Title

Applications of chemical ionization mass spectrometry to in vitro and in vivo metabolic studies on R, S, and RS alpha-methyldopa (12C and 13C-enriched)

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APPLICATIONS OF CHEMICAL IONIZATION MASS SPECTROMETRY TO *IN VITRO* AND *IN VIVO* METABOLIC STUDIES ON R, S, AND RS ALPHA-METHYLDOPA (^{12}C AND ^{13}C-ENRICHED)

by

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ABSTRACT

APPLICATIONS OF CHEMICAL IONIZATION MASS SPECTROMETRY TO *IN VITRO* AND *IN VIVO* METABOLIC STUDIES ON R, S, AND RS ALPHA-METHYLDOPA (^{12}C AND ^{13}C-ENRICHED)

by

MATTHEW M. AMES

UNDER THE SUPERVISION OF PROFESSOR NEAL CASTAGNOLI, JR.

Catecholamines represent an important class of small molecules in plants and animals. In mammals, they possess marked peripheral and central pharmacological activity, as do many of their synthetic analogs. Furthermore, dopamine and norepinephrine are chemical transmitters in many important neural pathways.

This thesis is concerned with certain aspects of the metabolism of the antihypertensive agent alpha-methyldopa, the alpha-methyl analog of the endogenous amino acid DOPA. The drug is of interest both as a potent pharmacological agent and as a structural analog of the endogenous catecholamines. Alpha-methyldopa contains a chiral center at the

alpha or C-2 side chain carbon atom, and thus exists as two isomers differing in configuration about the asymmetric center. Our studies have been concerned with the metabolism of the asymmetric molecule in both in vitro and in vivo systems.

Extensive pharmacological studies have shown that the mechanism of action is centrally mediated, and that decarboxylation appears to be a prerequisite to pharmacological activity. Metabolic studies have revealed marked differences in the fate of the two enantiomers of alpha-methyldopa. Pharmacologically important amine derivatives appear to be formed, via decarboxylation, only from the S isomer. Indeed, studies have shown that key activities (blood pressure reduction, endogenous amine depletion, decarboxylase inhibition) are associated only with the S isomer. While these studies have not shown any activity for the R isomer, no definitive studies on the important in vivo brain metabolism of the R, S, and RS alpha-methyldopa have been reported.

The quantitative estimation of these compounds, and their detection at very low levels, require both a sensitive and specific analytical assay. Procedures employed in the past, including the classical spectrophotofluorimetric catecholamine assays, do not provide the specificity and sensitivity necessary for these studies, especially in the determination of the endogenous amines as well as their alpha-methyl counterparts. Separation techniques, such as column chromatography, have not successfully overcome these problems.

We have developed analytical procedures based on chemical ionization mass spectrometry to provide the sensitivity and specificity needed to study the stereochemical aspects of alpha-methyldopa metabolism in the rat brain, as well as the concommitant effects on the levels of the

endogenous amines dopamine and norepinephrine. The mass spectrometer, when operated in the chemical ionization mode, allows the unambiguous determination of these molecules and their internal standards on the basis of mass discrimination, without prior separation techniques. The cims analysis has been coupled with the synthesis of stable isotopically labeled substrates and internal standards. In order to better investigate stereochemical aspects of metabolism, the ^{13}C-labeled alpha-methyldopa was synthesized, and then resolved into its optically active enantiomers. Optical purity was demonstrated by means of a new gas liquid phase chromatography assay for the enantiomers of alpha-methyldopa.

The above methods were utilized in the study of alpha-methyldopa metabolism in the caudate nucleus and hypothalamus of the rat brain after intraperitoneal or intraventricular administration of R, S, or racemic alpha-methyldopa as the ^{12}C, ^{13}C, or pseudo-racemic (^{12}C-R/^{13}C-S) compound. The results of these studies may be briefly summarized as follows. After intraperitoneal administration, the R isomer was not decarboxylated in the caudate nucleus or hypothalamus, nor did it effect the endogenous level of dopamine in the caudate nucleus. Further studies with the amino acid revealed that R-alpha-methyldopa did not reach the caudate nucleus after peripheral administration. Therefore, R and S drug were separately administered directly into the brain via intraventricular injection. No decarboxylation of the R isomer was observed, while the S isomer was decarboxylated as in the studies employing intraperitoneal administration of alpha-methyldopa. Thus, R-alpha-methyldopa was neither taken up into the caudate nucleus, nor decarboxylated after after direct administration into the brain.

DEDICATION

This thesis is dedicated to:

Kathleen, who shared too much of the worst of this work, and too little of the best. Her support, understanding, and love were an integral part of this work.

My parents, who have always provided the opportunity, encouragement, and interest so necessary for such undertakings as this.

Dr. Neal Castagnoli, Jr., whose guidance and enthusiasm not only provided the necessary direction for this research, but also contributed to a close and rewarding friendship.

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Patrick Callery, Wally Murray, Peter McGraw, Peter Coates, and the rest of my peers for hours of conversation and cups of coffee.

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Drs. Henry Rapoport and J. Cymerman Craig for their quick review of the manuscript.

Dr. Sid Nelson and his wife Karen for their kind hospitality and friendship upon my arrival at their doorstep.

With the practical action there goes another, namely finding pleasure in the action for its own sake - in the skill that one perfects, and perfects by being pleased with it. This at bottom is responsible for every work of art, and science too: our poetic delight in what human beings do because they can do it.

- Jacob Bronowski

There's no relief.

- Patrick Callery

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PART I

INTRODUCTION

Catecholamines represent an important class of small molecules in plants and animals. In mammals they possess marked central and peripheral pharmacological activity, as do many of their synthetic analogs. Furthermore, two of the endogenous catecholamines (dopamine and norepinephrine) have been shown to be chemical transmitters in many neural pathways. A massive amount of literature has been published with regard to catecholamine pharmacology, biochemistry, and metabolism. To acquire useful information about the biological properties of catecholamines, many studies have focused on their isolation, identification, and quantitation. In general these investigators have been hampered by analytical problems because of the lack of sensitivity and specificity possible with these chemically unstable molecules.

This thesis is concerned with certain aspects of the metabolism of the antihypertensive agent alpha-methyldopa, the alpha-methyl analog of the endogenous amino acid DOPA. The drug is of interest both as a potent pharmacological agent and as a structural analog of the endogenous catecholamines. Alpha-methyldopa has pharmacological, biochemical, and metabolic properties both similar to and different from its endogenous counterpart DOPA. The drug contains a chiral center at the alpha (C-2) side chain carbon atom, and thus may exist as two isomers differing in their configuration about the asymmetric center. Our studies have been concerned with the stereochemical aspects of alpha-methyldopa metabolism in both *in vitro* and *in vivo* systems.

Extensive pharmacological studies with alpha-methyldopa have resulted in several proposals for its mechanism of antihypertensive activity. The literature review will show that, while the detailed

mechanism is not known, the mode of action is centrally mediated. Furthermore, central metabolism of the drug appears to be required for pharmacological activity.

Metabolic studies have indicated marked differences in the fate of the two enantiomers of alpha-methyldopa. Pharmacologically important amine metabolites appear to be formed, via decarboxylation, only from the S isomer. The R isomer is absorbed from the gastrointestinal tract about 10% as readily as the S isomer, and does not seem to lead to urinary amine metabolites. Pharmacological studies have suggested that decarboxylase inhibition, endogenous amine depletion, and blood pressure lowering are associated with the S isomer. While these investigators suggested that the R enantiomer of alpha-methyldopa was both metabolically and pharmacologically inert, no definitive _in vivo_ studies on the brain metabolism of S,R, and racemic alpha-methyldopa have been reported. There have been some studies reported which were concerned with levels of endogenous dopamine and norepinephrine, as well as the appearance of their alpha-methyl counterparts, after administration of alpha-methyldopa.

The quantitative estimation of these compounds in the concentrations expected in the brain requires both a sensitive and specific analytical assay. The procedures employed in the past have consisted primarily of spectrophotofluorimetric assays, a classical method of catecholamine analysis. These assays unfortunately lack a reliable degree of specificity between the endogenous catecholamines and their alpha-methyl analogs. This problem can only be solved by by highly effective separation procedures. The column chromatography

methods most commonly used in conjunction with the fluorimetric assay procedures have been only partially successful in such separations.

