfuric acid was added dropwise with stirring to solubilize the amine. The contents of the tube were frozen in liquid nitrogen and the tube sealed. The reaction was run for 36 hours with the sealed glass tube encased in a metal tube immersed in an oil bath maintained at 90-95°. The dark-purple reaction mixture was then refrozen in liquid nitrogen and the sealed tube broken. Impurities were extracted with four 5 ml portions of methylene chloride and the remaining contents basified with KOH pellets. Product was extracted with three 10 ml portions of methylene chloride, combined extracts washed with water, and filtered through sodium sulfate. Solvent was removed under a stream of nitrogen gas and the product vacuum distilled at room temperature (5-10μ) over 8 hours using the high vacuum manifold to give 785 mg clear liquid, sp. act. ~0.80 mCi/mmole.

2) To tritiated 2,6-dimethylaniline (700 mg, 5.8 mmoles) in 40 ml methylene chloride was added chloroacetic acid-1-^{14}C (Amersham-Searle, 550 mg, 5.8 mmoles, sp. act. 0.15 mCi/mmole). DCCI (Aldrich, 1.24 g, 6 mmoles) in 10 ml methylene chloride was added dropwise to the stirred solution over 20 minutes and the reaction stirred for three hours at room temperature. The white precipitate of dicyclohexylurea was filtered off and the filtrate washed with two 10 ml portions of 10% sodium carbonate, 10 ml of water, and dried over anhydrous sodium sulfate. Removal of solvent and recrystallization to constant sp. act. from CCl_4 gave doubly-labeled ω-chloro-2,6-dimethylacetanilide (698 mg, ^{3}H-sp. act. corrected for quenching and crossover using internal standards

~0.82 mCi/mmole, ^{14}C-sp. act. corrected for quenching and crossover using internal standards ~0.13 mCi/mmole).

3) To doubly-labeled ω-chloro-2,6-dimethylacetanilide (198 mg, 1 mmole) in 20 ml benzene, was added a 70% aqueous solution of ethylamine (161 mg, 2.5 mmoles of ethylamine). After 3 days at 60° the reaction was washed with water and extracted with two 50 ml portions of 3N HCl and combined acid extracts backwashed with 25 ml of methylene chloride. Acid extracts were basified to pH 11 with 20% KOH and back-extracted with three 50 ml portions of methylene chloride. Combined extracts were washed with two 20 ml portions of water and dried over anhydrous sulfate. Evaporation of solvent gave a 175 mg crystalline mass which was recrystallized 3 times from purified n-hexane to constant sp. act. (112 mg, ^{3}H-corrected sp. act. 0.65 mCi/mmole, ^{14}C-corrected sp. act. 0.125 mCi/mmole). The purity of the product was confirmed by glc analysis and radiochromatographic analysis.

4) Doubly-labeled lidocaine was synthesized on a 1 mmolar scale by the same procedure outlined for doubly-labeled MEGX using diethylamine (190 mg, 2.5 mmoles). Recrystallization to constant sp. act. (4 times from purified pet ether) yielded 124 mg (^{3}H-corrected sp. act. 0.67 mCi/mmole, ^{14}C-corrected sp. act. 0.128 mCi/mmole). Purity was confirmed by glc and radiochromatographic scanning.

o. Reverse-Isotope Dilution Study with Doubly-Labeled MEGX

To 10 ml of pH 7.4 phosphate buffer was added doubly-labeled MEGX (~10μg, sp. act. ^{3}H-0.65 mCi/mmole ^{14}C-0.125 mCi/mmole)

and 50 μl of the resulting solution added to a 1 l aliquot of a total 36-hour human urine sample from a normal male subject dosed orally with 500 mg of lidocaine. The sample was placed in a refrigerator (10°) for 12 hours. Extraction and isolation of metabolites was carried out as previously described using aldehyde-free methylene chloride and the Carbowax glc system. Cyclic metabonate was collected, taken up in 1.5 ml of purified n-hexane, and its concentration determined by uv absorbance at 264 nm. Solvent was then evaporated under a stream of nitrogen and radioactivity determined by scintillation counting using the tritium channel. Sp. act. found for cyclic metabonate: 2.20 μCi/mmole. MEGX was collected and diluted to 2 ml in purified hexane. A 100 μl aliquot was counted using the tritium channel and a 1.5 ml aliquot used for uv determination of concentration at 264 nm. Sp. act. found for MEGX: 1.90 μCi/mmole. The reduced sp. act. found in the isolated metabolites occurred because of dilution of the radio-labeled MEGX standard with MEGX metabolite present in the urine.

2. Equilibrium and Kinetic Studies

a. Materials

Glc analyses were obtained on a Hewlitt-Packard Model 5750 gas chromatograph equipped with flame ionization detectors using a 6 ft X $\frac{1}{4}$ in X 1/8 in i.d. stainless steel column freshly packed with 3% OV-17 on A/W Chromosorb W DMCS 100-120 mesh. Conditions employed were: column temperature 185°; injector 280°; detector 280°; He gas

flow 25 ml/min; hydrogen and air adjusted to give maximum flame response; chart speed (HP Model 7127 Recorder) 0.25 in/min. A Beckman LS-230 scintillation counter was used for radioactivity determinations with ^{14}C-channel discriminator set from 25-100. All determinations (M. S. E. ± 1%) were made in 10 ml Aquasol (NEN) and corrected for quenching using internal standardization with toluene-^{14}C standard (NEN). All reactions were run in 0.1 M sodium phosphate buffer solutions of varying pH which was checked on a Beckman Model H2 pH meter calibrated with standard Beckman buffers. Reactions were carried out in 10 ml pyrex culture tubes fitted with Teflon-sealed caps which were shaken (100 oscillations/min) in an AO Model 2156 constant temperature water bath at various temperatures (± 0.5°). Benzene was purified by shaking successively with concentrated sulfuric acid, water, 5% sodium bicarbonate, and water, and finally distilled.

b. Acetaldehyde-1,2-^{14}C

This compound was prepared by gently distilling (30°) paraldehyde-1,2-^{14}C (NEN, 0.022 mmoles, sp. act. 10.9 mc/mmole) diluted with 0.125 ml of carrier paraldehyde and 10 µl of 1 M sulfuric acid in a micro-distillation apparatus.[37] The acetaldehyde evolved was trapped in a 5 ml pear-shaped flask containing 1 ml of distilled water. The amount trapped was weighed in the sealed flask by difference on a Cahn electrobalance Model M-10 and determined to be 61.5 mg. An aliquot of this sample was diluted in the 0.1 M phosphate buffer of appropriate pH to obtain solutions containing 615 µg/ml, 163 dpm/µg or ~10^5 dpm/ml.

c. Equilibrium Studies

One ml samples of stock solutions containing MEGX (36.5 mg of the hydrochloride/100 ml 0.1 M phosphate buffer pH 5.7, 6.5, 7.4 and 8.0) were pipetted into culture tubes and 0.1 ml of the acetaldehyde stock solution added to each tube. All samples were run in duplicate. The tubes were shaken for 24 hours at 25°, then 0.5 ml of saturated sodium bisulfite solution was added and the cyclic metabonate extracted with 1.5 ml of benzene by shaking for 0.5 min. A 400 µl aliquot of each extract was pipetted into a counting vial, 10 ml of Aquasol added, and the samples counted. Blank samples containing everything but acetaldehyde-^{14}C were used to determine background. Blank samples containing everything except MEGX were used to determine acetaldehyde carried through the extraction (~1% in 400 µl).

Calculations of the ratio of [cyclic metabonate]:[MEGX] were made by setting [cyclic metabonate] = dpm recovered after correction for extraction efficiency and acetaldehyde -^{14}C carried through the extraction. [MEGX] = Acetaldehyde-^{14}C remaining in solution which was equal to the total dpm at the beginning less the dpm recovered as cyclic metabonate.

The 1.1 ml of benzene extract remaining after using 0.4 ml for scintillation counting, was evaporated under a gentle stream of nitrogen. The residue was dissolved in purified methanol and analyzed by glc. Only cyclic metabonate was found to extract (retention time 8.0 min).

Collection of several of these samples and recrystallization from purified pet ether yielded a few mg of purified cyclic metabonate labeled with ^{14}C in the C-2 unit. This radio-labeled metabonate was used to determine the extraction efficiency (87%) of the compound in the procedure used, and was also used to check the equilibrium determinations in the reverse direction. At pH 7.4 the ratio of cyclic metabonate: MEGX was found to be 1.41 compared to 1.46 for the value obtained by reacting equimolar quantities of MEGX and acetaldehyde. Thus the two values compare to within 4%.

A second set of duplicate samples was analyzed by basifying the mixture to pH 8.0 with 0.64 ml of 5N NaOH and extracting with 1.5 ml of benzene. In this manner both MEGX and cyclic metabonate were extracted. Glc comparison of the extracts from equilibrium studies carried out in both directions showed the same ratio ($\frac{1}{2}$ peak height x width) of cyclic metabonate to MEGX, retention times of 8.0 and 8.8 min, respectively.

d. Kinetic Studies

All studies were run twice using duplicate samples. Stock solutions of the cyclic metabonate (10 mg/100 ml) were prepared in 0.1 M phosphate buffer of the appropriate pH just prior to use. One ml of each stock solution was added to culture tubes and at time t = 0, 0.1 ml of acetaldehyde-^{14}C stock solution was added using a Fibrosystem dispensing pipette. Samples were removed from the shaker-water bath

after 10, 20, 30, 40, 50, 60, 70, 80, 90, 120, 180, 240, 300 and 720 minutes. Each reaction was quenched with the addition of 0.5 ml of 40% sodium bisulfite solution followed by shaking each culture tube manually for 0.2 min. Then the cyclic metabonate was extracted by shaking with 1.5 ml of purified benzene for 0.5 min. The mixture was allowed to settle 0.8 min, and then 400 μl of the benzene was transferred by disposable pipette to a counting vial. Ten ml of Aquasol was added to each vial and radioactivity determined by scintillation counting.

Duplicate samples containing everything except acetaldehyde-^{14}C were treated in the same manner and used as background. Duplicate samples containing everything except cyclic metabonate were treated identically to determine the excess radioactivity extracted into the benzene layer from remaining acetaldehyde. Duplicate 0-time samples were quenched after addition of acetaldehyde-^{14}C and shaking for 0.1 min. All samples were counted for three 10 min cycles (M.S.E. ± 1%); counting efficiency calculated by internal standardization ranged between 91 and 94%.

To insure that the radioactivity being recorded was due only to cyclic metabonate, duplicate 0-time, 60 min, and 300 min sample extracts from a run at 26°, pH 7.4, were evaporated under a gentle stream of nitrogen and recrystallized (3-4 times from purified pet ether) to constant sp. act. with 100 mg of pure non-radioactive cyclic metabonate. Each scintillation counting was made on 10 mg of the recrystallized material. Results indicate that 97 ± 2% of the

radioactivity determined by the extraction procedure used is accounted for by the cyclic metabonate. Glc analysis of 400 μl aliquots from several time periods also indicated that the extractions consisted of pure cyclic metabonate.

Calculations of the amount of exchange at each time period were made as described by Duffield and Calvin[16] on the basis of the amount of radioactivity incorporated between the 0-time sample and the sample in question (x_t). The maximum value for exchange is the end point (x_∞). Calculations were then made of the quantity one hundred minus the percent exchange at the time period in question ($100 - \frac{x_t}{x_\infty} 100$). The log of this value was plotted against time (t) as shown in Figure 7, after a standard linear regression analysis of the data to determine slopes, intercepts, and correlation coefficients.

3. Microsomal Incubations

a. Materials

Doubly-labeled lidocaine (^3H and ^{14}C) was diluted with unlabeled lidocaine and recrystallized three times to constant sp. act. from purified pet ether (^3H sp. act. 0.057 μCi/μmole, ^{14}C sp. act. 0.011 μCi/μmole). NADPH was purchased from Worthington Biochemicals, Freehold, N.J. Homogenization was performed in a Potter-Elvehjem glass tube and Teflon pestle with 0.10-0.15 mm clearance. Centrifugation was carried out in polycarbonate tubes (1 in x 3.5 in at 10,000xg

and 5/8 in x 3 in at 105,000xg) in a Beckman L2-65B refrigerated ultracentrifuge. Incubations were carried out on an AO Model 2156 shaker-constant temperature water bath at 37 ± 0.5°. All solvents were purified to remove traces of aldehydes.[13,38] Tlc separations were made on Eastman #6060 silica gel GF plates 20 x 20 cm. Glc analyses and scintillation counting were carried out as previously described, p. 173.

b. Animal and Tissue Preparation

Three male Sprague-Dawley rats (130 ± 2 g) were stored in a large stainless steel cage for 7 days with sufficient food and water supply. The rats were programmed to a 12 hour on-off light cycle[39] and on the morning of the last day they were weighed (all weights ranged between 140 and 190 g for all runs). The animals were decapitated using a guillotine and exsanguinated. They were immediately carried to a cold room (0 ± 4°) and the livers excised and weighed (total weight of livers 20-25 g). After mincing the livers with scissors, homogenization was carried out in 3 volumes of cold 1.15% KCl-0.01 M sodium phosphate buffer using 5 upward and downward strokes of the pestle in 50-60 sec with the pestle attached to a stirring motor.

The large polycarbonate tubes were filled with cold homogenate and centrifuged for 20 min at 10,000xg (9500 rpm) in a pre-cooled size 42.1 rotor head. All centrifugation was carried out at 0 ± 2°. The supernatant was transferred to the small centrifuge tubes using a

pre-cooled syringe and centrifuged at 105,000xg (34,000 rpm) for 60 min in a size 50 T1 rotor head. This supernatant was discarded and the microsomal material resuspended in an equal volume of cold 1.15% KCl-0.01 M phosphate buffer by manually homogenizing (5 strokes) in the homogenizer. The resuspended microsomes were centrifuged again at 105,000xg for 60 min and finally resuspended in the same volume of isotonic buffer.

c. Protein Determination

Protein determinations were performed before each incubation on 1 ml of the microsomal suspension diluted to 100 ml with distilled water. The Lowry method outlined in LaDu[40] was used. The microsomal suspension was then diluted with the appropriate amount of KCl buffer to obtain a concentration of 5.0 mg protein/ml.

d. Incubation

To 25 ml erlenmeyer flasks was added in order: 1 ml of a stock solution of doubly-labeled lidocaine (585 μg/ml, 2.5 μmoles, ~3.16 x 10^5 dpm - ^{3}H and ~6.11 x 10^4 dpm - ^{14}C); 1 ml of a 0.1 M sodium phosphate buffer, pH 7.4; 1.5 ml of 1.15% KCl-0.01 M phosphate buffer; 1 ml of a freshly prepared solution of NADPH (5 mg/ml) in 0.1 M phosphate buffer. This 4.5 ml of solution was preincubated for 5 min (37°) followed by the addition of 0.5 ml of the pre-incubated (5 min) microsomal suspension to give a final volume of 5 ml/flask,

0.5 mg protein/ml, and 0.5 mM in substrate. Incubations were conducted in open flasks at 37° and 120 oscillations/min for 15 min. Reactions were terminated by pouring the contents of the flask into 20 ml culture tubes containing 10 ml purified methylene chloride and 0.5 ml of 0.25 N NaOH to bring the pH to 8.5. Control incubations were carried out using duplicate samples of everything but substrate, and duplicate samples of everything but microsomes.

e. Isolation and Quantification of Metabolites

The methylene chloride extraction was repeated twice with centrifugation at 800xg for 5 min to break emulsions. The combined extracts were filtered through anhydrous sodium sulfate and rotary evaporated at 20°. The residue was taken up in 200 μl of purified methanol and one half of each sample was counted in 10 ml Aquasol. The other half of each sample was subjected to tlc in two dimensions using 70:30:10 cyclohexane:chloroform:methanol to separate MEGX (rf = 0.37) from both lidocaine (rf = 0.82) and cyclic metabonate (rf = 0.77). The top half of the tlc plate was cut out and developed with 55:45 benzene: chloroform at right angles to the original development. This separated the cyclic metabonate (rf = 0.15) from lidocaine (rf = 0.43). Although MEGX and lidocaine could be distinguished under uv, the cyclic metabonate could not be easily visualized without the addition of cold cyclic metabonate as a standard. Each separated spot was then cut out,

dissolved in a small amount of methanol and quantitatively determined by scintillation counting.

To insure that the tlc regions being counted were free from other radioactive products, the following controls and experiments were run:

1) A second set of duplicate samples was separated by tlc and each radioactive area extracted with purified methanol and subjected to glc on 3% OV-17.