In order to provide the analytical capabilities necessary to investigate the stereochemical course of alpha-methyldopa metabolism in brain tissues, we have developed analytical methods based on chemical ionization mass spectrometry (cims). Mass spectrometry allows the discrimination of any molecules having different molecular weights, and thus offers the specificity required for studies involving chemically similar molecules. The use of stable isotopically labeled substrates and internal standards, isolation of metabolites by cation exchange chromatography, and derivatization of the amine metabolites and amino acid itself have allowed the identification and quantitative estimation of alpha-methyldopa, alpha-methylamine metabolites, and endogenous amines in the brain. With these methods we have studied the fate of S, R, and racemic alpha-methyldopa in specific regions of the rat brain.

HYPERTENSION AND ALPHA-METHYLDOPA

Hypertension is one of the most prevalant diseases in the United States. A Public Health Survey covering the years 1960-1962 found that 24 million adults were suffering from hypertension or hypertensive diseases.[1] A population study of 5,000 people conducted in the late sixties found 18% of the men and 16% of the women were hypertensive.[2] Thus, the management and treatment of this disease is of prime importance in health care.

There are a number of known causes of elevated systemic arterial blood pressure, including pheochromocytoma, Cushing's disease, renal tumors, aldosteronism, diabetes mellitas, and endocrine dysfunction.[3,4,5] In these cases, treatment of the causal factors is possible, and the hypertension may be alleviated permanantly. In most cases, however, no known cause can be detected, and the disease is labeled essential or primary hypertension. "It is a diagnosis of exclusion."[5] For these patients, therapeutic management of the high blood pressure is the only treatment.

Hypertension has been roughly categorized by the elevation of blood pressure.[5] Mild hypertension may be defined as an average diastolic pressure of 90-100 mm Hg. Such disease often is not accompanied by renal or cardiovascular complications. Although it can be treated with antihypertensive agents, often treatment consists of diet control and/or diuretics.[4] Moderate hypertension may be defined as a diastolic pressure of 100-120 mm Hg, and severe hypertension as as greater than 120 mm Hg diastolic blood pressure. For people suffering from moderate or severe hypertension, regular antihypertensive therapy is essential. Studies have verified that high blood pressure is definitely associated with increased mortality and morbidity, at all

age levels.[6,7,8] Complications increase with the severity of the hypertension. It therefore follows that management of essential hypertension in any degree of severity lessons the dangers associated with the disease. Treatment of even mild hypertension appears to be beneficial.[9,10] In a survey of over four million people, the Society of Actuaries found that life expectancy at any age level varied inversely with arterial blood pressure.[7]

The antihypertensive properties of alpha-methyldopa were first reported by Oates et al in 1960.[11] Further studies by Gillespie et al[12] confirmed these results. One study involved twelve hospitalized patients, all suffering from moderate to severe hypertension. Significant lowering of standing blood pressure was reported following treatment with S-alpha-methyldopa. Subsequent studies with alpha-methyldopa demonstrated a statistically significant reduction in blood pressure after treatment with the drug.[13]

Alpha-methyldopa is now in wide clinical use, both in hospital and outpatient treatment. The maximum antihypertensive effect is observed after 4-8 hours.[11,14] The duration of effect is approximately 24 hours,[11] but the drug is usually taken 3-4 times daily. A summary of hemodynamic studies[15] indicated that the fall in pressure is primarily via reduced vascular (arteriolar) resistance. Cardiac output is little changed, and neither renal function nor cerebral blood flow are decreased.

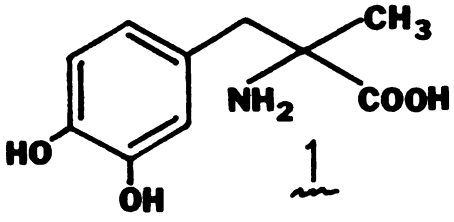
Side effects are observed with alpha-methyldopa therapy. Mild sedation or depression is sometimes seen, usually during the first 3-4 days of therapy.[3,5,16] Postural hypotension is much less severe

than with other antihypertensive agents used in moderate or severe hypertension.[3,5,17] Positive Coomb's tests and (rarely) hemolyic anemia have been observed.[3,5,17] Drug induced chronic hepatitus,[18] liver hypersensitivity,[19] fever,[14] and other liver abnormalities[14] have been detected after administration of the drug.

Alpha-methyldopa has been found to be a very effective antihypertensive agent, with excellent hemodynamic qualities. While its side effects are fewer and less severe than many other agents used in serious hypertension, there are several precautions which must be exercised in the use of this drug.

ALPHA-METHYLDOPA PHARMACOLOGY

As part of a large study, Sourkes[20] demonstrated the *in vitro* inhibition of DOPA decarboxylase by alpha-methyldopa (1) with the supernatant fraction of homogenized and centrifuged pig kidneys. Using DOPA (2) as the substrate, 1 was found to be the most effective inhibitor among a



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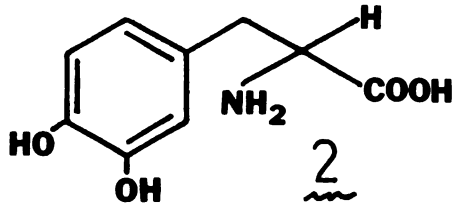

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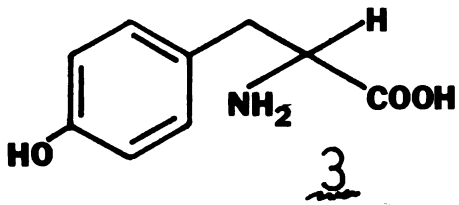
group of amino acid analogs tested for decarboxylase inhibition (98% inhibition at 5×10^{-4}M). Decarboxylase activity was determined by capture of metabolically formed carbon dioxide (CO_2) in a Warburg apparatus. The substrate was not isotopically labeled, nor was the possibility of decarboxylation of the amino acid analog inhibitors considered. The inhibition of DOPA decarboxylase by racemic 1 was found to be reversible at concentrations up to approximately 10^{-4}M. Higher concentrations produced inhibition which could not be characterized as strictly reversible or irreversible by kinetic parameters.

In vivo decarboxylase inhibition was then demonstrated in animals[21,22] and humans.[11,12] The most thorough of these was a human study by Gillespie *et al*.[12]. Patients were given oral loads of tyrosine (3) and the urinary output of tyramine (4) was determined before and after administration of racemic, R, and S alpha-methyldopa. After administration of either racemic or S-alpha-methyldopa, tyramine excretion was definitely reduced from



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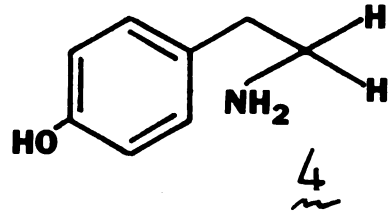
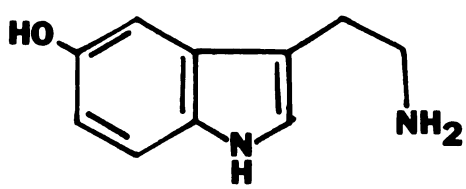


4

control values of ~500 micrograms/8 hr to less than 100 micrograms/8 hr. R-alpha-methyldopa values were ~300 micrograms/8 hr. To counter the possibility of l competing with tyrosine for transport across a membrane or with some other critical process a second experiment was conducted with patients. Urinary levels of tyramine without tyrosine loading were determined after R-or S-l was administered together with a monoamine oxidase inhibitor (N-benzyl-N-methyl-2-propynylamine hydrochloride, MO-911). With the S isomer, values of approximately 2000 micrograms/24 hr were reported, compared to approximately 900 micrograms/24 hr after the R isomer.

A brief examination of the analytical techniques employed in these studies will allow an evaluation of some of the problems encountered in early assays of catecholamines and related compounds. The tyramine study[12] determined levels of urinary amines by ion exchange column isolation and separation followed by fluorimetric assay. No experimental details were provided with regard to the fluorimetric assay, nor was the effectiveness of the column procedures reported. There was, however, a citation to an earlier assay procedure by Udenfriend et al[23] published in 1955. This article reported a new fluorimetric assay for 5-hydroxytryptamine (serotonin, 5) after treatment of the sample with 3N hydrochloric acid, based on the indolic nature of the compound. The activation wavelength was



5

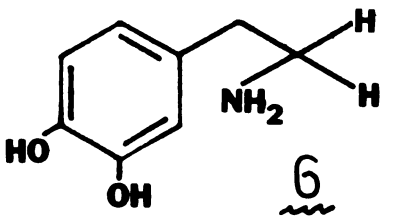
295 nm and the fluorescence was determined at 550 nm. Preliminary studies[24] on this procedure examined the fluorescence characteristics of a group of related molecules (Table I). The similarity of fluorescence properties, both with regard to activation and emission, point out the problems with the assay. The fact that caution must be exercised in interpreting data obtained by such procedures is emphasized by the characteristic instability of spectrophotofluorimeters. It must also be noted that at this time, there was no information concerning the metabolism of alpha-methyldopa itself, which is now known to yield metabolites similar to the endogenous catecholamines.

Table I

Fluorescence Characteristics of Endogenous Amines and Amino Acids (Ref. 24)

Compound	pH	Excitation Wavelength (nm)	Emission Wavelength (nm)
Serotonin	2-11	295	330
5-Hydroxytryptophane	2-11	295	330
DOPA	0.01N H_2SO_4	275	320
Norepinephrine	0.01N H_2SO_4	275	320
Epinephrine	0.01N H_2SO_4	275	320
Tryptamine	2-11	275	360
5-Hydroxyindoleacetic acid	2-11	295	330
Tyrosine	0.01N H_2SO_4	270	300-330

At that time, the mechanism of antihypertensive activity of alpha-methyldopa was thought to be mediated through the inhibition of DOPA decarboxylase. The production of dopamine (6) and norepinephrine (7) was thought to be reduced in various organs resulting in a general loss of sympathetic tone.



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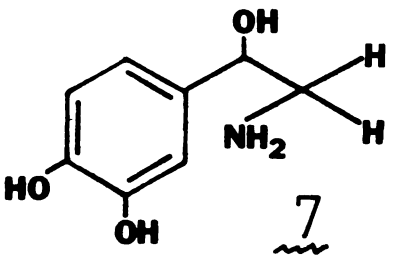


7

These preliminary studies led to investigations of the effects of alpha-methyldopa on the tissue levels of the three endogenous amines 5, 6, and 7. It was assumed that the tissue levels of these compounds would vary in a pattern similar to the variations in blood pressure and decarboxylase inhibition after treatment with 1. Hess et al[21] found that whole brain levels of dopamine and serotonin fell to levels less than 50% of the normal values, and returned to the normal values (0.55 micrograms/gram and 0.30 microgram/gram, respectively) within 18-24 hours. Norepinephrine (heart and whole brain), however, was depleted for as long as 100 hours to values of approximately 50-75% of the control values (1.0 microgram/gram and 0.2 microgram, respectively). No data were provided for the R isomer, but the authors stated these effects were observed only with the S isomer.

No analytical details were published, only the general methods followed by citations from earlier literature studies. Thus, "serotonin was determined by the fluorimetric procedure of Bogdanski et al[25] (1956)."

The Bogdanski procedure is an extension of the Udenfriend et al[23] assay earlier cited by Gillespie et al.[12] Tissues were homogenized in 0.1N hydrochloric acid, and the pH adjusted to 10 with sodium carbonate. The basic solution was then extracted with butanol, acidified, and heptane added to return the serotonin to the aqueous phase. Fluorimetric assay was then accomplished by reading the excitation and emission at 295 nm and 550 nm , respectively. The authors stated that counter current distribution curves of isolated material were similar but not identical to authentic serotonin. They estimated seven per cent of their reading values were due to impurities, and the assay dealt in microgram quantities of serotonin.

The Hess et al study[21] reported norepinephrine determinations "according to the procedure modified by Crout et al[26] (1961) in which the protein is precipitated with trichloroacetic acid (TCA), the catecholamine is adsorbed on alumina, eluted, and converted to the highly fluorescent trihydroxyindole with iodine and alkaline ascorbate." This citation[26] reported the identification and quantitation of catecholamines by isolation on alumina at pH 8.4 followed by elution with acetic acid (0.2N). The analysis was completed by reaction of the column isolate with iodine followed by fluorimetric estimation. No further details were given and no mention was made of possible interfering compounds, of the effectiveness of the separation by alumina chromatography, nor of control values.

To return to the Hess et al[21] paper, the authors noted that dopamine could have contributed about 12 per cent of the fluorescence of a comparable amount of norepinephrine. This was corrected for by using "appropriate standards during the assay for norepinephrine and by determining the dopamine content by the procedure of Drujan et al[27] (1959) except that

the fluorescence was developed by warming the final solution for 50 minutes at 45°." Again, no consideration was given to the possible production of alpha-methyldopa metabolites. Alpha-methyldopa was analyzed according to the method of von Euler and Floding,[28] an earlier report on the analysis of norepinephrine relying on ferricyanide oxidation and fluorescence determination. By this procedure, Hess et al[21] found 1 in the brain, kidneys and heart.

Careful reading of studies like those of Hess et al,[21] including review of the earlier published methods of analysis, reveal the inherent problems these investigators faced in the use of fluorimetric assay procedures. The authors extracted much valuable information with the methods available to them and these studies are still often quoted in recent literature reports. Some of the more recent studies still rely on analytical procedures which were developed more than 20 years ago. It is clear, however, that these procedures suffer from many shortcomings and limitations.

The findings of Hess et al[21] were corroborated by Henning[29] in 1967. After one 400 milligram/kilogram dose of racemic alpha-methyldopa, the blood pressure in renal hypertensive rats fell for a period of one to twelve hours. In that same time period whole brain levels of dopamine and serotonin fell to 75% of control values. Interestingly, the amine levels were determined in normotensive rats. Norepinephrine levels were not maximally depleted until after 24-48 hours, and did not return to normal until 72 hours had passed.

No experimental details were included in Henning's paper.[29] Norepinephrine determination was attributed to the 1958 method of Bertler et al.[30] The citation provides a thorough review of available fluorimetric procedures as well as an extensive study of modifications of these

procedures. It will be worthwhile to summarize this paper since a large number of catecholamine studies based their analytical methods on this report.

The isolation procedure followed by Bertler et al[30] involved homogenization of the tissue with perchloric acid followed by pH adjustment to a value of about 4. The solutions were then passed through a strong cation exchange column (Dowex 50, Na+ form), which retained the amines but not the amino acids. Norepinephrine and epinephrine were eluted together (1N hydrochloric acid) while dopamine was eluted later with 2N hydrochloric acid. Dopamine was not assayed fluorimetrically in this report. After treatment of the eluants with base, potassium ferricyanide was added and the reaction allowed to proceed for two minutes. Then more base and ascorbate were added, and five to thirty minutes later the sample was assayed in a spectrophotofluorimeter. The excitation wavelengths were 410 to 425 nm for norepinephrine and epinephrine, respectively, and the emission wavelengths 535 and 545 nm, respectively. The close similarity of the emission wavelengths was taken into account by a "differential estimation of the two amines by measuring the fluorescence intensity at different activating wavelengths (e.g. 410 and 455 nm). Readings are performed at a fluorescent wavelength of 540 nm." The reason for reading at a value between the noted emission wavelengths for the two compounds was not clear. The analysis thus depended on algebraic manipulations of fluorescence values of samples, blanks and standards. The authors also noted that approximately 1.5% of the norepinephrine values obtained were due to incomplete separation of the dopamine fluorophore.

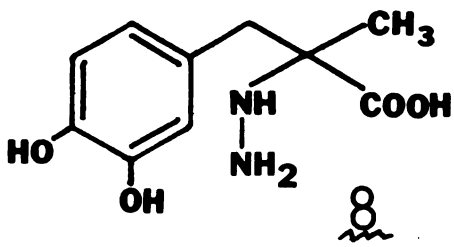
An interesting observation with regard to this analysis is that while Bertler et al[30] had no reason to suspect the decarboxylation of alpha-methyldopa, or even be concerned with it, Henning[29] was well aware of the production of amine metabolites from l. As will be discussed shortly, the decarboxylation of alpha-methyldopa was well established by 1967. Yet Henning[29] made no mention of possible interference by these close structural analogs, and did not discuss any modifications of the analytical procedures used to determine norepinephrine.