2) Duplicate radioactive lidocaine samples were incubated with all components except microsomes, and extracted and chromatographed two-dimensionally as previously described. The lidocaine, MEGX, and cyclic metabonate rf areas were then counted and subjected to glc as before. In one sample, cold MEGX and cyclic metabonate were added to insure that the proper tlc areas were being extracted.

3) Radio-labeled MEGX (^{14}C and ^{3}H) was incubated with everything but microsomes, then cold cyclic metabonate added and the extract subjected to identical extraction, tlc, and counting procedures as previously described.

All of these controls showed that the cyclic metabonate was formed during the incubation and not an artifact of the experimental conditions.

The bottom half of each tlc plate was developed in the second dimension with 90:5:10 benzene:cyclohexane:methanol to separate possible minor metabolites. This procedure was carried out both with and without the prior addition of non-radioactive carrier for better uv visualization. After final development, all areas were cut out, placed in scintillation

vials with 400 ul of purified methanol, and shaken manually for 1 min followed by the addition of 10 ml of Aquasol. All samples were then subjected to scintillation counting and efficiency determinations. Controls were run by adding all of the cold metabolites to extracts from incubations of lidocaine without microsomes and carrying out the same tlc isolation and counting procedures. This insured that no stray radioactivity was being counted in the various separated regions.

Extraction recoveries from the microsomal suspension were 78-80% based on the amount of radioactivity initially present in each incubate and the amount recovered. The remaining 20% was water soluble material of undetermined composition. Recoveries from both control and incubate tlc plates was 80-85%.

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CHAPTER IV

SPECULATION ABOUT PATHWAYS IN LIDOCAINE METABOLISM

A. INTRODUCTION

The purpose of this chapter is to assemble the information presented in Chapters I, II and III concerning lidocaine metabolism, and speculate about possible biochemical pathways involved in the sequence. Hopefully, the consequences of this course of action will be to:

1) clarify the known basic routes of *in vivo* lidocaine metabolism
2) generate enthusiasm for research into the vague and otherwise unresolved areas
3) serve as a transition between *in vivo* lidocaine metabolism and more general theories of microsomal metabolism of nitrogen containing compounds, the topic to be discussed in the final chapter.

B. KNOWN ROUTES OF LIDOCAINE METABOLISM

Although the quantities of metabolites vary greatly from species to species, certain basic routes of biotransformation have been established for lidocaine.

1. Oxidative Pathways

The primary route of metabolism in all species studied, including man, is N-deethylation (refer to Chapter I and references therein). Other primary oxidative routes include oxidation to yield phenols of lidocaine and MEGX, and possibly the N-hydroxyamides of each. Oxidation of 2,6-dimethylaniline to the p-hydroxy compound is also significant, especially in man. Oxidation of the aromatic methyl groups (benzylic oxidation) and of the tertiary amine group to an N-oxide have not been found, although these metabolites have been looked for in human urine. All of these oxidations can occur in liver microsomes, and most are cytochrome P-450 dependent.[1,2,3]

2. Hydrolytic Pathways

In contrast to other species studied (possibly with the exception of the Rhesus monkey), 60-70% of lidocaine metabolism in man appears to involve hydrolysis of the amide linkage to presumably yield 2,6-dimethylaniline and N,N-diethyglycine from lidocaine, and 2,6-dimethylaniline and monoethylglycine from MEGX. This seems surprising considering the chemical stability of this sterically hindered amide towards either acid or base hydrolysis.[4] However, a microsomal amidase was partially purified from rabbit liver by Hollunger[5] which rapidly hydrolyzed MEGX, and slowly hydrolyzed lidocaine.

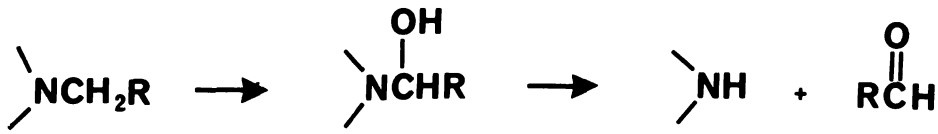
3. Conjugations

Secondary metabolic routes include conjugations of the phenolic and phenolic-like (N-hydroxyamides) metabolites of lidocaine to form glucuronides and sulfates. All of these basic reactions are shown in Scheme 1.

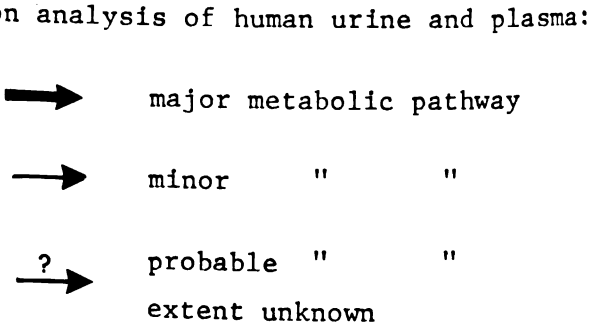
C. SPECULATION ABOUT BIOCHEMICAL MECHANISMS INVOLVED IN METABOLISM OF LIDOCAINE

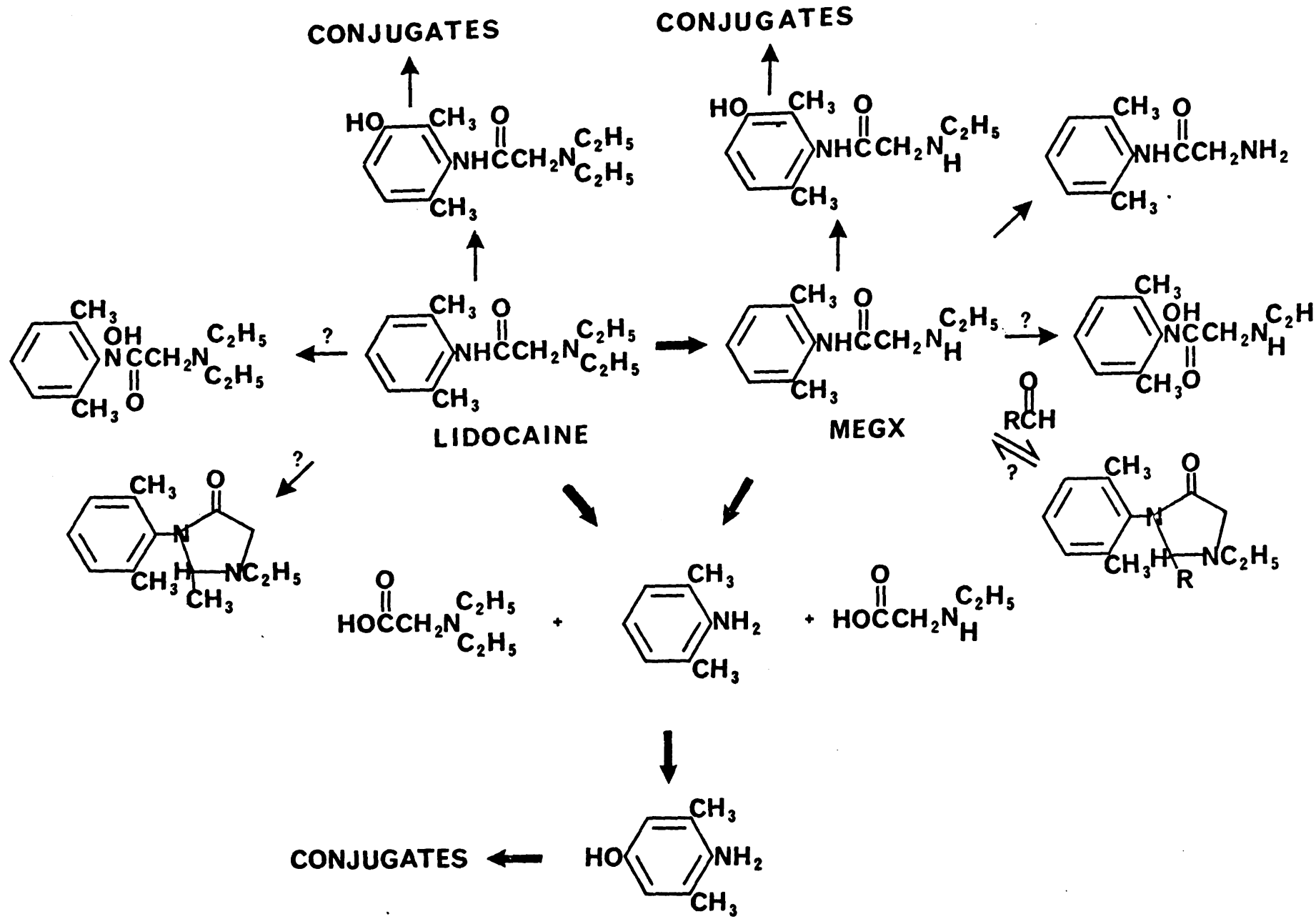
1. Possible Routes to N-Dealkylation

The bulk of evidence to date indicates that the N-dealkylation of amines results from cytochrome P-450 mediated direct attack of oxygen on the α-C-H bond to produce an unstable carbinolamine intermediate which then breaks down to dealkylated amine and an aldehyde.[6]

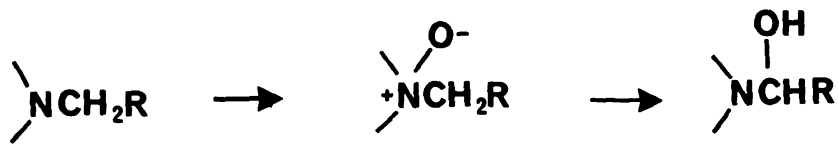


Scheme IV-1. Basic pathways of lidocaine metabolism.




CONJUGATES
CONJUGATES
LIDOCAINE
MEGX
CONJUGATES
?
?
?
?
RCH
+
+

Other evidence suggests that N-oxidation may occur first, followed by an intramolecular Polonovski-type rearrangement to give the carbinolamine.[7,8]



Our results with lidocaine and lidocaine N-oxide do not clarify the issue, but they seem to indicate that direct C-oxidation is operative. Lidocaine N-oxide was not detected in human urine as a lidocaine metabolite, although rearrangements of the N-oxide did occur under mild conditions leading to the formation of several products some of which are lidocaine metabolites, i.e., MEGX, cyclic metabonate, and lidocaine itself. This is in accord with evidence presented by Craig, et al.,[9] on similar rearrangements of nicotine N-oxide to produce 6 products which are nicotine metabolites.

2. Potential Significane of Arene Oxide Intermediates

In recent years the intermediacy of arene oxides in aromatic hydroxylation reactions has been well-established.[10] Assuming that

lidocaine is oxidized by microsomes in a similar manner, the intermediate arene oxide has certain special properties. First, it can be converted to m- and p-hydroxylidocaine, both of which have been found as metabolites in man and other animals.[11, 12] Based on electrophilic substitution reactions of this aromatic system* and on intermediate carbonium ion stability upon cleavage of the epoxide to the oxocarbonium ion (considered to be the rate-determining step in formation of certain phenols from arene oxides),[13] one would predict that m-hydroxylidocaine would predominate. This appears to be the case in man.[11]

Secondly, the arene oxide can be visualized as playing a role in amide hydrolysis. In man the major terminal biotransformation products of lidocaine appear to be conjugates of 4-hydroxy-2, 6-dimethylaniline and the N-substituted amino acids, N, N-diethylglycine and monoethylglycine, which arise from hydrolysis of the amide linkage. Considering the great stability of the amide bond in lidocaine towards chemical hydrolysis,[4] some sort of activation process of this linkage must take place for hydrolysis in the enzymatic system. The activation energy for hydrolysis of the amide linkage in the intermediate arene oxide is lowered in two ways. In the first place, the oxiryl group has been shown to be electron-withdrawing (σ = 0.14),[14] which makes the carbonyl group somewhat

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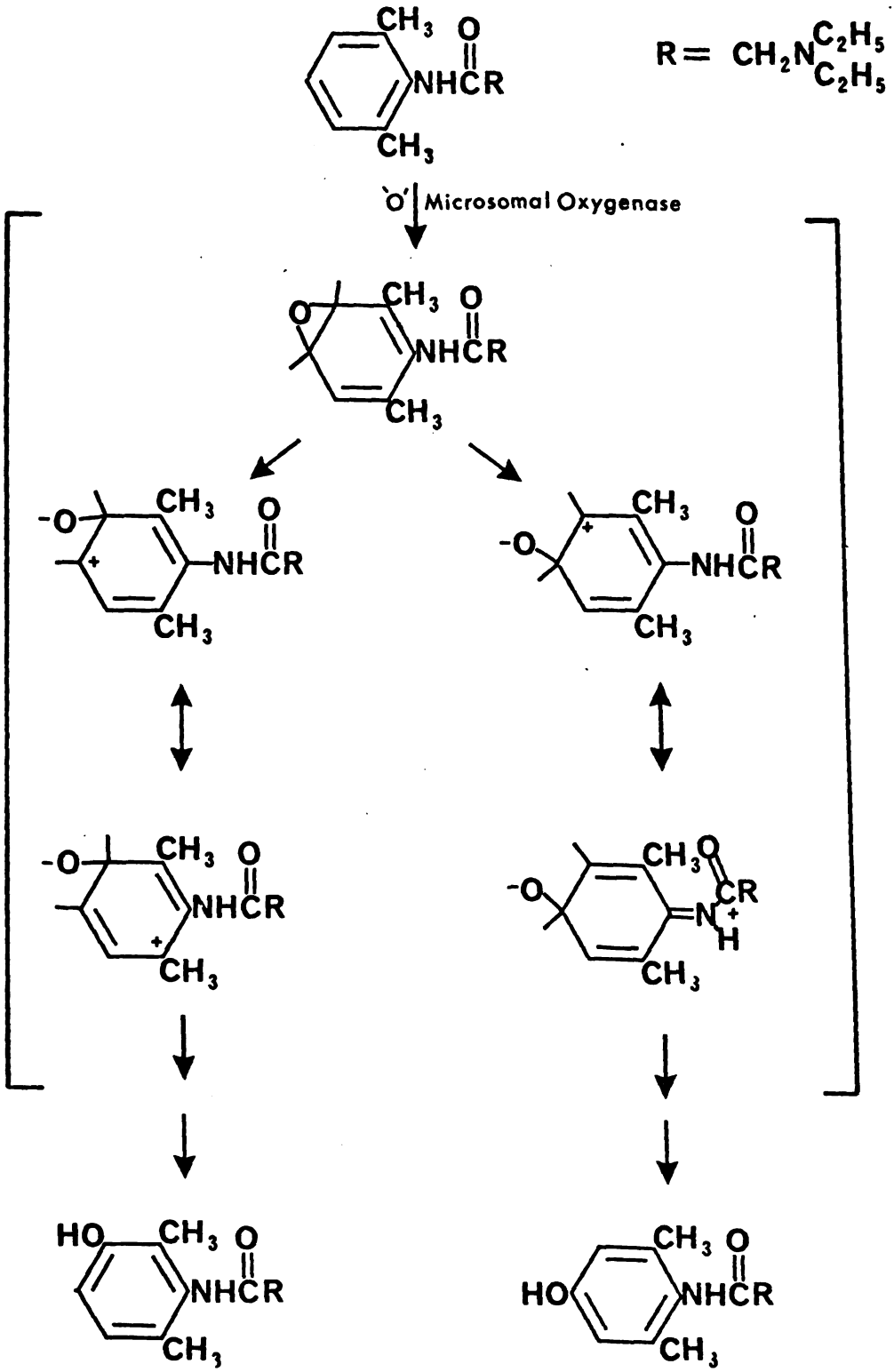
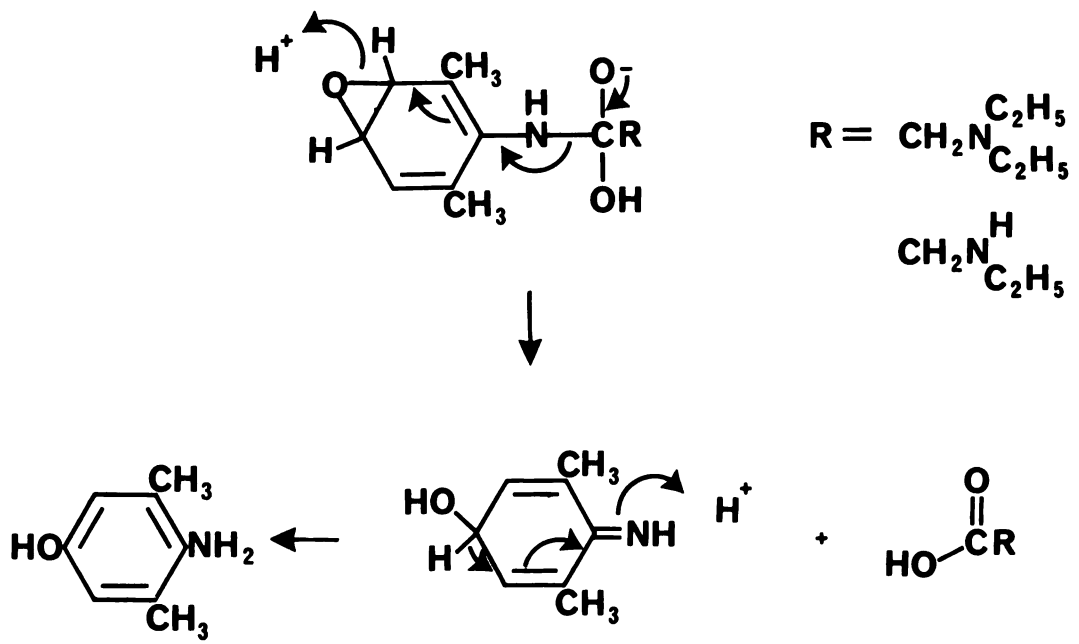
*The two aromatic methyl groups must sterically hinder amide resonance stabilization of any carbonium ion intermediate so that the aryl methyl groups dominate. Note that lidocaine gives m-nitrolidocaine when subjected to electrophilic nitration.[11, 12] Refer to Scheme 2.