The Henning paper[29] further cited Carlsson and Lindqvist[31] and Anden and Magnusson[32] (1962 and 1967, respectively) for the assays of dopamine and serotonin. The Carlsson and Lindqvist[31] citation unfortunately described only modifications of earlier procedures, including the Bertler et al[30] method just discussed. The dopamine assay was based primarily on a procedure developed by Carlsson and Waldeck[33], a companion report to the Bertler et al[30] norepinephrine and epinephrine assay. Carlsson and Lindqvist[31] did provide a glimpse of the problems associated with the alpha-methyl analogs in assays for catecholamines:

> "Differential estimation of alpha-methyldopamine, i.e. the decarboxylation product of alpha-methyldopa, could be performed by utilizing the finding, that this amine behaves essentially like dopamine on the DOWEX 50 column and can thus be separated from norepinephrine, whereas it behaves like norepinephrine in the assay with ferricyanide and thus can be distinguished from dopamine."

The Anden and Magnusson[32] serotonin assay is based on the Bertler et al[30] norepinephrine and epinephrine assay (p.16), but using a weak cation exchange column (Amberlite CG 50). They reported detection of serotonin in the 25 ng range, with a 50 ng minimum for quantitative work.

These studies by Hess et al[21] and Henning[29] suggested that there was no correlation in time between (1) decarboxylase inhibition, (2) norepinephrine depletion, and (3) changes in blood pressure due to administration of alpha-methyldopa. A review by Muscholl on adrenergic transmitters[34] in 1966 also pointed out that more potent decarboxylase inhibitors such as the hydrazine, N-amino-alpha-methyldopa (8) did not lower blood pressure of hypertensive patients.



8

Porter et al[35] also found that the order of potency of a group of decarboxylase inhibitors (RS-alpha-methyl-meta-tyrosine, RS-alpha-methyldopa, and RS-alpha-methyl-2,3-dopa) was inversely related to their ability to deplete the brain of serotonin. Although no data were reported, they stated that only the R isomers of the compounds were active in their studies. Their serotonin assay was that of Bogdanski et al.[25]

It was apparant that decarboxylase inhibition could explain neither the antihypertensive nor endogenous amine depleting characteristics of alpha-methyldopa. In retrospect, this was not a surprising finding. The biosynthetic pathway to norepinephrine (7) from DOPA (2) involves three enzymatic steps. Tyrosine hydroxylase has been shown to be the rate-limiting step in normal norepinephrine synthesis.[36,37,38]

Enzyme	K_m (Ref. 36)
Tyrosine Hydroxylase	4×10^{-6}
DOPA Decarboxylase	4×10^{-4}
Dopamine-beta-hydroxylase	5×10^{-3}

The K_m value may be taken as that substrate concentration at which the enzyme velocity is one half maximal. Thus, inhibition of DOPA decarboxylase would not be likely to significantly alter production of dopamine and norepinephrine.

While studies of endogenous amine depletion and decarboxylase inhibition were being carried out there was a growing interest in the fact that alpha-methyldopa was a substrate for DOPA decarboxylase. In 1960, Udenfriend *et al*[39] presented preliminary findings that l was decarboxylated by *in vitro* preparations of DOPA decarboxylase. The same group published a more complete report[40] later that year. Some of their data are shown in Table II. Using a 10-20 fold purification of the enzyme, the authors found that alpha-methyldopa was a substrate, albeit a poor one, for DOPA decarboxylase.

Neither of these reports[39,40] included detailed experimental data. Amine formation was determined by the 1963 colorimetric method of Dietrich.[41] The *in vitro* preparations were passed through an activated Permutit column which was developed with 20% potassium chloride, and the eluant allowed to react with Folin's reagent.[42] The resulting solutions were assayed colorimetrically. The alpha-methyl amines were, however, also identified by paper chromatography in several solvent systems. The Dietrich paper[41] reported recovery and identification of

Table II

Decarboxylation of Various Amino Acids

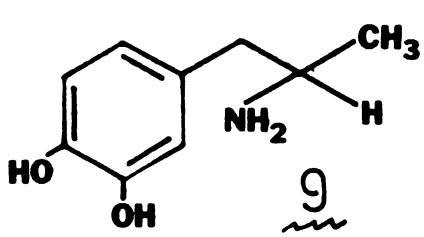
And Alpha-methyl Amino Acids (Ref. 40)

Substrate	Specific Activity microgram/milligram protein/hour
DOPA	8,000
5-Hydroxytryptophane	2,930
Tryptophane	300
Alpha-methyldopa	95
Alpha-methyl-5-Hydroxy-tryptophane	38
Alpha-methyltryptophane	16
Tyrosine	15

Incubations: total volume 3 ml, carried out in 20 ml beakers at 37^{o}, contained enzyme (0.2 - 2 mg protein), 10^{-3}M substrate, 50 micrograms pyridoxal phosphate, 250 micromoles Tris buffer pH 8.5

10 micrograms from their column isolation procedure.

After these initial reports, studies were initiated to examine the formation of alpha-methylamines in animal tissues. Early evidence that these amines might be involved in some of the pharmacological events associated with administration of alpha-methyldopa came from Porter et al[35] in a study on the depletion of endogenous amines. Without studying the decarboxylation of 1 itself, they found that administration of racemic alpha-methyldopamine (9) produced longer depletion of heart norepinephrine than administration of alpha-methyldopa. The effect of 9 on



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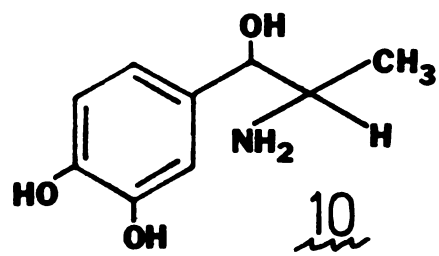

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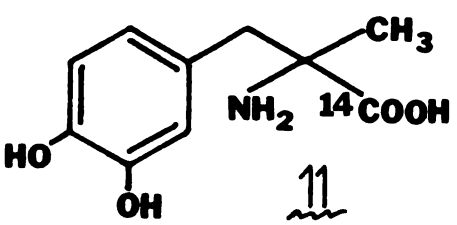
blood pressure was not studied. Thus it appeared even more certain that decarboxylase inhibition was not the cause of endogenous amine depletion.

Carlsson and Lindqvist[31] then published their findings on the in vivo formation of alpha-methyldopamine (9) and alpha-methylnorepinephrine (10) in the brains of mice and rabbits, following administration of alpha-methyldopa. The isolation and identification of these compounds as metabolites will be discussed in detail in the metabolism section. The authors[31] reported whole brain levels of 9 to be about 0.5-1.0 microgram/gram. Whole brain levels of 10 were stated to be approximately 0.4 microgram/gram. These workers further noted that norepinephrine could be totally depleted in the brain by repeated ad-

ministration of alpha-methyldopa, and that six hours after treatment with 1 , alpha-methyldopamine levels in the brain were approximately equal to dopamine levels.

As was mentioned earlier (p.18), Carlsson and Lindqvist[31] relied on analytical methods published by Bertler et al[30] and Carlsson and Waldeck.[33] After column isolation, alpha-methyldopamine was identified by fluorescence procedures. The difficult separation of alpha-methyldopamine from dopamine and norepinephrine was described and quoted earlier (p.18), both from the standpoint of column isolation, and fluorescence characteristics. Alpha-methyldopamine was also identified by paper chromatography methods. Alpha-methylnorepinephrine (10) was identified by paper chromatography following acidic elution from a cation-exchange column. The quantitative data with regard to 10 was evidently obtained fluorimetrically by spraying the paper chromatography spots with a ferricyanide solution. No further details were provided in the report.

Porter and Titus[43] then reported the collection of $^{14}CO_2$ after administration of ^{14}C-labeled alpha-methyldopa(11). Up to 50% of a five milligram/kilogram intraperatoneal (ip) dose was recovered as expired $^{14}CO_2$. With the label in the alpha (or C-2) position (12), these workers could isolate a radioactive substance which was identified as alpha-methyldopamine (9) by paper chromatography.