Scheme IV-2. Possible aromatic oxidation pathway for lidocaine.


R= CH2N C2H5 C2H5
'O' Microsomal Oxygenase

more susceptible to nucleophilic attack. Secondly, hydrolysis of the amide linkage in the arene oxide will lead to formation of a tetrahedral intermediate which can rapidly tautomerize to the more stable aromatic system as shown below. This provides a driving force for the hydrolysis and will produce the requisite products.



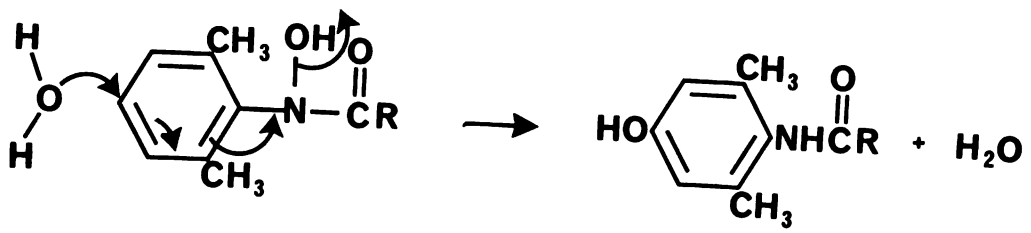
One such system which has already been identified in microsomes is the cytochrome P-450 oxidase-epoxide hydrase couple.[10] The proposed oxidation-hydrolysis mechanism might help explain why trimecaine (p-methyl-lidocaine) has such a long duration of action.[15, 16] The

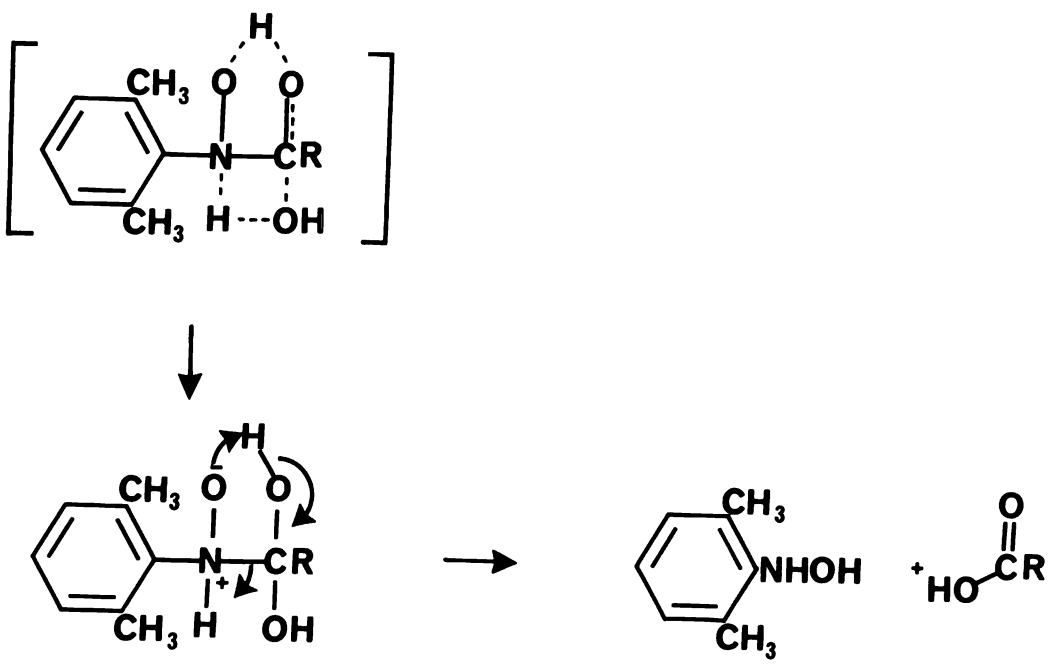
additional methyl group in the para position would not be expected to have any great effect on a straight-forward amide hydrolysis, but could greatly effect the rate of formation of arene oxide and re-tautomerization in the oxidation-hydrolysis mechanism.

3. Potential Significance of N-Hydroxyamides

Rearrangements of aromatic hydroxylamines to ortho- and para-aminophenols were recognized by Bamberger[17] as early as 1894. In biological systems, various workers have shown that hydroxylamine derivatives, such as N-hydroxyamides can rearrange to phenols. The Millers[18] demonstrated in rat that both 1- and 3-hyroxy-N-2-fluorenylacetamide could be derived from N-hydroxy-N-2-fluorenylacetamide. Booth and Boyland[19] showed that a soluble fraction from rabbit liver converted several N-hydroxyamides to ortho-acetamidophenols. Although the N-hydroxyamides of lidocaine and MEGX have no ortho-position available for such an enzymatic conversion, the small amounts of p-hydroxylidocaine found by Thomas and Meffin[12] in human urine after an oral dose of lidocaine may simply result from an intermolecular Bamberger-type rearrangement such as that shown on page 196.

The N-hydroxyamides also provide an activated intermediate for amide hydrolysis. This results from increased polarizability of both the carbonyl C-N and C=O bonds through the N-OH bond as depicted on p. 196, and will therefore lower the activation energy for hydrolysis.


H
O
H
CH3
OH
O
N
CR
CH3
CH3
HO
NHCR
O
CH3
+ H2O


H
CH3
O
O
N
CR
CH3
H---OH
H
CH3
O
O
N
+
CR
CH3
H
OH
NHOH
CH3
+HO
CR
O

Similar mechanisms involving internal general acid-base catalysis have been suggested for similar reactions only in the reverse direction.[20] The 2, 6-dimethylphenylhydroxylamine can then further rearrange to p-hydroxy-2, 6-dimethylaniline as discussed in Chapter II.

In conclusion, we must truthfully say that although most of the major metabolites of lidocaine have been determined, much still remains unanswered about the biotransformation process involved. Hopefully, future work will resolve some of the ambiguities in these still poorly understood pathways so that the results may be applied to the search for an orally effective antiarrhythmic agent with fewer toxicities.

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CHAPTER V

MECHANISMS IN MICROSOMAL NITROGEN METABOLISM

A. INTRODUCTION

Discussion to this point has concerned the metabolism of lidocaine in terms of the various products formed. This final chapter will consider more fully the problem of how the substrate may be converted to product, with attention primarily focused on intermediates in the microsomal metabolism of the various nitrogen containing function groups involved. Obviously, before any proposals concerning specific pathways are made, the information available about microsomal oxygenases should be carefully reviewed. This review will be followed by a rather esoteric application of molecular orbital theory in probing some possible intermediates in microsomal amine metabolism. The question of N-dealkylation will be further analyzed using some results of microsomal experiments with specifically deuterium-labeled lidocaine substrates, and finally a few wild schemes will be divulged in an attempt to correlate some of the information.

B. GENERAL THEORIES RELATING TO AMINE OXIDATIONS

Since Garfinkel[1] and Klingenberg[2] first noticed a strong absorption band at 450 nm after reducing a liver microsomal suspension and

subsequently treating it with carbon monoxide, a wealth of information and speculation has been published concerning the nature and properties of both cytochrome P-450 dependent and other microsomal reactions.[3] Most of the available information concerning the oxidation of nitrogen containing compounds can be categorized in the following manner:

1. Nature of Reactants and Products

a. Primary Amines → Hydroxylamines and Oximes

b. Secondary Amines → Hydroxylamines and Primary Amines

c. Tertiary Amines → Tertiary Amine N-Oxides

↓ ↙ ?

Carbinolamines → Aldehyde + Secondary Amine

d. Imines → Oximes

e. Aromatic Amides → N-Hydroxyamides

The oxidations of secondary amines to hydroxylamines and tertiary amines to N-oxides are the only non-cytochrome P-450 mediated reactions. These oxidations are catalyzed by another microsomal flavin requiring enzyme isolated by Ziegler and co-workers.[4]

2. Binding of Substrate to Enzyme

Most primary and secondary amines bind to microsomal cytochrome P-450 in a different manner than tertiary amines which leads to observable differences in the position of maxima and minima in the uv spectra.[5] Most recent studies support the idea that the Type II spectrum observed

with primary and most secondary amines is due to direct ligand interaction of the amine with the iron atom of the cytochrome.[6, 7] Heteroaromatics such as pyridine and imidazole as well as imines[8] also give Type II spectra. The Type I spectra of tertiary amines appears to be caused by binding at some other interacting site.[5] Aromatic amides, and possibly other nitrogen containing compounds, appear to give mixed Type I and Type II spectra or Type I and "Reversed" Type I spectra. This indicates multiple interacting sites which is apparently dependent on both amine group basicity and the nature of the interacting ligand.[9]

3. Cofactors Involved

For maximal activity both NADPH and oxygen are required with the NADPH supplying reducing equivalents in the oxidation. An FAD containing enzyme (cytochrome-C reductase) is also necessary although no exogenous FAD need be added since it is regenerated during the course of the reaction. Likewise, in the oxidation of secondary amines to hydroxylamines and tertiary amines to N-oxides, the flavin-protein oxidase involved requires NADPH.[4]

4. Nature of the Reactive Groups in the Enzymes

a. Ziegler's flavoprotein oxidase has as the main reactive site the flavin moiety with no heavy metal or cytochrome requirement.[4]

b. Coon and associates[11] have separated the microsomal fraction into 3 basic components necessary for activity—a flavin containing

cytochrome P-450 reductase, a cytochrome P-450 containing fraction, and a phospholipid containing fraction. Cytochrome P-450 itself is considered to be an iron containing porphyrin similar to cytochrome-C, with a sulfide group as one of the axial ligands.[12] The cavity in which the substrate binds contains the heme and is apparently hydrophobic as determined by spin-labeling the active site.[13]

5. Nature of the Active Oxygenating Species

Although many model oxidation reactions have been developed to mimic the activity of the microsomal mixed-function oxygenases,[14] none has yielded results which exactly define the nature of the active oxygenating species. From the most recent experimental evidence three lines of thought are emerging, none of which are mutually exclusive:

a. The Oxenoid Mechanism

This mechanism was originally postulated by Ullrich and Staudinger[15] and independently by Hamilton.[16] Basically the evidence for the mechanism comes from the similarity between the many substrates oxidized and their resultant products, and the known chemical peracid oxidations and insertion reactions of carbenes and nitrenes. The reaction is therefore thought to be a concerted electrophilic reaction between an electron deficient oxygen atom and a lone pair of electrons, a π-electron system, or a σ-C-H bond.

b. Superoxide Anion

Evidence for the formation of superoxide anion ($O_2^{\cdot -}$) in cytochrome P-450 mediated oxidations has been reported by three

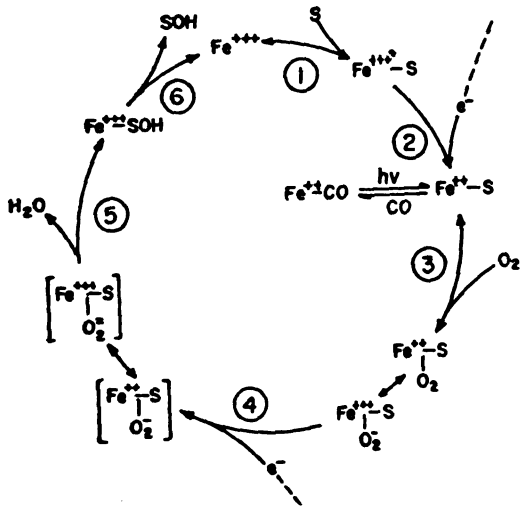
independent groups within the last two years.[17a, b, c] This anion radical can result from a one-electron reduction of iron-bound molecular oxygen. It can then react in an oxenoid manner by either a polarization mechanism or radical mechanism as described by Ullrich and Staudinger.[15]

c. Singlet Oxygen

Discovery of microsomal chemiluminescence induced by NADPH in the absence of oxidizable substrate, suggested to Howes and Steele[18] the presence of singlet oxygen in microsomes. Superoxide anion, by loss of an electron of appropriate spin can yield singlet oxygen ($O_2^{\cdot -} \rightarrow {}^1O_2 + e^-$), and this reaction has been observed chemically with protoporphyrin IX and oxygen.[19] More recent studies[20a, b] have shown that singlet oxygen is probably not involved in oxidation of normal substrates, based on the pattern of oxidation products formed. However, this does not exclude the possibility that in the absence of a suitable substrate singlet oxygen is produced. This singlet oxygen can react with microsomal lipids to produce lipid peroxides which have been detected in the absence of various protecting substances.[21]

6. Isolated Steps in Cytochrome P-450 Mediated Oxidations[22]

a. In the isolated state of microsomes, epr evidence suggests that the cytochrome P-450 is a low-spin ferrichemoprotein, which upon addition of substrate reverts to a high-spin ferrichemoprotein-substrate complex (Step 1) with perturbation of the sulfur ligand, and displacement



A proposed scheme for the cyclic reduction and oxidation of cytochrome P-450 during interaction with oxygen and a substrate.
The valence state of the heme iron of cytochrome P-450 is indicated by the superscript associated with Fe. The organic substrate to be hydroxylated is labeled *S*.

of another postulated but unknown ligand away from the sixth coordination site of the iron.[23]

b. The ferric-substrate complex is reduced by NADPH (Step 2) through cytochrome P-450 reductase to a ferrous-substrate complex. This step requires phospholipid, possibly to insure the stability or mobile nature of an unknown "electron carrier."

c. The ferrous-substrate complex can react with carbon monoxide to form the familiar complex absorbing at 450 nm, or it can react with oxygen to form a ternary complex of heme, oxygen, and substrate (Step 3).

d. What happens in the next sequence (Steps 4, 5 and 6) is not understood, and where the electron enters is not really known. All that is known is that a second one-electron reduction occurs: one atom of the oxygen is reduced to water and the second oxidizes the substrate.

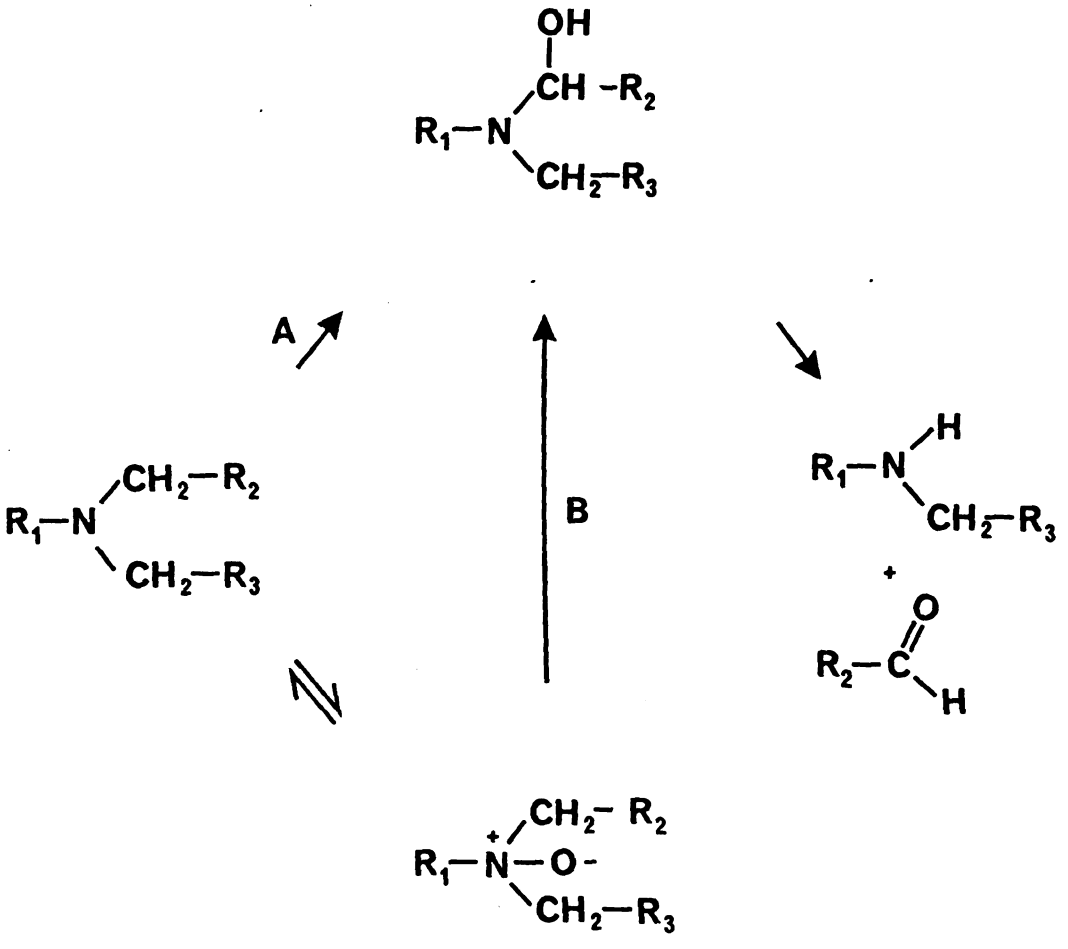
Thus, with this "biochemical Carnot Cycle" completed let's look at some of the intermediate steps involved in amine metabolism.