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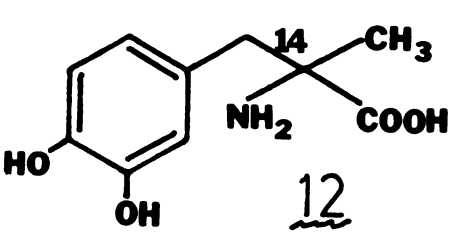

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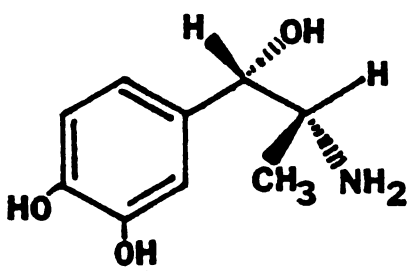
Independently, Creveling et al[44] reported that alpha-methyldopamine (9) was a substrate for an in vitro preparation of dopamine beta-hydroxylase. Relative to the best substrate for the system (para tyramine), dopamine had 85-90% activity and alpha-methyldopamine 50-60% activity. The hydroxylated products were assayed fluorimetrically directly from the incubation mixtures, each substrate being incubated with the enzyme preparation separately. No consideration of stereochemistry was involved in the study. Several other studies reporting the conversion of alpha-methyldopa to 10 relied on the effect of tissue extracts containing 10 on the blood pressure of pithed rats.[45,46] There were also additional reports utilizing fluorimetric assay procedures for the identification of 10.[46,47,48]

As already discussed, the difficulty in assaying alpha-methyldopamine in biological fluids is due in part to its chemical similarity to dopamine and norepinephrine in terms of column isolation and fluorimetric assay procedures. These considerations also apply to the determination of alpha-methylnorepinephrine. Despite these methodological problems, one of the studies cited above[47] provided no experimental details. Another study relied on the blood pressure activity of tissue extracts for catecholamine content, and paper chromatographic (no details reported) identification of 10 with a potassium ferricyanide spray. Another study[46] employed both the pithed rat assay and "differential fluorimetry." This procedure was not discussed, but Bertler et al[30] were cited yet again for their assay of norepinephrine and epinephrine. The isolation procedure employed in the assay[46] utilized alumina adsorption, as did the final paper in this group of alpha-methylnorepinephrine studies.[48] Both cited von Euler and Orwen[49] for the method. This 1955 citation, of course, did not consider the problem of separation of the alpha-methylamines from their endogenous

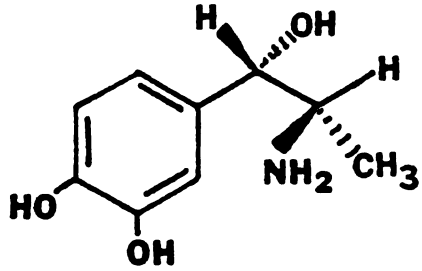
counterparts. It appears that this series of studies may be taken in summation to definitely indicate the formation of the alpha-methylamines in tissues, but that the quantitative data cannot be regarded as reliable.

A more recent fluorimetric assay for alpha-methylnorepinephrine by Waldeck[50] indicates that these problems still exist. The author noted a need for "a more sensitive method" and the "importance to make a differential estimation of alpha-methylnoradrenaline and noradrenaline since these two compounds are not easily separated by cation exchange chromatography." He might also have included alpha-methyldopamine in his statement, as it too is difficult to separate by column procedures.

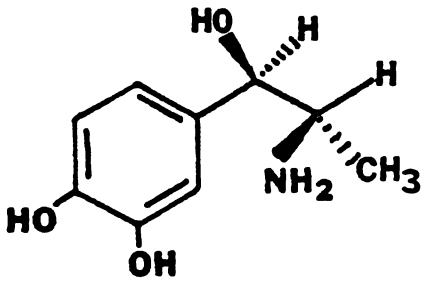
The absolute configuration of the erythro pair (10a, 10b) has been determined,[51] but we could not find that information for the threo pair (10c, 10d). Waldeck's assay depended on two facts reported earlier in a citation by Hallhagen and Waldeck.[52] First, the erythro pair can be converted to the threo pair in aqueous acid. Second, the fluorescence of the erythro and threo pairs, when treated according to the methods of Bertler et al[30] are quite different. The paper[52] reported the preparation of tritium labeled alpha-methylnorepinephrine (Figure 10, 15) by reduction of the precursor ketoxime 16 with tritium gas. After separation of reaction products by cation exchange chromatography, two of the components were found to be inseperable from authentic alpha-methylnorepinephrine by paper chromatography in several solvent systems. After mixing one component with authentic erythro 10, and the other with authentic threo alpha-methylnorepinephrine, the components were rechromatographed on the cation exchange columns. "The radioactive elution pattern coincided with the fluorescence elution pattern of the respective authentic substances in both samples."



10a



10c



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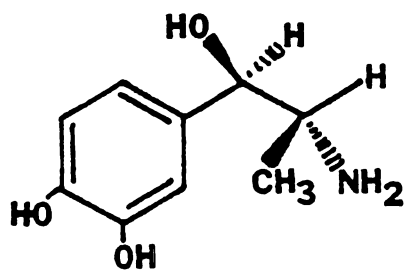

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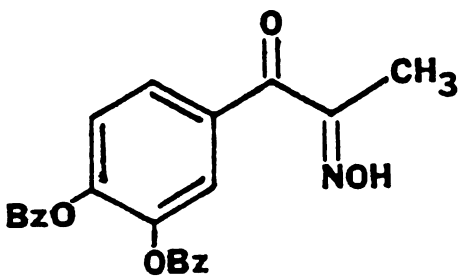

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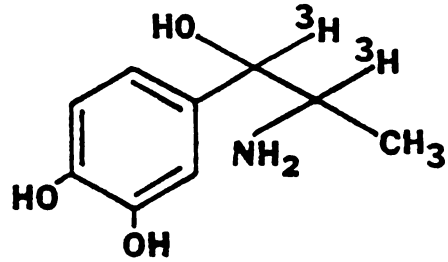

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It was further reported that when authentic threo-10 and erythro-10 were treated by a specific fluorimetric procedure (Bertler et al[30]), the threo compound had a fluorescence similar to that of norepinephrine, and 30-40 times stronger than the erythro-alpha-methylnorepinephrine pair 10a, 10b. Upon comparison with authentic samples, the fluorescence characteristics of the two components isolated from the cation exchange column, it was found that they differed in a similar pattern to the authentic compounds. Thus, Hallhagen and Waldeck,[52] on the basis of cation exchange separation, paper chromatography, and fluorescence characteristics satisfactorily identified and separated the erythro and threo pairs. However, the only mention of the acid catalyzed conversion of erythro to threo was a sentence in the discussion section noting that when the acidic eluate from the cation exchange column was evaporated, the erythro compound was converted to the threo compound.

On the basis of the information summarized above, Waldeck[50] treated erythro-10, threo-10, and norepinephrine with boiling 2N hydrochloric acid, and reported the following results after fluorimetric analysis:

	threo-10	erythro-10	norepinephrine
Unboiled	100 ± 5	6 ± 1	103 ± 5
Boiled	69 ± 1	69 ± 2	92 ± 2

Relative fluorescence based on erythro-10 as 100% (Ref. 50).

The conclusion was that an equilibrium was established after the acidic treatment between the erythro and threo isomers. No further experimental results were reported except for a linear response of fluorescence for both boiled and unboiled erythro compound as a function of concentration.

Although the need for separation techniques was alluded to in the preliminary discussion of the assay, no details were reported. The

problems of interference from other amines were not resolved, either from the standpoint of separation of or fluorimetric analysis. Further, the assay does not depend upon converstion of one isomer to the other, but rather an equilibrium between two isomers. Finally, as will be discussed in the metabolism section, the configuration of metabolically formed alpha-methylnorepinephrine has not been unambiguously determined.

The earlier mentioned studies all pointed to the metabolic formation of alpha-methyldopamine (9) and alpha-methylnorepinephrine (10). There were also reports of neuronal uptake,[53] concentration in granules,[54] and release upon nerve stimulation of alpha-methylnorepinephrine.[46] A second proposal for the mechanism of action of alpha-methyldopa was formulated on the basis of these and other reports. The hypothesis is usually jointly attributed to Carlsson and Lindqvist[31] and to Day and Rand.[55] The proposal, as reported by Day and Rand[55] in 1964, stated that *in vivo* production of alpha-methylnorepinephrine would:

> "serve in place of noradrenaline to mediate responses to sympathetic nerve impulses. However, alpha-methylnoradrenaline is less potent than noradrenaline. It may be that in hypertensive patients treated with alpha-methyldopa the substitution of alpha-methylnoradrenaline for noradrenaline diminishes the effectiveness of sympathetic impulses to the blood vessels. Nevertheless, responses to sympathetic impulses persist, so that severe postural hypotension and other unpleasant consequences of complete sympathetic blockade occur only rarely."