C. MOLECULAR ORBITAL CALCULATIONS ON SUSPECTED INTERMEDIATES IN OXIDATIVE AMINE METABOLISM

As previously pointed out, many biologically important amines such as nicotine,[24] morphine,[25] and others[26] undergo oxidative N-dealkylation and N-oxidation reactions which often represent primary metabolic pathways for such compounds. These metabolic processes have been the subject of a tremendous amount of research in the last ten years.[27] One area of continuing interest and debate is the oxidative N-dealkylation of a variety of tertiary N-alkyl compounds, possibly with the exception of t-butyl tertiary amines,[28] with regard to the intermediacy or non-intermediacy of N-oxides.[29] The postulated nondetailed mechanisms are shown in Scheme 1. The major question is whether pathway A and/or B is viable <u>in vivo</u>, and if so, to what extent.

There is evidence supporting both mechanisms and the dispute is still unresolved although most evidence favors path A with some recent work by Bickel[30] indicating that both pathways are operative. If the N-oxide is a true intermediate, the rearrangement to carbinolamine must involve an intramolecular migration of the N-oxide oxygen atom to a neighboring carbon atom since the product aldehyde contains ^{18}O when the initial source of oxygen is $^{18}O_2$.[31] N-oxides have been synthesized and are fairly stable unless heated near their melting point at which time

Scheme V-1. Possible pathways for the oxidation of tertiary amines.


OH
CH -R2
R1—N
CH2—R3
A
B
CH2—R2
R1—N
CH2—R3
H
R1—N
CH2—R3
+
O
R2—C
H
CH2- R2
+
R1—N—O-
CH2—R3

they decompose into radicals where one of the possible recombination products is a carbinolamine.[32] Most acyclic carbinolamines spontaneously break down into an amine and an aldehyde. This surprising reactivity of carbinolamines has never been well explained. It was hoped that some insight into the process of N-dealkylation might be gained by looking into the electronic distributions and relative stabilities of postulated intermediates and products using molecular orbital methods.

We performed CNDO/2 calculations[33] on trimethylamine N-oxide, N, N-dimethylcarbinolamine, the isomeric N-methyl-N-ethylhydroxylamine, and formaldehyde plus dimethylamine (Figure 1). Hydroxylamines were included in the calculations since certain secondary and primary amines are known to be metabolized to hydroxylamines,[34] and because we wished to compare the energies of the various C_3H_9NO isomers. The conformation and bond distances for trimethylamine N-oxide were taken from crystal structure data;[35] for the hydroxylamines, the O-H was taken to be <u>cis</u> to the nitrogen lone pair, as found by Radom, et al.,[36] and Giguere and Liu;[37] for the carbinolamines the completely staggered conformation, with the OH <u>trans</u> to the nitrogen lone pair was found to be the lowest energy. The atomic populations calculated are relatively insensitive to conformational changes involving rotation around single bonds. As can be seen, the N-oxide was found to be some 41 Kcal/mole less stable than the corresponding carbinolamine. The N-oxide has a high electron density on the oxygen atom (Mulliken population on $O \equiv \zeta\ (0) = 8.504$) with the positive charge smeared primarily over

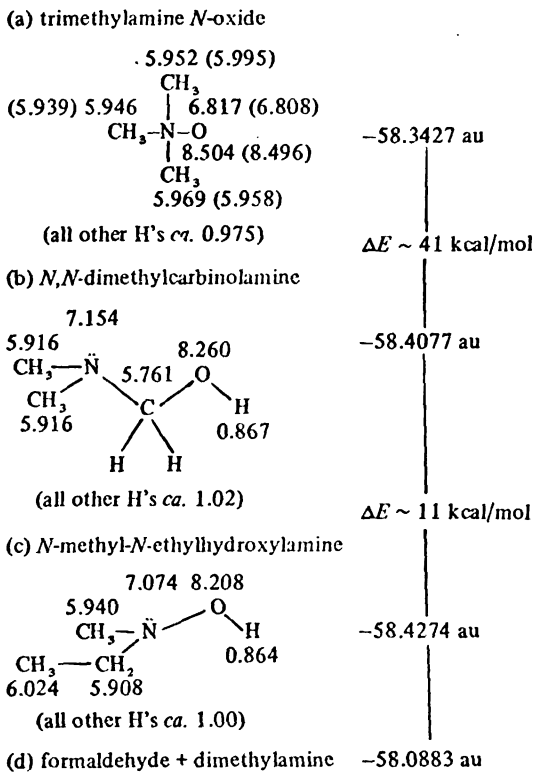


Figure 1. Mulliken atomic populations and energies of C_3H_9NO isomers calculated by the CNDO/2 method. Bond lengths and angles for trimethylamine *N*-oxide from ref 35. All C-H distances 1.083 Å with other bond lengths and angles taken from "Handbook of Chemistry and Physics," 51st ed, R. C. Weast, Ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1970, pp F-154 ff. Numbers in parentheses for the N-oxide are charge densities with the molecule solvated.

the nitrogen and hydrogens of the rest of the molecule. Since the carbon atoms bear little of the positive charge, $\Delta H^{\dagger}$ for the intramolecular transfer of oxygen from the nitrogen to the carbon atom would probably be significant. Thus, from a kinetic point of view, there would appear to be no a priori tendency for intramolecular rearrangement. However,

perturbing the system could alter the situation and a mechanism whereby the oxygen with its excess negative charge would polarize one of the C-N bonds during attack on the carbon is conceivable. As the oxygen approached the carbon, the carbon would gradually transfer charge to the nitrogen, becoming more electrophilic and facilitating C-O bond formation. On the other hand, in the thermodynamically more stable carbinolamine, the carbinol carbon is very electron deficient which could render this intermediate quite reactive towards fragmentation to formaldehyde and dimethylamine, a fact which is observed experimentally. Although fragmentation to formaldehyde plus dimethylamine appears to be an energy requiring process from the calculations, the results in Figure 1 do not accurately describe the thermodynamics of the reaction since no term corresponding to the energetically favorable entropy change is included in the calculations. *

These electronic structure calculations were carried out on the gas phase molecules and one needs some estimate of the effects of solvation on both the relative energies and the charge distribution of such species. In aqueous solution the very large negative charge of the N-oxide oxygen should allow considerable solvation energy due to the protons of nearby water molecules. We estimate a solvation energy of approximately

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*The difference in energy between the carbinolamine and the aldehyde plus amine is greatly exaggerated in the CNDO/2 studies; the ab initio studies of the CH_5NO isomers more accurately reflect these energy differences. Radom, et al.,[36] find the CH_5NO carbinolamine to be 14 kcal/mol more stable than formaldehyde and ammonia, thus the difference is less but still does not include the favorable entropy change.

40 kcal/mole from this interaction.* Moreover, the carbinolamine and hydroxylamine should form four H-bonds of a total strength of approximately 15-20 kcal/mole.† CNDO/2 calculations on the solvated trimethylamine N-oxide with three waters approaching the oxygen indicate little change in the charge distribution of the N-oxide (charge densities in parentheses in Figure 1). At neutral pH, the carbinolamine will be largely protonated, increasing its solvation energy.

From the above analysis, it appears unlikely that solvation will change the relative energetics of the carbinolamine and N-oxide, although the energy gap may be smaller. It is of interest that an empirical estimate of the energy difference between the carinolamine and the N-oxide predicts the carbinolamine to be more stable by 51 kcal/mole[39] and it is in accord with the relative stabilities predicted by this theoretical treatment.

The electronic structure of the protonated carbinolamine is similar to the neutral species, with most of the positive charge shared by the nitrogen and the methyl and methylene hydrogens. Surprisingly, the Mulliken population on the carbinol carbon increases slightly from 5.761 to 5.785 electrons upon protonation. An alternative rationalization of the carbinolamines susceptibility to fragmentation might be provided by

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*This is slightly more than half the solvation energy of OH^- in the gas phase due to three water molecules.[38] Since the oxygen in the N-oxide is considerably less negative than that in OH^-, one would expect weaker hydrogen bonds with water.

†A reasonable average H-bond strength for the four hydrogen bonds is 5 kcal/mole, the N···HOH being stronger and the OH···OH_2 weaker.

the relative bond orders of the N-oxide, hydroxylamine, and neutral and protonated carbinolamine. The ab initio C-N bond orders for the CH_5NO isomers are 0.610, 0.645, and 0.660 for the N-oxide, hydroxylamine, and carbinolamine, and the corresponding CNDO/2 results for C_3H_9NO isomers are 0.625, 0.636 and 0.647. Protonation of the carbinolamine lowers its bond order to 0.616. This change in bond order is small and not much can be made of this difference; however, good empirical evidence that protonation is an important step in carbinolamine breakdown comes from the stability of amide[40] and carbazole[41] carbinolamines, both of which have much less basic nitrogens.

Thus, the evidence appears strong in support of the thermodynamic instability, but kinetic stability (observed empirically) for the N-oxide. These electronic structure calculations provide some rationalization why the N-oxide is relatively kinetically stable and the carbinolamine very difficult to isolate. The calculations cannot, however, answer the question of whether the N-oxide is an intermediate in vivo. Even if one could carry out a precise potential surface study for the oxygen transfer in the isolated molecule, this question could not be answered with any confidence, because neither the mechanism for oxygen transfer nor the solvation effects on the transfer is known.

To get a more precise estimate for the energy difference, ab initio calculations were carried out using an STO-3G basis set[42] as well as CNDO/2 calculations on the isomers of formulae CH_5NO. The charge distribution and relative energies for these species are presented in Figure 2.

Figure V-2. Mulliken atomic populations and energies of CH_5NO isomers.

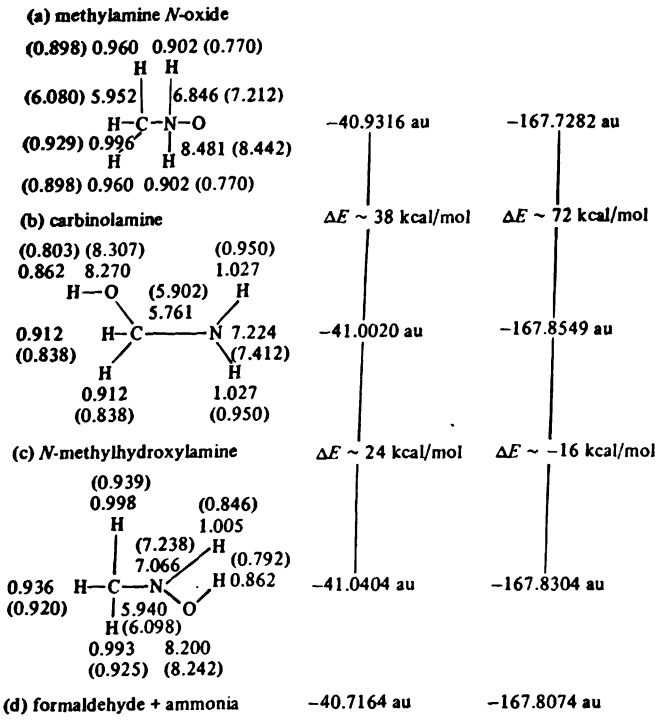


Figure 2. Mulliken atomic populations and energies of CH_5NO isomers found in the CNDO/2 and *ab initio* calculations. Molecular conformations and bond lengths and angles are the same as those of the corresponding C_3H_9NO isomers. The numbers in parentheses are the Mulliken populations from the *ab initio* calculations.

The charge distributions predicted by the ab initio and CNDO/2 methods are very similar, but the ab initio energy difference between the N-oxide and carbinolamine is almost twice as large as that predicted by CNDO/2, supporting previous conclusions on the relative energies of the two types of species. The relative energies of hydroxylamine and carbinolamine are reversed. More precise ab initio calculations support the greater stability of the carbinolamine than the hydroxylamine; Radom et al.,[36] find an energy difference of 38 kcal/mole between the two species. The CNDO/2 and ab initio charge distributions, however, show why the carbinolamine is kinetically unstable. The ab initio atomic populations (Figure 2 in parentheses) show the same trends as the CNDO/2, but the hydrogens generally have less charge and the "heavy" atoms, C, N and O more charge in the ab initio calculations.

A CNDO/2 calculation on the trimethylamine N-oxide radical cation showed that the majority of the charge loss was on the oxygen, [ζ (O) = 7.99), ζ (N) = 6.79, ζ (C) = 5.96], with the unpaired electron spending considerable time on the methyl carbons. Thus, a possible radical mechanism for the N-oxide to carbinolamine is quite feasible. This seems more likely than heterolytic cleavage and recombination, although a definitive statement on this cannot be made with these calculations. It is noteworthy that free radicals have been observed using CIDNP in thermal rearrangements of N-oxides, such as the Meisenheimer rearrangement.[43]

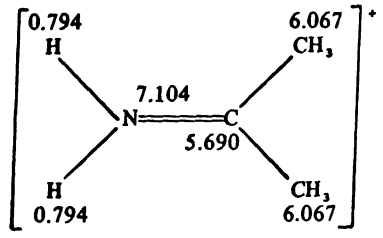
Recent work on oxygenation mechanisms of xenobiotics by microsomal mixed function oxygenases have indicated that epoxides are intermediates in certain aromatic hydroxylations.[44] Although no such intermediates have been detected in amine oxidations, one might speculate that imminium ions or ion radicals may be generated in microsomal systems by some form of hydrogen abstraction with subsequent oxygenation to reactive oxaziridinium ions or ion radicals. Results of CNDO/2 calculations on N, N-dimethyl imminium ion, isopropyl imminium ion, and N, N-dimethyloxaziridinium ion are shown in Figure 3.

The postulate of an intermediate oxaziridinium ion is not completely unfounded considering the work of Parli, et al.,[45] who alternatively suggested a direct cytochrome P-450 mediated oxidation of an imine to an oxime. Although none of the recovered products of the in vitro oxidation was an oxaziridine, one might speculate that such a species may be an intermediate as outlined in Scheme 2. As shown, the mechanism might involve oxidation to the oxaziridine followed by "protonation" at either the oxygen or nitrogen, possibly by an enzyme as suggested by Watabe and Suzuki[46] for the hydrolysis of aziridines.

Another possibility for the breakdown of the oxaziridine not shown in Scheme 2, but suggested by Watabe and Suzuki's work on aziridines, is attack by other nucleophiles such as a hydroxyl group of water to give directly a carbinol hydroxylamine which could then dehydrate to give

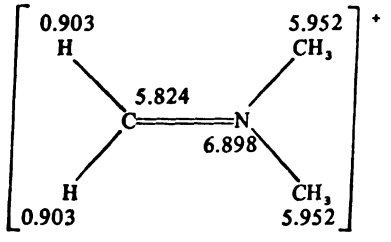
Figure V-3. Mulliken atomic populations and energies of imminium and oxaziridinium ions.

(a) isopropylimmonium ion



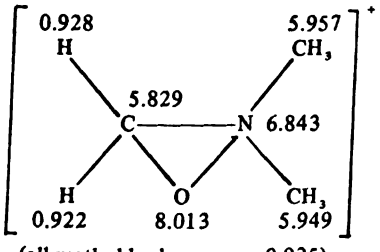
−38.7337 au

(b) trimethylimmonium ion



−38.7352 au

(c) *N,N*-dimethyloxaziridinium ion

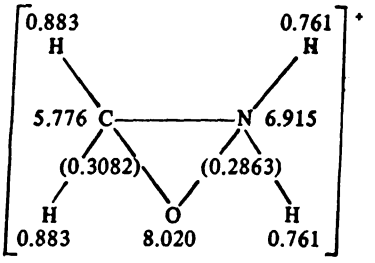


−57.3205 au

(all methyl hydrogens *ca.* 0.925)

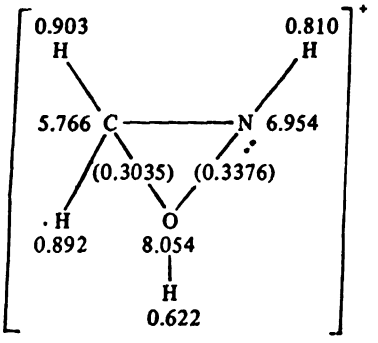
Figure 3. Mulliken atomic populations and energies of immonium ions and an oxaziridinium ion calculated by the CNDO/2 method.

(a) oxaziridinium ion (N-protonated)



−39.9237 au

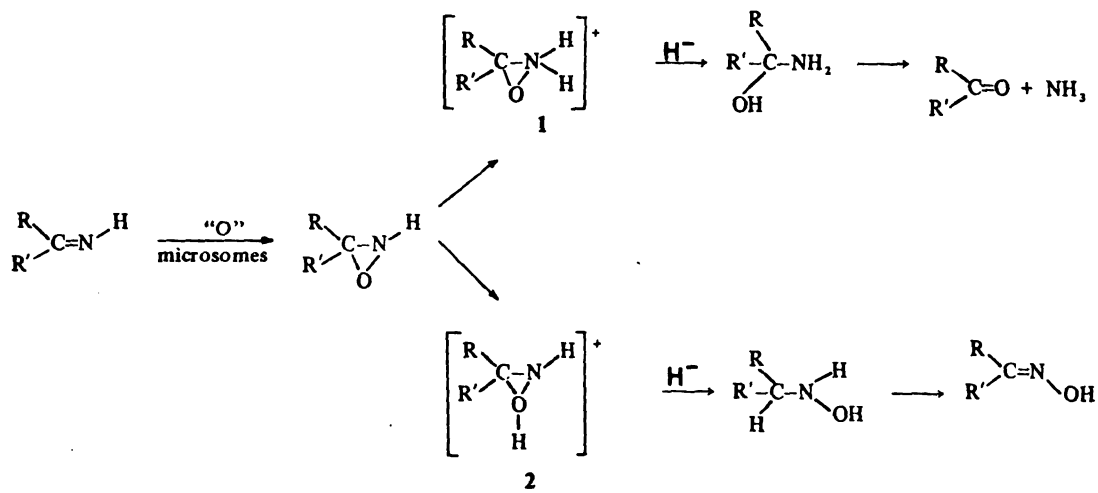
(b) oxaziridinium ion (O-protonated)



−39.8331 au

Mulliken atomic populations and energies and the C–O, N–O bond orders (in parentheses) of N- and O-protonated oxaziridinium ion. The oxaziridine geometry used in the calculations was taken from J. M. Lehn, B. Munsch, Ph. Millie, and A. Veillard, *Theor. Chim. Acta*, **13**, 313 (1969).

Scheme V-2. Postulated oxaziridine formation and breakdown.


"O"
microsomes
H⁻
H⁻
1
2
+ NH₃

oxime or ketone. The CNDO/2 calculations (Figure 3) of the two protonated species 1 and 2 indicate favored N-protonation and differences in the C-O vs. N-O bond orders (numbers in parentheses) which might lead to preferential opening of the protonated oxaziridine as shown in Scheme 2 to ultimately produce the observed ketone and oxime.[45, 47, 48]

The substituent effects on imminium ions are very interesting. Substituting methyl groups on the positive carbon (isopropyl imminium ion) makes the carbon more positive relative to the unsubstituted imminium ion, where ζ (C) = 5.75 and ζ (N) = 7.01,[49] and the nitrogen more negative. Substitution of methyl groups on nitrogen makes the nitrogen more positive and the carbon more electron rich. These results show that the simple electron-donating inductive model to describe the substituent effect of methyl groups does not work for imminium ions and probably should be applied with caution to any system involving heteroatoms.

In conclusion, these electronic structure calculations on some postulated intermediates in amine metabolism have suggested that if the N-oxide is an intermediate in oxidative dealkylation, it is probably subjected to electron loss prior to forming the carbinolamine, and that oxaziridinium species may be involved in imine oxidation and possibly in N-dealkylation processes.

D. ISOTOPE EFFECTS IN N-DEETHYLATION REACTIONS

1. Introduction

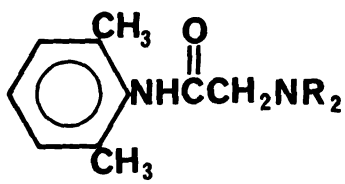
Theoretical calculations[50] have shown that the effects of isotopic substitution on reaction rates are caused by changes in the vibrational frequencies associated with the process of activation of a molecule from its ground state to a transition state. Only two types of isotope effects will be considered here—primary and secondary isotope effects on the breaking of a C-H vs. C-D bond where the effect arises from a difference in the zero-point energy of a C-H and C-D bond.

Primary isotope effects are observed when a bond to the isotopic atom itself is broken in the rate-determining step. The magnitude of a "normal" kinetic primary isotope effect is k_H/k_D = 6-10, but theoretically and experimentally this ratio may vary considerably. In fact, an unusually low or high primary isotope effect may actually assist in a description of the transition state.[51] A secondary isotope effect on the rate of a reaction may be observed when the isotopic atom is in a position (usually near to the reacting center) from which transfer of the atom does not take place, but changes in vibrational frequencies do occur.

Since the early 1960's, isotope effects on reaction rates have been used extensively in probing enzymatic mechanisms both *in vivo* and *in vitro*.[52, 53] Of particular interest to the work presented here,

are deuterium isotope effects observed in enzymatic reactions involving microsomal oxygenases. Mitoma, et al.,[54] found that O-demethylation of o-nitroanisole by rat liver microsomes was accompanied by a k_H/k_D between 1.5 and 2.8 with a 50% decrease in the Km value for the deuterated analog. The same group[55] found an in vivo isotope effect of $k_H/k_D = 2.28$ on the sleeping time of mice given 5-n-butyl-5-ethylbarbituric acid labeled with deuterium in the 3'-carbon atom of the butyl side chain, which is the position oxidized. The in vitro effect with the post-mitochondrial supernatant fraction from mouse liver was $k_H/k_D = 1.59$. They also reported an in vivo $k_H/k_D = 1.47$ when the 4'-position adjacent to the oxidized site was labeled with deuterium. Although the authors could not explain this result, a secondary isotope effect might be involved. Björkhem,[56] studying the 11-hydroxylation of lauric acid by rat liver microsomes, found a primary isotope effect on V_{max} which decreased by a factor of 3.6 with deuterium substitution in the oxidized position, and an effect on the Km which decreased by a factor of 1.5. Finally, Elison, et al.,[57a,b] determined that the N-demethylation of $N\text{-}CD_3$ morphine occurred with an in vitro isotope effect on the initial velocity of $k_H/k_D = 1.40$ and a $Km^D/Km^H = 1.43$. The in vivo isotope effect on analgetic potency in mice ranged from 1.5 to 3.0 depending on the route of administration. The indication from all of these studies is that a C-H bond is being broken in a rate-determining step in the hydroxylation mechanism.

We have now carried out a series of experiments on a rat liver microsomal N-deethylation reaction using lidocaine (1) specifically labeled with deuterium in the α-methylene positions (2 = lidocaine-d_4) and on the β-methyl groups (3 = lidocaine-d_6).



	R
1	CH_2CH_3
2	CD_2CH_3
3	CH_2CD_3

2. Materials and Methods

a. Chemicals

Lidocaine was supplied as the free base by Astra Pharmaceutical Products, Inc. (Worcester, Mass.). Lidocaine-d_4 (89% overall deuterium incorporation determined by EI and CIMS) and lidocaine-d_6 (94% overall deuterium incorporation) were synthesized as previously described in the experimental sections of Chapters I and III, respectively. The 3-methyl-2-benzothiazolone-hydrazone hydrochloride was obtained from Aldrich Chemical Co., Inc. (Cedar Knolls, N. J.). NADP,

glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, Mo.).

b. Tissue Preparation

Hepatic microsomes were obtained for each experiment from three male albino Sprague-Dawley rats (140-190 g). The animals were decapitated and exsanguinated. All further preparations and transfers were carried out in a cold room (0 ± 4°). The livers were removed, weighed, pooled and minced, and suspended in 3 volumes of cold 1.15% KCl-0.01 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogenizer. After centrifugation (Beckman L2-65B) at 10,000xg for 15 min at 0° to remove cell debris and nuclei, the supernatant was recentrifuged (0°) at 105,000xg for 60 min. The microsomal pellet was resuspended manually with the homogenizer in a volume of cold isotonic buffer equal to that of the supernatant discarded, and centrifuged again for 60 min at 105,000xg. The final pellet was resuspended in an equal volume of the 1.15% KCl buffer and protein determined by a modified Lowry procedure[58] on a 1:100 dilution of the microsomal suspension in water. The final microsomal suspension was diluted with the isotonic buffer to a concentration of 5.0 mg protein per ml of suspension.

c. Incubation Mixture

The reaction mixture contained phosphate buffer, pH 7.4, (200 μmoles); magnesium chloride (10 μmoles); NADPH-generating system consisting of glucose-6-phosphate (20 μmoles), glucose-6-phosphate dehydrogenase (5 units), and NADP (2 μmoles); lidocaine, lidocaine-d_4, or lidocaine-d_6 (0-7.5 μmoles); microsomal suspension (0.5 ml); 1.15% KCl-0.01 M phosphate buffer (to give final volume, 5.0 ml).

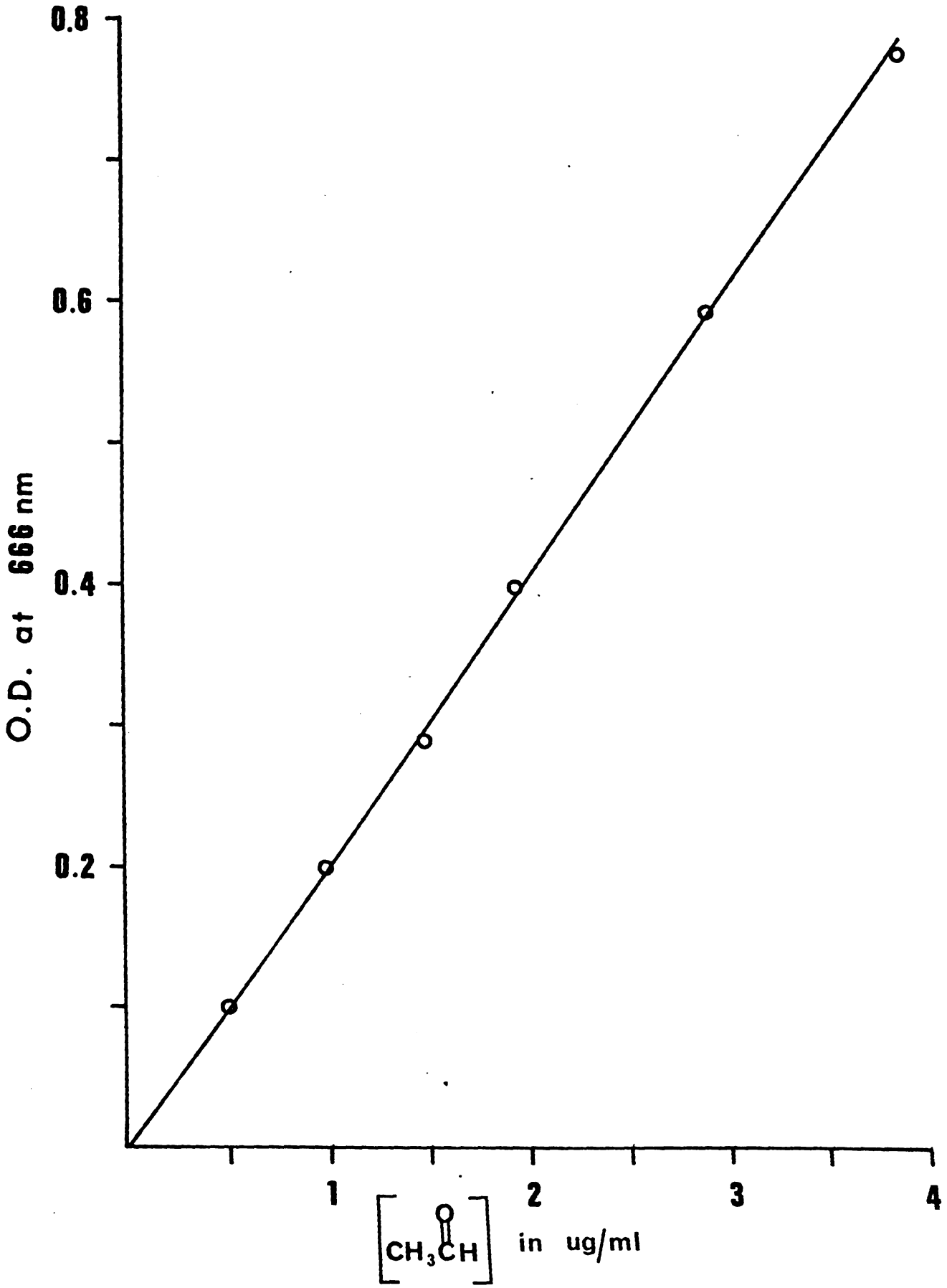
Incubations were conducted in open 25 ml erlenmeyer flasks at 37° and 120 oscillations/min. Incubation times were 7.5 min for Km determinations and 0, 2.5, 5.0, 7.5, 10 and 15 min for initial velocity determinations. Reaction rates were essentially linear for 10 min (linear regression correlation coefficient, $r \geq 0.990$) although determinations were made from 0-7.5 min ($r \geq 0.995$). A solution containing everything but the microsomal suspension was preincubated for 5 min and then 0.5 ml of the preincubated microsomal suspension added to each flask at 10 sec intervals. Reactions were terminated in the same sequence at 10 sec intervals by adding 2 ml of 15% zinc sulfate solution, followed approximately 5 min later by pouring the suspension into 20 ml pyrex culture tubes, each containing 2 ml of saturated barium hydroxide solution. Each flask was rinsed with 1 ml of distilled water.

d. Acetaldehyde Determination

As shown in the microsomal experiments described in Chapter III, the primary mode of metabolism of lidocaine by hepatic microsomes isolated from rat liver involves oxidative N-deethylation of lidocaine to MEGX plus acetaldehyde. The MEGX formed can react further by other mechanisms, therefore, the best method to follow the reaction is to measure the acetaldehyde produced. A convenient, sensitive colorimetric assay was devised, based on the 3-methyl-2-benzothiazolone hydrazone test of Sawicki, et al.[59] The method was linear from 0.25 μg of acetaldehyde formed per ml of incubate to 4 μg/ml with absorbances ranging from 0.06 to 0.76. The usual range of absorbance found necessary for monitoring acetaldehyde production from the N-deethylation of lidocaine was from 0.15 to 0.50 at 666 nm. A standard curve obtained from 0.25 to 4 μg/ml of acetaldehyde in the concentration of buffer used in the microsomal incubations is shown in Figure 4.

The test was performed in the following manner: Each denatured microsomal sample was centrifuged at 2500 rpm for 15 min to impact the white precipitate of barium and zinc salts, and 5 ml ($\frac{1}{2}$) of the supernatant pipetted into an appropriately labeled 50 ml pyrex culture tube. To each sample was added at 30 sec intervals, 2 ml of 0.5% 3-methyl-2-benzothiazolone hydrazone hydrochloride reagent with brisk shaking after each addition. After a 30 min reaction period, 2.5 ml of a 1% ferric chloride solution was added at 30 sec intervals

Figure V-4. A standard curve of optical density (O. D.) at 666 nm vs. acetaldehyde concentration for the 3-methyl-2-benzothiazolone hydrazone test.