In pursuing this concept, several aspects were of obvious importance for study. These included estimations of the potencies of the metabolites (alpha-methyldopamine had not been excluded) as neurotransmitters, the response of the sympathetic nervous system to alpha-methyldopa treatment, the effect of the metabolites themselves on the sympathetic nervous system, and finally, the effect of blockage of amine metabolite formation on the response to alpha-methyldopa treatment.

Day and Rand[55] showed that cat nictitating membrane contractions were reduced, but not abolished, by alpha-methyldopa. They further demonstrated that alpha-methyldopa and its amine metabolites 9 and 10 restored tissue response in reserpine treated animals upon sympathetic stimulation. They found alpha-methylnorepinephrine to have from one-half to one-ninth the activity of norepinephrine in such experiments. Tyramine response was partially restored in reserpine treated animals with alpha-methylnorepinephrine. Alpha-methyldopamine (9), the precursor of alpha-methylnorepinephrine (10), had only about 1% the activity of norepinephrine.[56] Thus, there was some information to support the claim that the amine metabolites were "false" neurotransmitters, i.e., weaker than norepinephrine. The cardiovascular characteristics of 1 , along with the side effects, were seen as indirect evidence supporting the hypothesis. Bradycardia, postural hypotension, and reduced vascular resistance were all interpreted as signs of replacement of norepinephrine with a weaker neurotransmitter (such as 9 or 10) in the sympathetic nervous system.

Further study of the false neurotransmitter theory tended to disprove the hypothesis. The evidence against the proposal that the mechanism of action of 1 was dependent upon replacement of norepinephrine by 9 or 10 has been well summarized by Henning,[57] and also by Muscholl[34] in his review on adrenergic blockade.

Henning[57] summarized a variety of studies concerned with alpha-methylnorepinephrine as a false neurotransmitter. He concluded that while 10 was a weaker transmitter than norepinephrine, it could not account for the physiological responses to alpha-methyldopa. Reasons cited by Henning included the fact that most organs have compensatory mechanisms to allow higher rates of firing (2-4 times) that would easily alleviate the loss in sympathetic activity due to alpha-methylnorepinephrine.

Henning also noted there is less loss of sympathetic tone after administration of l than after administration of reserpine and other adrenergic blockers. Day and Rand[54] themselves had observed that the response to tyramine was not altered in cats after two days of alpha-methyldopa treatment.

Goldberg et al[58] found that sympathetic responses in unanesthetized cats were little altered during the time of antihypertensive response (4-6 hours) to 100-200 milligram/kilogram doses of alpha-methyldopa administered intravenously (iv). Henning and Svensson[59] thoroughly investigated adrenergic nerve function in rats after alpha-methyldopa treatment. Their results, when taken with other evidence, suggested to them that "alpha-methyldopa may indeed suppress peripheral sympathetic function at short intervals . . . such a blockade, if it occurs, is always considerably less than that observed after adrenergic neurone blocking drugs or reserpine." They concluded that the false neurotransmitter effect of the drug could not account for its antihypertensive activity.

Other information failed to support the false neurotransmitter theory. The lack of correlation between the depletion of norepinephrine and antihypertensive effects mentioned earlier (p. 14,16) are relevant to this hypothesis also. Since the levels of norepinephrine fell long after the lowering of blood pressure, replacement of the transmitter by alpha-methylnorepinephrine could not have occurred during the antihypertensive period.

It was noted earlier[21,29] that the depletion of dopamine (and its subsequent return to control values) correlated in time to the fall and rise in blood pressure after treatment with alpha-methyldopa, while norepinephrine depletion occurred much later. The same relationship held for the inhibition of DOPA decarboxylase. Based on these findings, Farmer[60] proposed that the accumulation of alpha-methyldopamine might be partially responsible for the antihypertensive activity of 1. The study[60] demonstrated that alpha-methyldopamine definitely impaired sympathetic nerve responses. Further, a summary of the literature was presented which suggested that the antihypertensive action observed with monoamine oxidase inhibitors may be due to an accumulation of dopamine. This proposal has received little further attention in the literature.

Further arguments against the false neurotransmitter theory included the finding that a much weaker neurotransmitter than alpha-methylnorepinephrine, metaraminol, did not lower blood pressure in animals except in very high doses.[57] Direct administration of either 9 or 10 was found to elicit hypertensive responses in rats[61] and monkeys.[62] In another study by Henning,[63] the effects of decarboxylase inhibitors on the pharmacological responses of animals to treatment with alpha-methyldopa were investigated in detail. One of these inhibitors, the hydrazine N-amino-alpha-methyldopa (8), was characterized by Henning[63] and others[64] as a peripheral decarboxylase inhibitor, one which prevented decarboxylation in the periphery, but not in the central nervous system. It was found that 8 did not prevent the antihypertensive action of alpha-methyldopa.

All of the studies suggested that replacement of norepinephrine by alpha-methylnorepinephrine in the peripheral sympathetic nervous

system could not explain the antihypertensive effect of 1. It then became necessary to explain the dramatic effects of alpha-methyldopa on the cardiovascular system without a corresponding loss of general sympathetic tone. Such selective effects suggested some form of control by the nervous system.

Further studies by Henning[63] with decarboxylase inhibitors led to the proposal of a centrally-mediated mechanism of action for alpha-methyldopa. The key finding was that the central (and peripheral) decarboxylase inhibitor seryl-2,3,4-trihydroxybenzyl-hydrazine (R 4-4602)[65,66] did prevent the antihypertensive effect of 1, while the earlier mentioned peripheral decarboxylase inhibitor 8 did not. Both inhibitors prevented formation of alpha-methyldopamine in the heart, but only the central decarboxylase inhibitor prevented formation of 9 in the brain. Henning and van Zwieten[67] reported that a 20 milligram/kilogram infusion of 1 into the vertebral artery of the brain was as effective as a 200 milligram/kilogram intravenous infusion in cats in eliciting an antihypertensive response. The former dose, when applied intravenously, was innefective. Studies with monkeys[68] also demonstrated that smaller doses, when administered centrally, were effective at lowering blood pressure, compared to peripheral administration. These studies suggested that alpha-methyldopa owed its antihypertensive activity to events taking place in the central nervous system, and that decarboxylation of the parent drug was a necessary prerequisite for that activity.

Ingenito *et al*[69] found that vascularly isolated brains of unanesthetized cats, when perfused with alpha-methyldopa, caused hypotension, bradycardia, and decreased hind limb vascular resistence. The authors, however, disagreed with Henning's conclusions[57,62] that metabolism of the

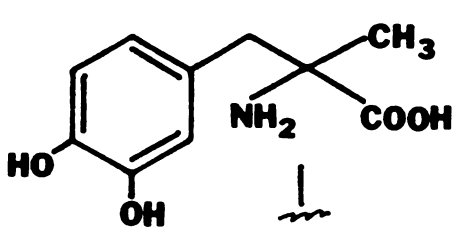
drug is a necessary prerequisite for its antihypertensive activity. Using column isolation and fluorimetric procedures similar to those employed by Henning in his work, Ingenito's group[69] found very little depletion of norepinephrine in the brain during the time of antihypertensive response to alpha-methyldopa. Further, the onset of action time was quite short, about five minutes, leading to the proposal that the parent drug was the active pharmacological agent. The fact that administration of alpha-methylnorepinephrine elicited a hypertensive response in animals[61,62] supported that hypothesis.

It is clear from the above review of the literature that the mechanism of action with regard to the antihypertensive effect of alpha-methyldopa is not well understood. It has, however, been shown that decarboxylase inhibition and replacement of norepinephrine with alpha-methylnorepinephrine cannot explain the action of the drug. While recent work has indicated that the mode of action is central in nature, the details of such a process are not understood. Based on the above considerations, it was apparant that further studies on the metabolism of l in the central nervous system were required to better understand the events leading to the antihypertensive effects of the drug. The reported studies on the central mechanism of action[63,67,68,69] have led to a particular interest in the decarboxylation of l in the brain, and to the relationship of the production of alpha-methylamine metabolites to the endogenous amines in the brain. While the analytical procedures employed in the pharmacological studies reviewed in this section provided much valuable information (including that used to formulate our research plans), they presented major problems with regard to sensitivity, and especially with regard to

specificity. In view of these limitations, and our interest in the details of alpha-methyldopa metabolism in the brain, we felt it necessary to develop more satisfactory methods for the analysis of compounds in the brain. These procedures will be described later in this thesis.

ALPHA-METHYLDOPA METABOLISM

To a large extent the metabolism of alpha-methyldopa (1) is determined by its structural similarities and differences to its endogenous analog DOPA (2). A review of DOPA metabolism is therefore useful for



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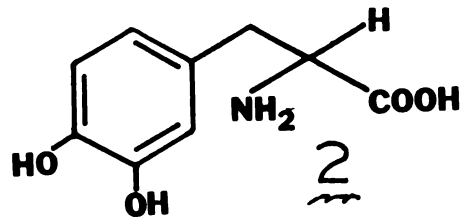

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comparison with the biotransformation of 1 .

There are hundreds of literature citations pertaining to the metabolism of DOPA, primarily due to its role as the precursor of the sympathetic transmitter norepinephrine (7a) and the neural hormone epinephrine (17a), as shown in Figure 1 . The metabolism of 2 has, however, been well summarized by many authors, and the review presented here is taken from several such summaries.[36,70,71] The biotransformations of DOPA can be divided into two general categories, the biosynthetic type reactions leading to the various amines (including 6,7, and 17) and bioinactivation reactions leading to inactive metabolites readily excreted by the organism.

The first general category which starts with the amino acid precursors phenylalanine (18) and tyrosine (19), is illustrated in Figure 2. The enzymes involved in these reactions have been thoroughly characterized. An excellent summary of each enzyme can be found in Nagatsu's volume on the catecholamines.[70] All of the minor amine products

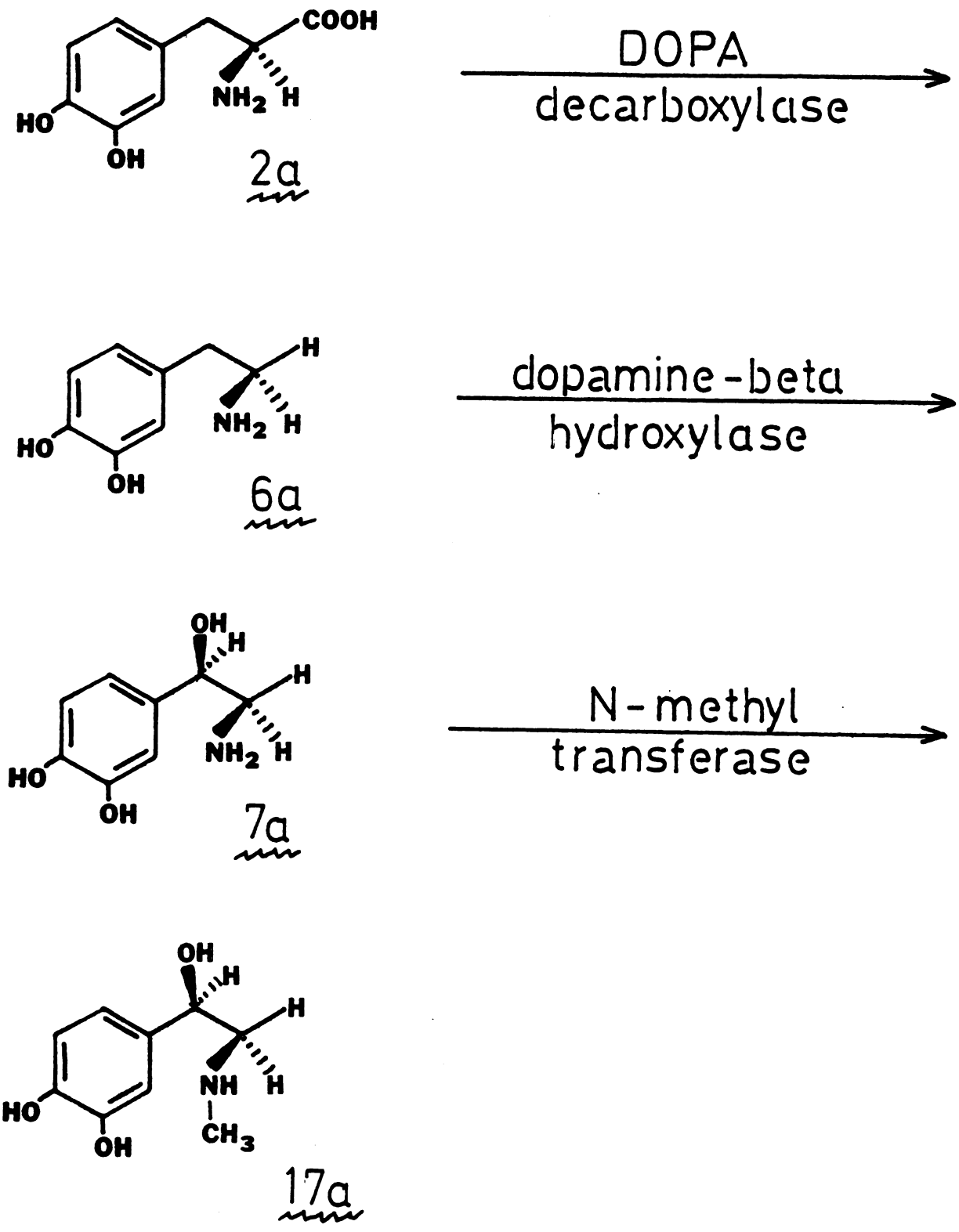


Figure 1. Enzymatic production of the neurotransmitter norepinephrine (7a) and the neural hormone epinephrine (17a).