0.8
0.6
0.4
0.2
O.D. at 666 nm
1
2
3
4
[CH3CH=O] in ug/ml

in the same order as the first addition. This resulted in the formation of a pale green to dark blue color. After another 30 minute reaction period, 30 ml of a 2:1 mixture of reagent grade acetone: water was added in the same sequence at 30 sec intervals to stop the reaction with subsequent formation of a fine precipitate. This precipitate was separated by centrifugation at 2500 rpm for 5 min, or was allowed to settle overnight in a refrigerator (~ 5°) with retention of 90-95% of the original absorbance. The optical density of each sample was then read (666 nm) against reaction mixtures from each time period containing everything except microsomes (= substrate blanks). A Perkin-Elmer Coleman 101 spectrophotometer was used for the readings.

e. Calculations

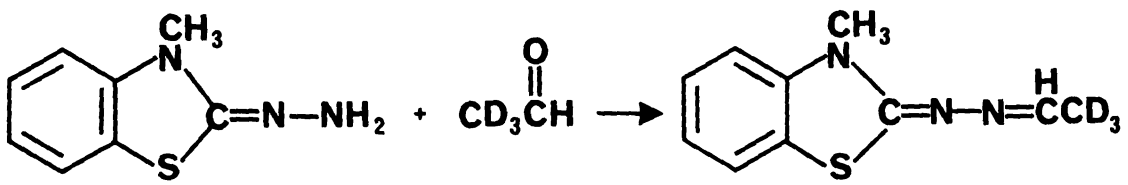
Aldehydes present in the microsomes (0.1-0.6 μg/ml) were determined simultaneously at each time interval using incubation mixtures containing everything but substrate. The quantity of this native aldehyde was subtracted from that value obtained for the reaction mixtures at the same time period. Apparent Km values were determined from the amount of acetaldehyde formed at several substrate concentrations (0.125 mM, 0.250 mM, 0.500 mM, 1.00 mM, and 1.50 mM) during a 7.5 min incubation period. The data was fitted to the equation $S/v = S/Vm + Km/Vm$ by linear regression analysis of S/v on S. Initial velocity determinations were made using the

amount of acetaldehyde evolved at 0, 2.5, 5 and 7.5 min from reactions 1.00 mM in substrate. A least squares fit of the data to a straight line was used to obtain the slopes and thus the k_H/k_D values. All velocities of deuterated substrates were corrected for less than 100% deuterium incorporation (lidocaine-d_4, 89% and lidocaine-d_6, 94%).

f. Mass Spectral Determination of Trapped Acetaldehyde

Because of the rather large isotope effect on the initial velocity using lidocaine-d_6 as the substrate (refer to Results and Discussion), an experiment was carried out to determine if any deuterium was being lost from the β-methyl groups in the acetaldehyde released. A microsomal experiment was performed with lidocaine-d_6 as previously described, and the acetaldehyde released during a 7.5 min incubation was reacted with 100 μl of the 3-methyl-2-benzothiazolone hydrazone reagent (0.5%) in the same procedure used for colorimetric determination. However, the intermediate azine formed (see below) was not further oxidized with ferric chloride, but extracted into purified ether at pH 6.5-7.0. This extract was filtered through anhydrous magnesium sulfate and rotary evaporated to yield a yellow residue which was subjected to CIMS at 140°. Peaks occurred at m/e = 180 (MH^+ of unreacted reagent), m/e = 209 (MH^+ of the trideuterated azine), and m/e = 241 (MH^+ of a small amount of lidocaine-d_6 carried through the extraction). This showed that no deuterium was being

released from the β-methyl groups of the substrate or its acetaldehyde product.



MH^+ = m/e 209

Trapping of Acetaldehyde with the Hydrazone Reagent

3. Results

Apparent Km values for the three substrates are shown in Table 1. From the apparent Km values, we find that $Km^{D-4}/Km^{H} = 1.23$ and $Km^{D-6}/Km^{H} = 0.92$.

TABLE V-1

Apparent Km Values for Lidocaine, Lidocaine-d_4, and Lidocaine-d_6 Data were derived from Lineweaver-Burk Plots employing five concentrations of substrate for each plot. Concentrations for each substrate were 0.125 mM, 0.250 mM, 0.500 mM, 1.00 mM and 1.50 mM. Calculations were made from 4 determinations in two separate microsomal experiments. (Mean ± standard deviation)

Compound	Apparent Km (mM)
Lidocaine	0.338 ± .090
Lidocaine-d_4	0.415 ± .077
Lidocaine-d_6	0.312 ± .031

A comparison of the initial velocities of N-deethylation of lidocaine and its deuterated analogs showed a significant slowing of the rate with both of the deuterated compounds. Plots of the amount of acetaldehyde formed vs. time is shown in Figure 5. Each reaction was linear from 7.5 to 10 min. From 0-7.5 min relative rates of deethylation were $k_H/k_{D4} = 1.49 \pm .11$ and $k_H/k_{D6} = 1.52 \pm .10$

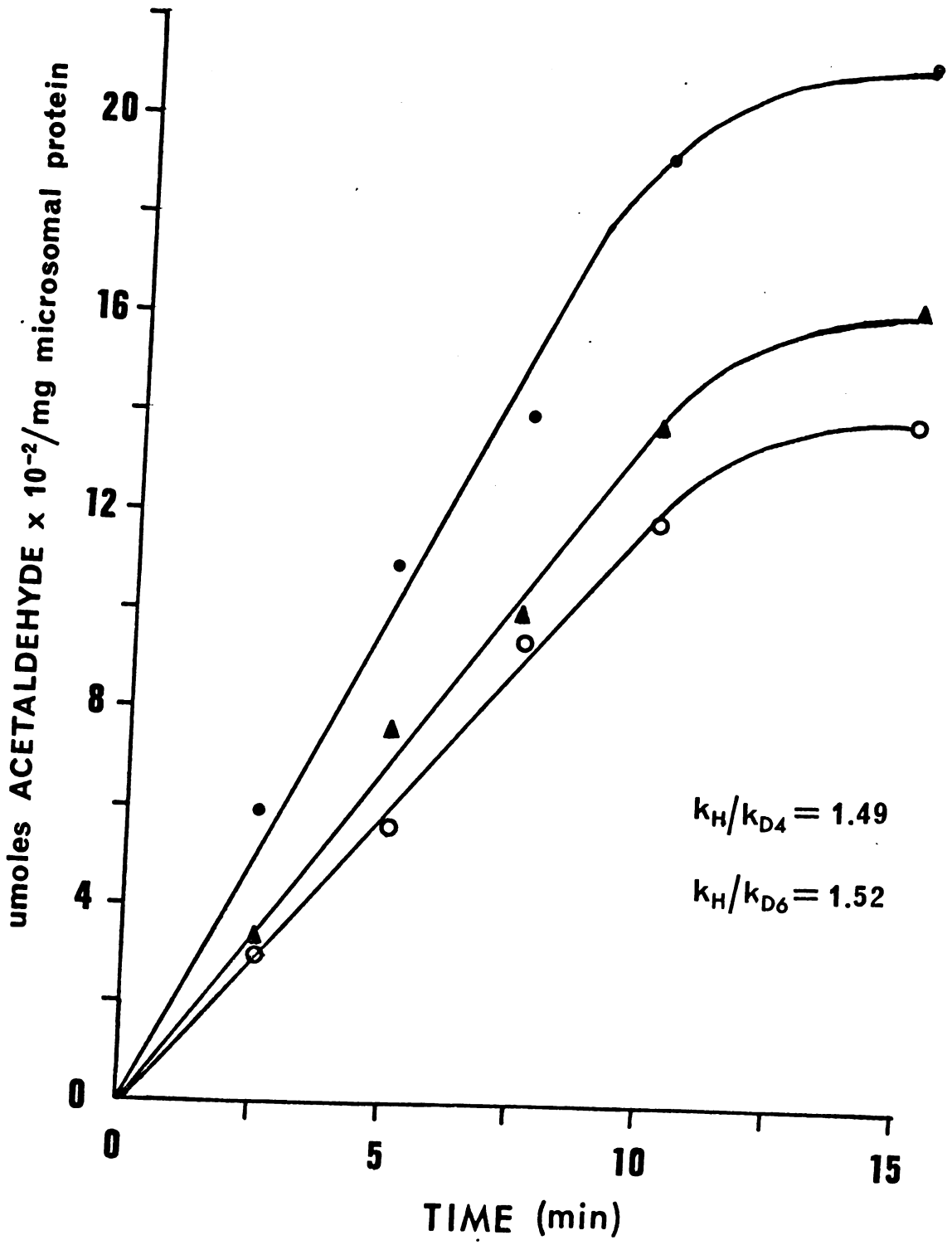
4. Discussion

a. Primary Isotope Effects

The results obtained with lidocaine-d_4 are similar to those obtained by Elison, et al.,[57] for the N-demethylation of morphine and its deuterated analog. Both cases show isotope effects on the initial velocities and both show increased Km values for the deuterium labeled substrate. We feel that the isotope effects observed in these oxidative N-dealkylations reflects C-H bond-breaking in a rate-determining step.

This interpretation requires some explanation. First of all, small isotope effects on rate-determining steps, such as those observed here, have been observed in several chemical reactions such as the Canizarro reaction ($k_H/k_D = 1.8$). For a normal isotope effect the assumption is made that the zero-point energy of a C-H and C-D bond are equal in the transition-state complex. This may not be true if: 1) the transition state is non-linear, 2) the transition state is

Figure V-5. Curves showing the rates of N-deethylation of lidocaine (●——— ●), lidocaine-d_4 (▲——— ▲), and lidocaine-d_6 (o ——— o). Each point represents the mean of 4 determinations from two separate microsomal experiments.


umoles ACETALDEHYDE x 10^{-2}/mg microsomal protein
20
16
12
8
4
0
0
5
10
15
TIME (min)
$k_H/k_{D4} = 1.49$
$k_H/k_{D6} = 1.52$

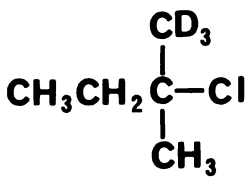
asymmetric and 3) the hydrogen atom involved is not undergoing translation at the saddle point.[51,60]

Secondly, the isotope effect on the Michaelis constants suggests that binding forces are used in the formation of the enzyme-substrate complex forcing the substrate to resemble the transition state. This results in changes in vibrational frequencies of the C-H bond to be broken in the transition state. Such an analysis has been made by Belleau and Moran[61] to explain their results with deuterated tyramine interactions with monoamine oxidase. Thus, in the N-deethylation of lidocaine, the bond-breaking process is already quite advanced in the enzyme-substrate complex since $Km^{D-4}/Km^{H} = 1.23$ and $k_H/k_{D-4} = 1.49$. Another possibility suggested by Jencks[51] is that the Michaelis constant may contain significant kinetic terms as well as the dissociation constant.

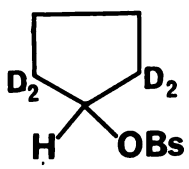
b. Secondary Isotope Effects

An α-secondary isotope effect arising from that deuterium which is not reacting, is probably operative in the methylene carbon undergoing the oxygenation in lidocaine-d_4, but compared to the primary isotope effect will be very small.[50] However, for lidocaine-d_6 the observed kinetic β-secondary isotope effect, $k_H/k_{D-6} = 1.52$, with only a small effect on the Michaelis constant $Km^{D-6}/Km^{H} = 0.92$, is consistent with the interpretation that the α-C-H bond is being broken in

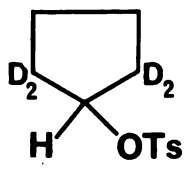
the transition state. The large β-secondary effect is similar to values obtained in solvolysis reactions involving the formation of carbonium ions. For example, Shiner[62] found a β-secondary effect k_H/k_D = 1.40 for the rate of solvolysis of 4, and 1.52 for the solvolysis of 5. Streitweiser, et al.,[63] found k_H/k_D = 2.06 for cyclopentyl tosylate-d_4 (6).



4



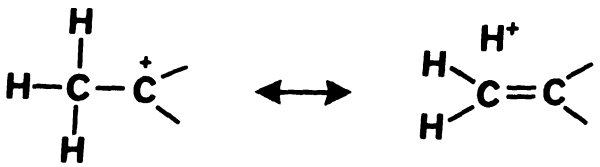
5



6

The kinetic β-secondary deuterium isotope effect observed in these reactions is best explained by the lowering of force constants to the C-H and C-D bonds adjacent to a carbonium-like center in the transition state through hyperconjugation.[50] In terms of molecular orbital theory the effect results from small contributions to the overall wave function of a molecule from a linear combination of atomic orbitals relating to species such as those shown on page 233. Thus, in going

from an sp^3 configuration to an sp^2 configuration, substitution of deuterium for hydrogen at a contiguous center will lead to $k_H/k_D > 1$.



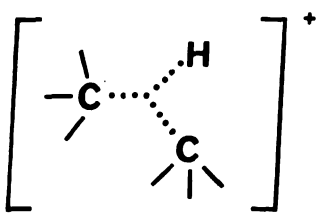
c. Conclusions

The inference to be made from the small primary isotope effect and relatively large secondary isotope effect in the oxidative N-deethylation of lidocaine is that an assymetric and/or non-linear transition state is involved in the removal of an α-methylene hydrogen, and that the carbon atom from which the hydrogen is removed has considerable sp^2 character in this transition state. This hypothesis is supported by several lines of reasoning:

1) The transition state for hydrogen removal is very likely asymmetric considering that the hydrogen atom is probably being transferred between two very different atoms, carbon and oxygen. That is, the hydrogen will probably not be equidistant between these two atoms in the transition state.

This will, therefore, decrease the primary isotope effect.

2) McMahon and Craig[64] found a kinetic isotope effect of 1.8 for the microsomal oxidation of α-deuterio-ethylbenzene and also showed that the hydroxylation was stereospecific with retention of configuration, i.e., the oxygen was inserted on the same side of the carbon as the hydrogen was removed. For such an event to occur, the reaction probably involves an electrophilic reaction of an oxenoid species on the C-H bond. Analogies to this reaction have been studied in detail by Olah[65] in his prolific work on carbenium ion insertions into single bonds via two-electron three center bonds, like that shown below.



Of particular importance to the discussion is that such electrophilic reactions involve non-linear transition states which will considerably decrease the magnitude of the primary isotope effect.[60]

3) In the special case of oxidative N-dealkylation, the nitrogen can considerably lower the activation energy for the breaking of the α-C-H bond by stabilization of an electron deficient intermediate through inductive and resonance participation.[49] The consequences of such stabilization will be to lower the magnitude of the primary isotope effect and increase the amount of sp^2-character in the transition state. This might account for the large β-secondary isotope effect observed.

4) Another piece of evidence in favor of the generation of an sp^2-like configuration in the transition state of microsomal N-dealkylation reactions comes from the strong inhibitory effect on this reaction observed with N-cyclopropyl compounds.[66] These compounds probably act as transition state analogs since both the α- and β-carbon atoms of the cyclopropyl ring have sp^2-character in the ground state. Since catalysis in many enzymes appears to be due to tighter binding of the transition state,[67] it follows that the N-cyclopropyl group should be tightly bound in its ground state, and therefore act as an inhibitor. This has been observed in the case of monoamine oxidase inhibitors.[68] This will, of course, only be true if our interpretation of the sp^2-character of the N-alkyl group in the transition state is correct.

The validity of such a hypothesis is subject to some speculation and this hypothesis, as well as others, will be discussed more fully in the final section of this chapter.

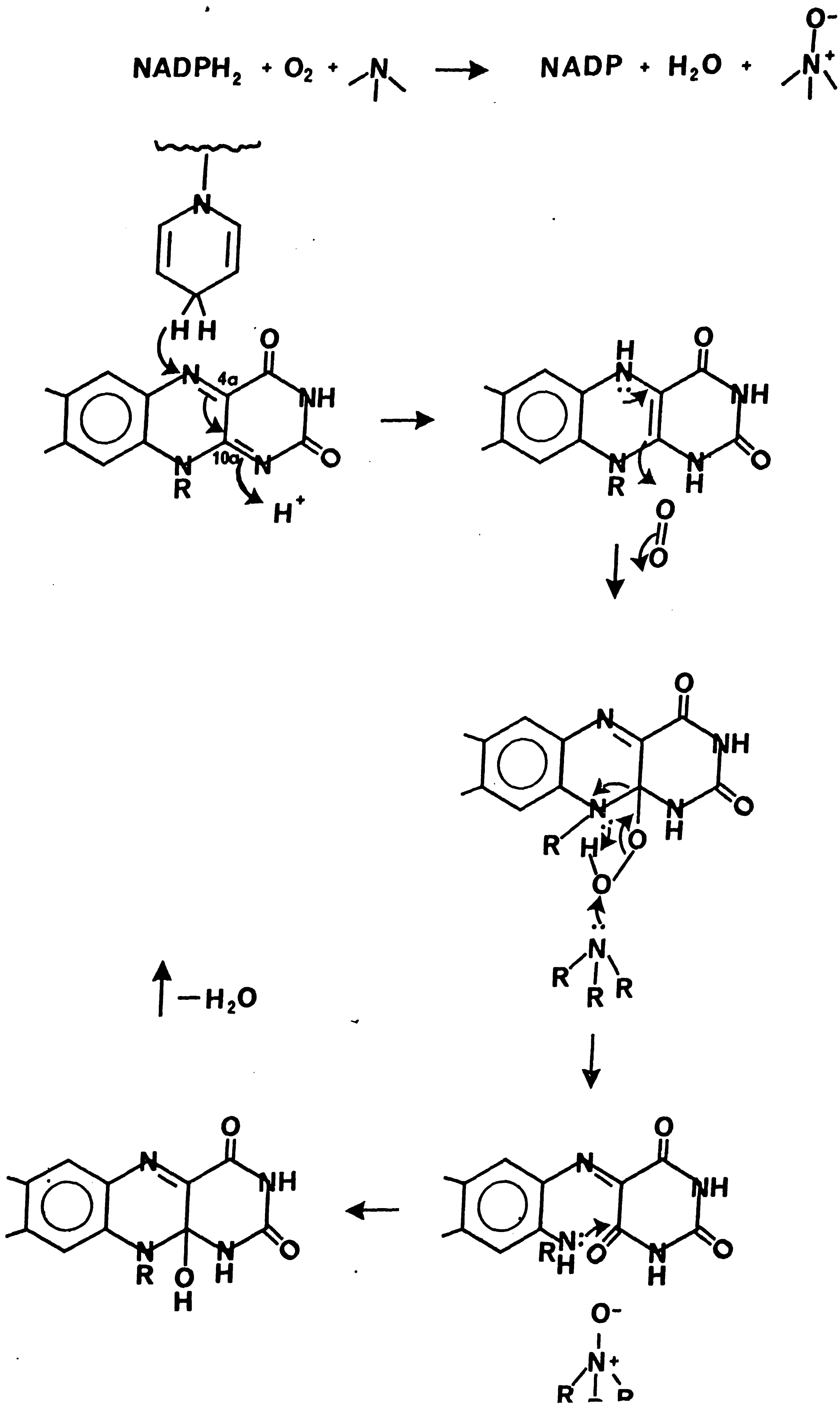
E. POSSIBLE SCHEMES FOR MICROSOMAL OXIDATIVE AMINE METABOLISM

In this concluding section an attempt will be made to outline some possible schemes involved in microsomal oxidative nitrogen metabolism as they might apply to certain postulated and known biotransformation routes. One technique used for such a purpose is the biochemical spinning wheel like that pictured on p. 205. However, since this technique obscures any hint of underlying chemical mechanisms, the more pictorial chemical schemes have been supplied in hopes of generating more insight into the problem. Of course, just as the biochemical spinning wheel may be too sketchy, the pictorial scheme is usually much more detailed than is justifiable with limited information.

1. Flavin Mediated Tertiary- and Secondary-Amine Oxidations

Ziegler[69] and Uehleke[70] have presented evidence that N-oxidation of secondary and tertiary amines proceeds via NADPH and oxygen dependent flavin mediated oxidation to produce hydroxylamines and N-oxides, respectively. The enzyme involved is located in the microsomal fraction, but is distinct from the cytochrome P-450 enzymes. Scheme 3 illustrates a reasonable mechanism for this

Scheme V-3. Postulated mechanism for flavin mediated N-oxidations.


NADPH2 + O2 +
NADP + H2O +
4a
10a
H+
−H2O

process based on available evidence and can be described in 4 major steps.

1) The first step involves a two-electron hydride transfer from NADPH to the flavin moeity. The proposed direction of the hydride transfer comes from evidence presented by Brüstlein and Bruice.[71]

2) Formation of an oxygenated flavin intermediate is proposed in the next step based on the initial postulate of Mager and Berends[72] for a similar reaction in non-enzymic systems. Hamilton,[73] on the other hand, prefers formation of peroxy intermediate at the 4a position as opposed to the 10a position of the flavin ring because he feels the 10a peroxy-flavin is similar to an alkyl hydrogen peroxide. However, a close examination of the 10a carbon atom of the peroxy-flavin compound reveals that it is in the same oxidation state (-3) as the carbonyl carbon atom of an organic peroxy acid such as m-chloroperoxybenzoic acid. Since these organic peracids react with secondary amines to form hydroxylamines and tertiary amines to form N-oxides, the same type of reactivity might be expected from the peroxy-flavin intermediate shown. Recent mass spectral evidence on a model compound[74] also supports the 10a peroxy-flavin intermediate.

3) The oxidation of the amine itself is strictly analogous to the concerted "ene" mechanism for peracid oxidations.[73]

4) The final step involves resynthesis of the oxidized flavin and is identical to the final rapid step in the chemical synthesis of most flavins and has been similarly proposed by Hamilton.[73]

2. Tertiary Amine N-Oxide Dealkylation

There is evidence both for and against this particular metabolic route.[29] As discussed in section C of this chapter, if the reaction takes place in vivo it probably proceeds by a free radical mechanism. Scheme 4A shows a likely pathway involving the heme portion of cytochrome P-450 and proceeds in 4 major steps.

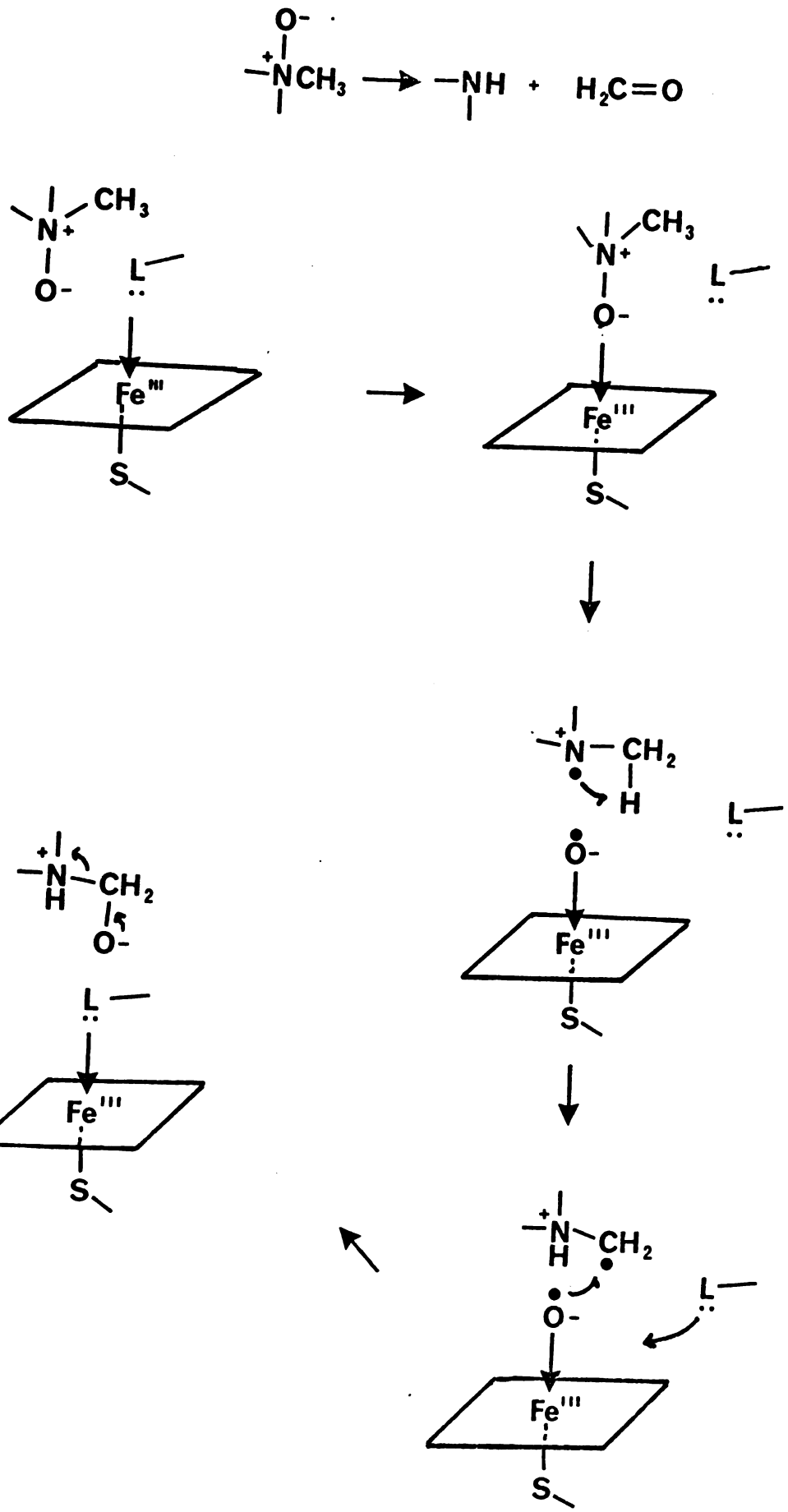
1) The N-oxide will probably bind to the iron since a certain amount of polar character is present in the coordinate covalent N $\rightarrow$ O bond. The CNDO calculations in section C actually indicate a reasonable amount of the π character in this bond in trimethylamine N-oxide which will make ligand interaction more favorable due to π^*-d overlap with ferric iron.

2) Homolytic cleavage of the weak N-O bond can occur as suggested from CIDNP evidence to form a free radical caged-type intermediate.[43]

3) Radical recombination can then take place with formation of the thermodynamically more stable carbinolamine.

4) Fragmentation of the kinetically unstable carbinolamine to aldehyde and secondary amine occurs in the final step.

Scheme V-4a. Postulated cytochrome P-450 mediated tertiary amine N-oxide dealkylation.


O-
N+
CH3
NH
H2C=O
L
Fe III
S
CH2
H

Another possible route is depicted in Scheme 4b wherein the heme iron is reduced leaving an N-oxy radical. This can rearrange to the carbon radical followed by a concerted intramolecular rearrangement to carbinolamine and reoxidation of the iron. The carbinolamine breaks down as previously described.

3. Aromatic Amide Oxidation

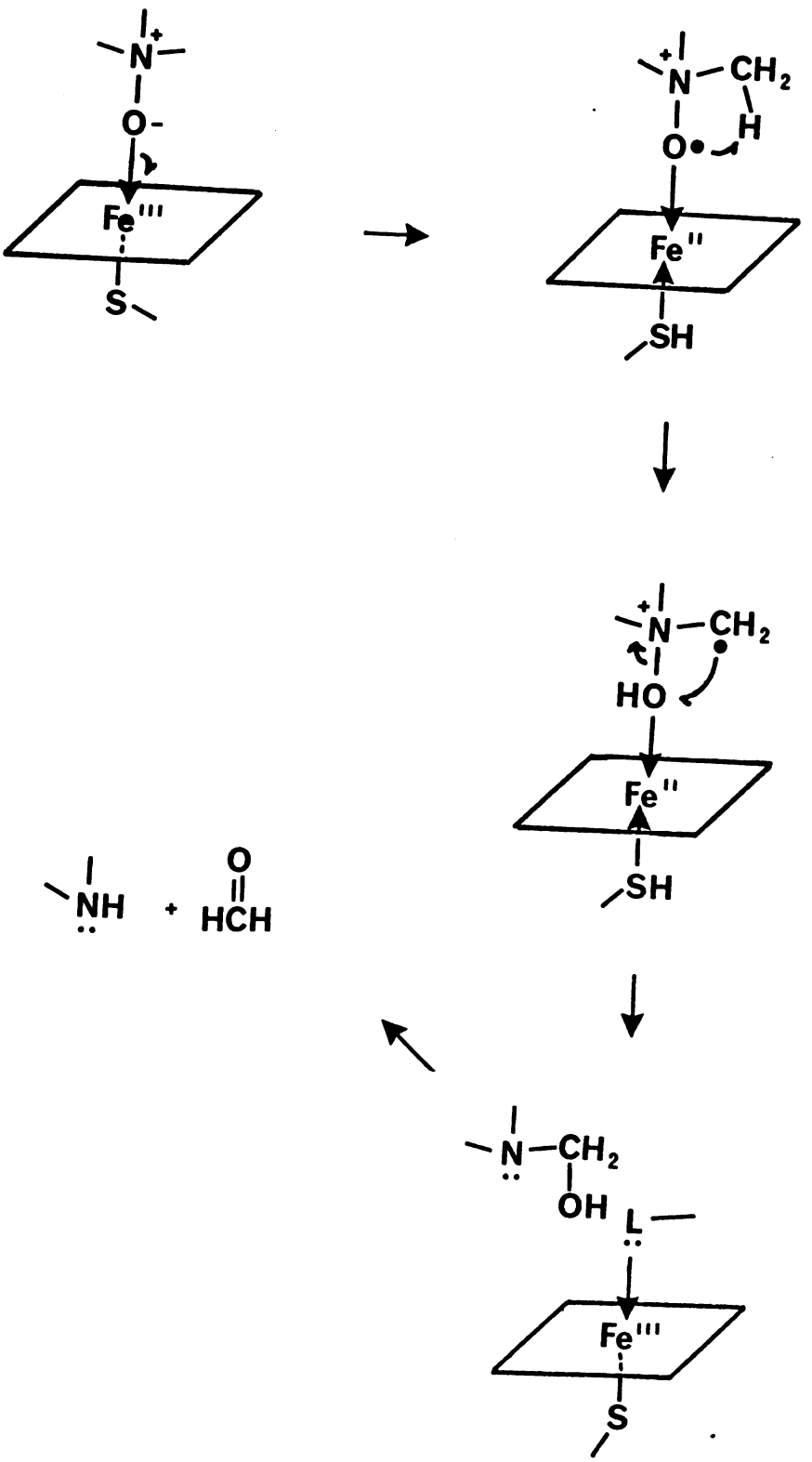
Most evidence supports a cytochrome P-450 mediated oxidation of the aromatic amido group to an N-hydroxyamide.[75] Clues to a possible enzymatic mechanism come from direct synthetic approaches to N-hydroxyamides from the corresponding amides. Only two such methods have been investigated, one involving the use of an oxomolybdenum (VI) complex,[76] and the other, peracid oxidation of amide imino-ether derivatives.[77]

The mechanism as outlined in Scheme 5 will be described in 3 parts.

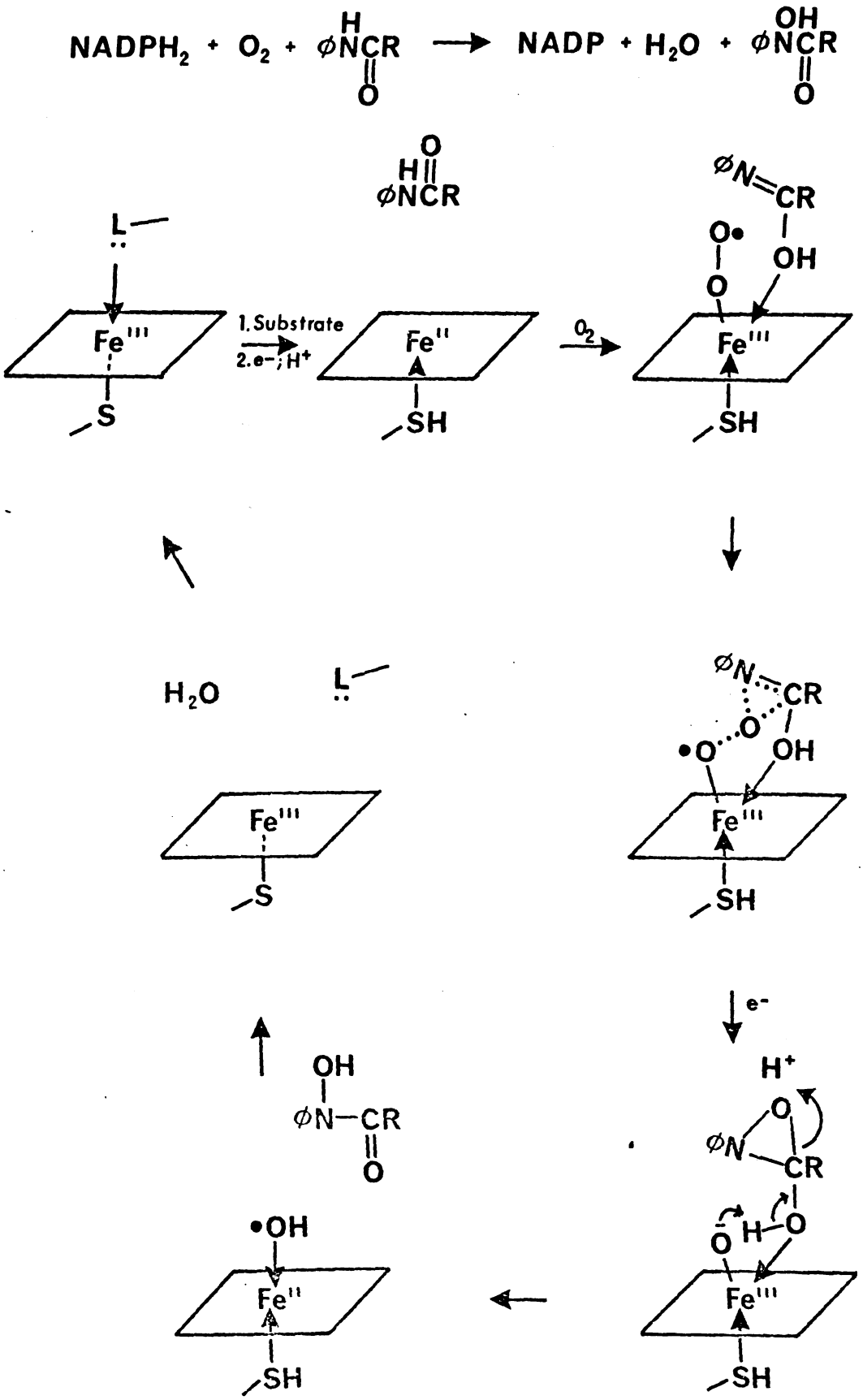
1) A ternary complex composed of cytochrome, oxygen, and imino-enol is formed as shown. Since the pKa of the perhydroxyl radical is 4.8,[78] the superoxide anion is shown as the reacting species.

2) If this intermediate is formed, a peracid-type oxidation of the imino double-bond can occur with the superoxide species to produce an intermediate oxaziridine[77] which breaks down to the N-hydroxyamide as shown.

Scheme V-4b. Alternative mechanism for tertiary N-oxide dealkylation.


N
O-
Fe'''
S
N
CH2
H
O•
Fe''
SH
N
CH2
HO
Fe''
SH
NH
O
HCH
N
CH2
OH
L
Fe'''
S

Scheme V-5. Postulated mechanism for aromatic amide hydroxylation.


NADPH2 + O2 + ØNCR → NADP + H2O + ØNCR
1. Substrate
2.e-; H+
O2
e-
H+
H2O
Fe III
Fe II
S
SH
•OH

3) This is followed by the second one electron reduction of cytochrome P-450 to a ferro-hydroxy radical intermediate which has been shown to undergo rapid internal oxidation-reduction to give water and regenerate the Fe (III) state.[80]

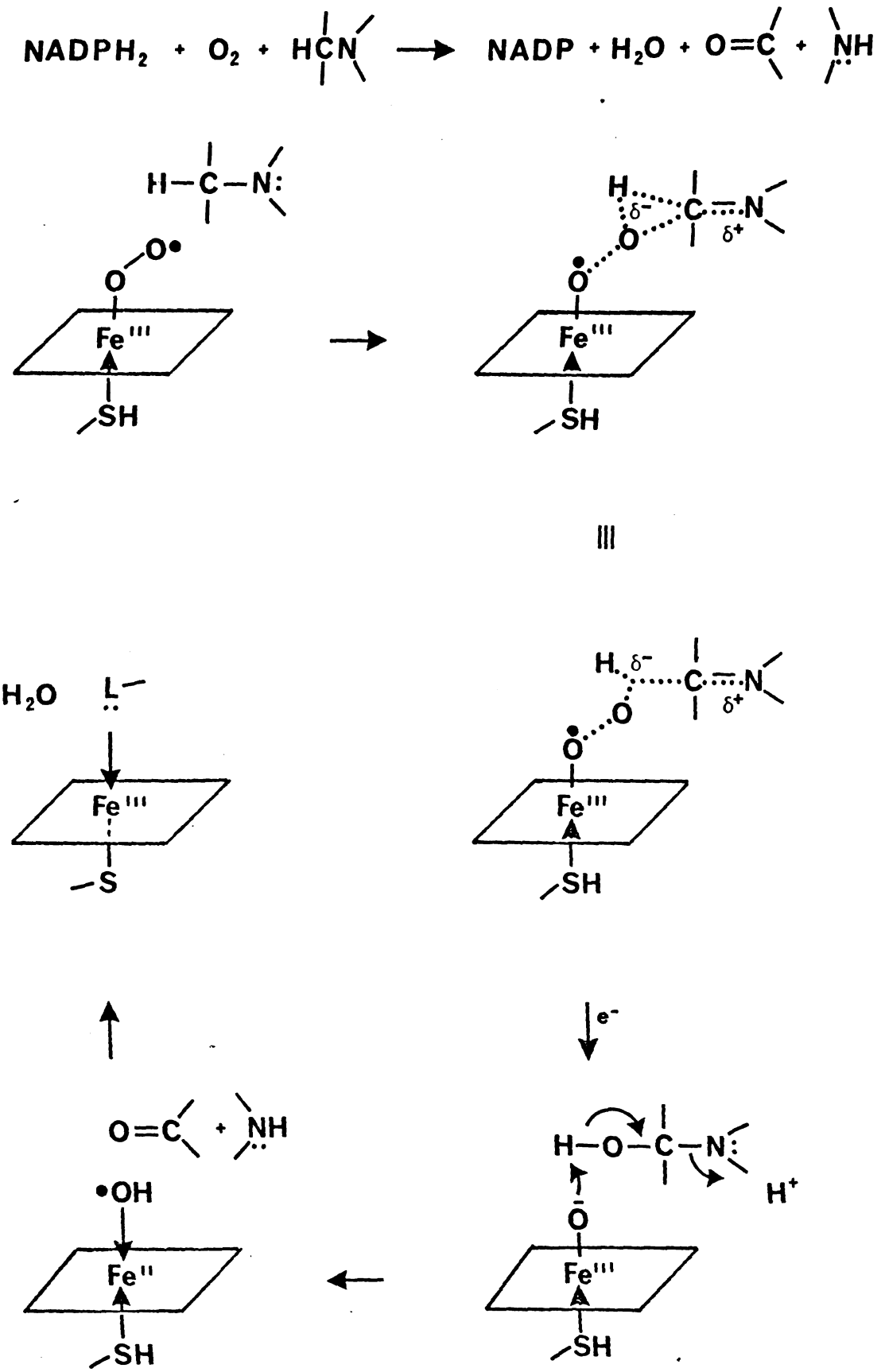
4. N-Dealkylation via Carbinolamine Formation

The final reaction to be considered will be the N-dealkylation mechanism via intermediate carbinolamine formation. This appears to be the major pathway in the metabolism of most tertiary amines.[40] The mechanism will be discussed primarily in terms of potential intermediates in the oxygenation process using the results obtained with specifically deuterated substrates.

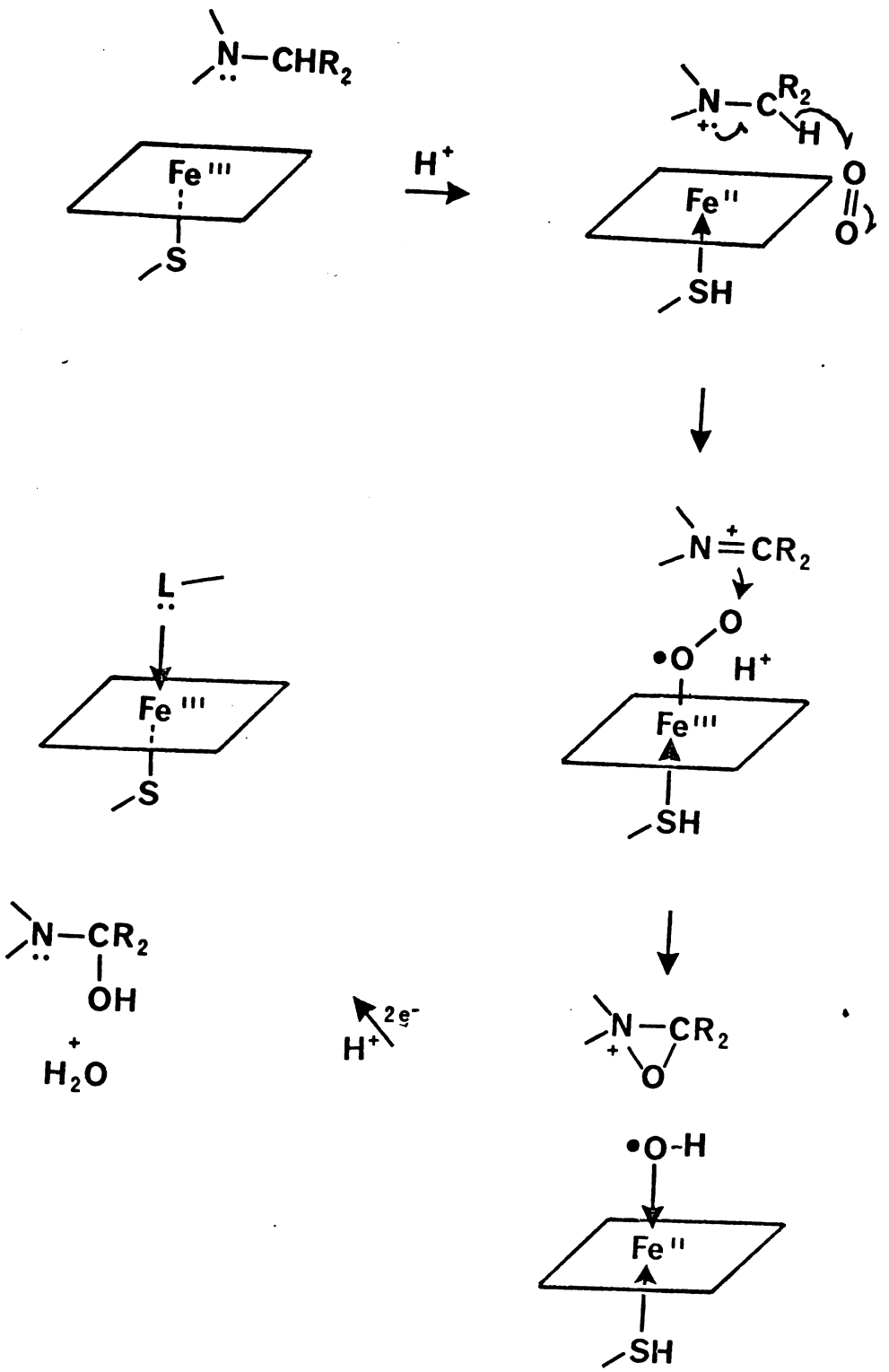
Scheme 6a is the most logical pathway based on a correlation of all of the available data on cytochrome P-450 mediated reactions. With amine substrates, a concerted polar insertion mechanism such as that shown, is likely involved, whereas the mechanism of "oxene" insertion into isolated C-H bonds in the other alkane groups is assumed to be more like a free radical insertion. The transition-state depicted in Scheme 6a for amine N-dealkylation accounts for the various isotope effects discussed in section D of this chapter since it is non-linear, asymmetric, and sp^2 in character.

The mechanism shown in Scheme 6b also accounts for the isotope effects observed since the intermediate imminium ion formed

Scheme V-6a. Postulated cytochrome P-450 mediated N-dealkylation reaction.


NADPH2 + O2 + HCN → NADP + H2O + O=C + NH
H—C—N:
O•
O
Fe III
SH
H δ− C N δ+
O
O
Fe III
SH
III
H2O L
Fe III
S
H δ− C N δ+
O
O
Fe III
SH
e−
O=C + NH
•OH
Fe II
SH
H—O—C—N:
H+
O
Fe III
SH

Scheme V-6b. Alternative mechanism for N-dealkylation.


N—CHR2
Fe III
S
H+
N—C
R2
H
Fe II
O
O
SH
N+=CR2
O
•O
H+
Fe III
SH
L
Fe III
S
N—CR2
OH
+
H2O
H+
2e-
N—CR2
O
•O-H
Fe II
SH

is strictly sp^2-hybridized. The formation of the oxaziridinium ion and its reduction to carbinolamine is analogous to aziridinium ion synthesis and reduction using diazomethane.[81] However, the overall pattern of one-electron reductions does not follow the accepted sequence of reductions as explained in section C of this chapter.

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SUMMARY

Lidocaine has been shown to be extensively metabolized both *in vivo* and *in vitro*, presumably by liver enzymes located in the microsomal fraction. Based on an analysis of lidocaine and its metabolites in human urine using a relatively new CIMS-stable isotope dilution technique, the major biotransformation processes were found to include N-dealkylation, aromatic oxidation, and amide hydrolysis. The sequence of events leading to some of the products found in the urine remains an open question and requires more study. Benzylic oxidation and direct N-oxidation products were not detected as urinary metabolites. However, the potential importance of the N-oxidation pathways in evoking serious toxicological responses requires that much more extensive work be carried out to either prove or disprove our results. This is particularly true since other researchers have presented evidence for the production of substantial quantities of both N-hydroxylidocaine and N-hydroxyMEGX in man. We do feel that the mass spectral methods used in our search for the N-hydroxyamide metabolites, combined with the available synthetic N-hydroxyamide standards, makes our results more reliable.

Further evidence for the paucity of direct N-oxidation and benzylic oxidation pathways comes from our results with the microsomal metabolism of radio-labeled lidocaine. The results obtained in these

experiments agree with those obtained in vivo supporting N-dealkylation as the major primary metabolic route.

Another route of lidocaine metabolism in man and Rhesus monkey has now been established showing that amide hydrolysis of both lidocaine and MEGX occurs to yield the amino acid metabolites N, N-diethylglycine and N-monoethylglycine. The results are qualitative and further quantitative work is necessary. This route may be of significance in clarifying the CNS toxicity observed with lidocaine therapy if these metabolites are found to inhibit glycine uptake into inhibitory synapses, a process which might lead to convulsive episodes.

The presence of a new metabolite of lidocaine, the cyclic metabonate, has been observed both in vitro and in vivo. Quantification of both in vitro and, particularly, in vivo levels of this metabolite has been shown to be extremely difficult due to equilibration with another metabolite of lidocaine, MEGX, plus acetaldehyde. The importance of this apparently minor pathway should not be under-estimated. An electrophilic imminium ion, such as that produced as an intermediate in the formation of the cyclic metabonate, can possibly effect critical enzyme or receptor sites. Further work needs to be carried out in vitro on the possible binding of such intermediates to macromolecular fractions. The ease with which MEGX, a major metabolite of lidocaine, reacts with aldehydes (such as acetaldehyde to form the cyclic metabonate) implicates this process as possibly being responsible for some of the efficacious or toxicological responses associated with lidocaine therapy.

Both observed and potential metabolic pathways involved in lidocaine and other xenobiotic nitrogen metabolism were discussed in terms of possible intermediates and mechanisms. Molecular orbital calculations support the hypothesis that if N-oxides are involved in N-dealkylation reactions, a free radical mechanism is probably involved. These calculations also pointed out the thermodynamic stability but kinetic instability of carbinolamine intermediates which are probably involved in the process of oxidative N-dealkylation. Isotopic substitution with deuterium in the α-methylene and β-methyl carbon atoms of the terminal amino group of lidocaine, presumably revealed both primary and secondary isotope effects in the N-deethylation sequence.

Based on all of the results calculated and experimentally observed, various chemical schemes were invoked to portray possible mechanisms involved in the microsomal metabolism of nitrogen-containing compounds. The intermediates and reaction sequences were explained with reference to a multitude of related topics found in the literature. A peroxy-flavin intermediate has been described for the N-oxidation of secondary and tertiary amines; imino-enol and oxaziridinium-like intermediates have been suggested in aromatic amide oxidation; and an asymmetric, non-linear transition state involving a 3-center, 2-electron sigma bond, with substantial sp^2-character on the interacting carbon atom, has been portrayed for oxidative N-dealkylation.

Although the mechanisms may be too detailed with the information available, we believe that they better elucidate potential biochemical mechanisms than the more simplistic reaction diagrams used by many investigators. These diagrams were, of course, essential to describe the initial discoveries concerning microsomal reactions, but with the accumulation of knowledge in the last few years, reaction mechanisms can now be discussed on a more molecular level.

So the gamut from theoretical calculations to analytical techniques with clinical significance has been covered in an attempt to learn something of the biotransformation of lidocaine and its relation to oxidative nitrogen metabolism. As with most thesis work, more questions have arisen than have been answered.

> Beware the man who works hard to learn something, learns it, and finds himself no wiser than before. He is full of murderous resentment of people who are ignorant without having come by their ignorance the hard way.
>
> From "Cat's Cradle, The Books of Bokonon," by Kurt Vonnegut, Jr.

COPY 1