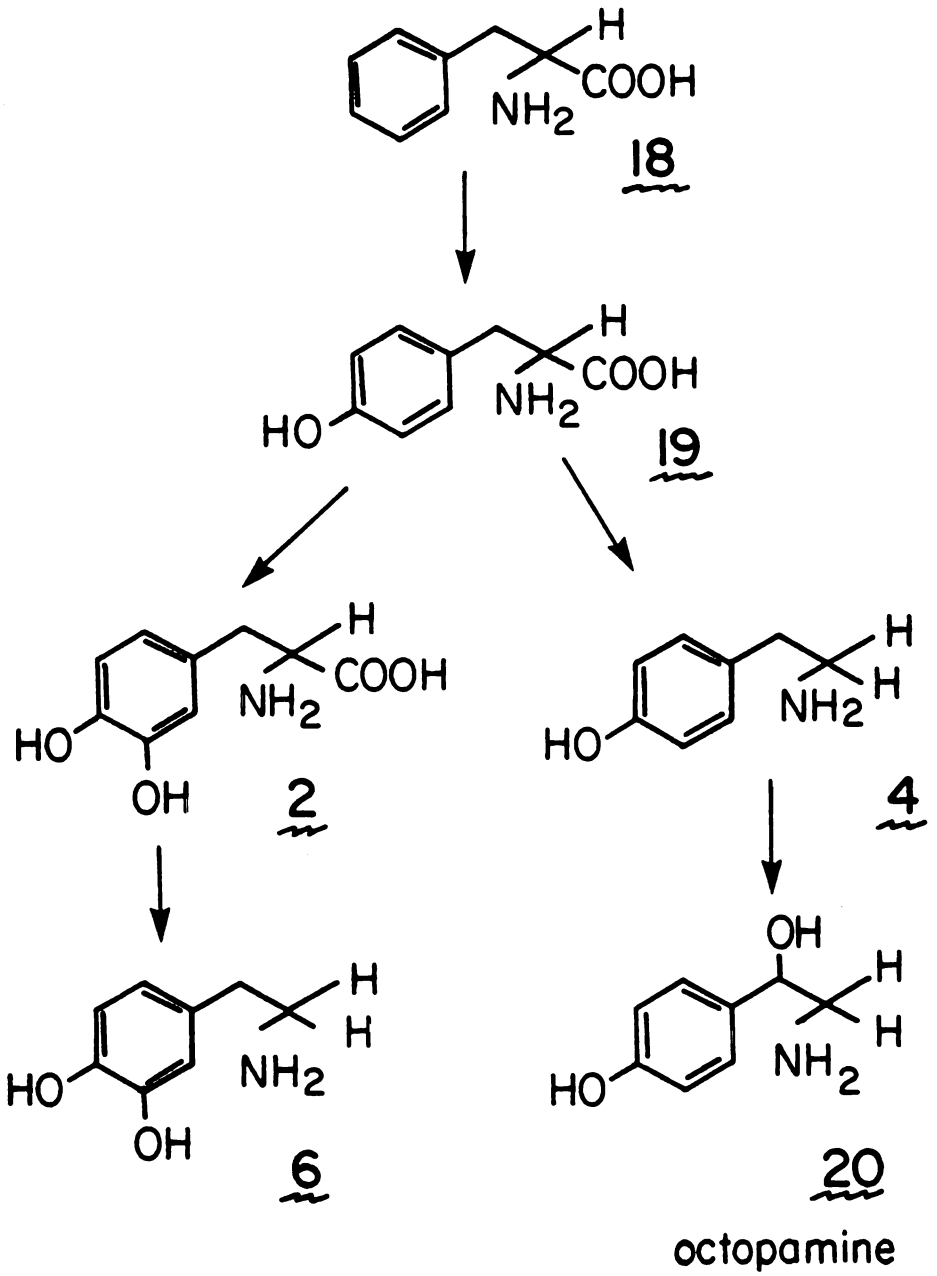


Figure 2. Production of amines from precursor amino acids, as a part of DOPA metabolism (Ref. 36, 70).

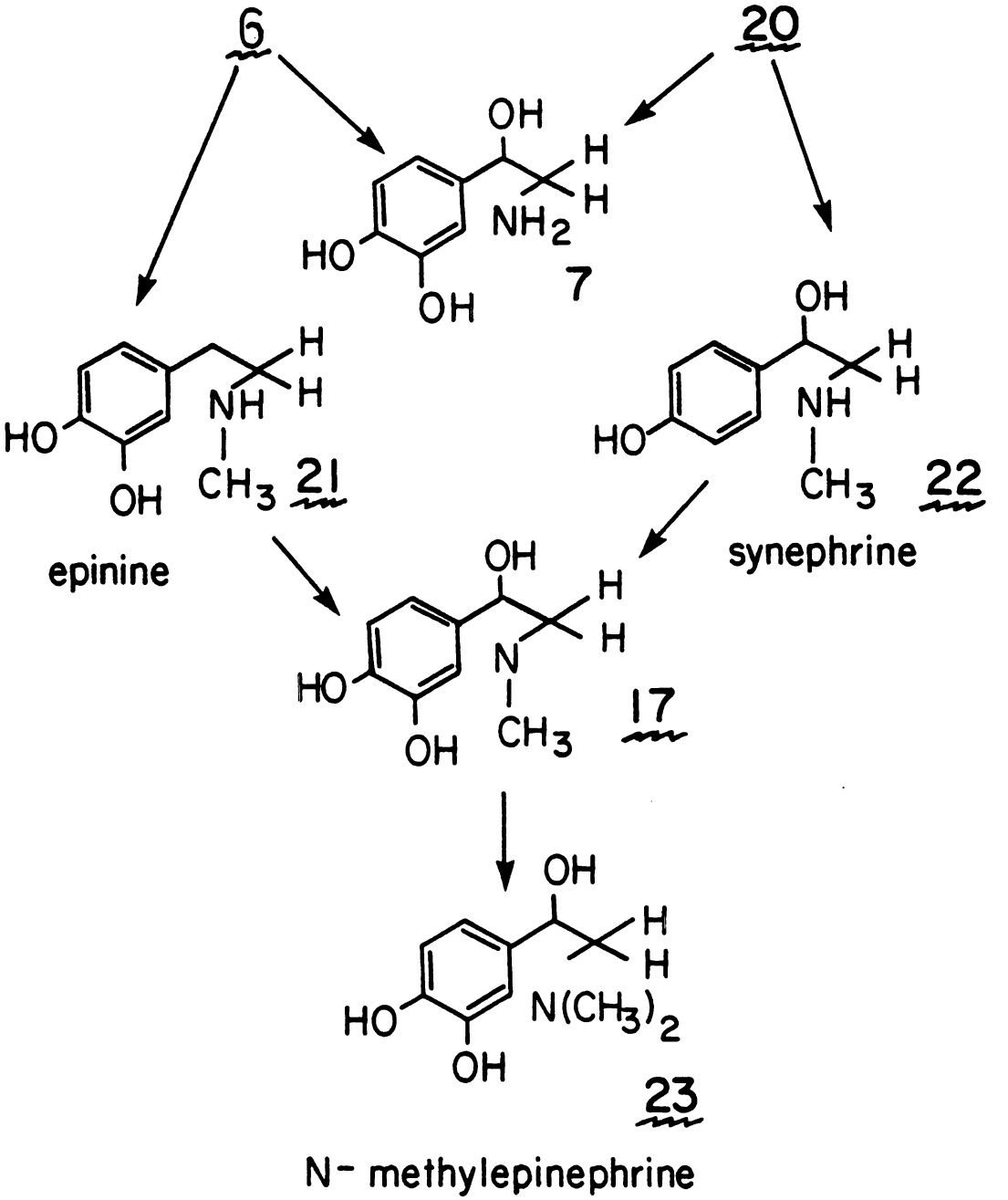
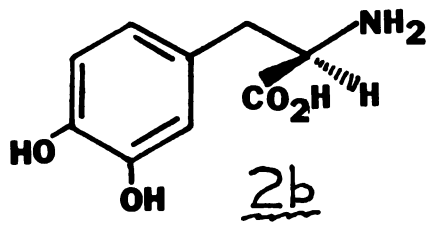


Figure 2, cont'd.

(octopamine, epinene, etc.) have been identified in urine or tissue. Many of the biotransformations depicted in Figure 2, such as side chain oxidation and decarboxylation, also describe the metabolism of alpha-methyldopa.

DOPA decarboxylase has been reported to decarboxylate the S isomers of amino acids, but not the R isomers.[72] However, R-DOPA (2b) was re-



2b

ported to yield significant amounts of dopamine and its metabolites.[73,74] A follow-up study by Sourkes et al[75] confirmed these results using carboxyl labeled ^{14}C-DOPA. Although no experiments were conducted with regard to the pathway for the formation of the decarboxylated metabolites of 2b, it was suggested that the configuration of 2b undergoes inversion (via D-amino acid oxidase and transamination) to form the S enantiomer. On the basis of such a mechanism, this transformation is not possible with alpha-methyldopa because of the lack of an alpha hydrogen atom. Thus, production of amine metabolites would not be expected from the R isomer of alpha-methyldopa.

The naturally occurring product of dopamine beta-hydroxylation, norepinephrine has been shown to be levorotatory.[76,77] The absolute configuration was determined to be R as shown in Figure 1 (7a),[78] as was the N-methylation product epinephrine (17a).[79] As will be discussed below, it has frequently been assumed, on the basis of the above information,

that alpha-methyldopamine undergoes stereospecific beta-hydroxylation in the same manner as does dopamine.

The further metabolism of these compounds, as shown in Figure 3, is usually discussed in the context of inactivation since most of these products are pharmacologically inactive and many are readily excreted. Side chain oxidation or reduction, O-methylation, or combinations of the two account for most of the biotransformation products. Although not shown in these schemes, catecholamines are also conjugated, yielding both sulfate and glucuronide derivatives.[36,70,71] In this second category of biotransformation (Figure 3), the most important fact with regard to alpha-methyldopa metabolism is that monoamine oxidase does not act upon 1-phenyl-2-aminopropanes.[80] Therefore, the pathways which lead to phenylacetic acids, phenylacetaldehydes, and phenylethanols are not viable pathways for the biotransformation of alpha-methyldopa.

The metabolic fate of alpha-methyldopa (1) has been studied both in animals and man. Many of the early studies[31,43,45,46,47,48] were concerned with the pharmacological implications of the production of alpha-methyldopamine (9) and alpha-methylnorepinephrine (10) in tissues, and were discussed in the pharmacology review. In vitro enzymatic studies demonstrating 1 to be a substrate for DOPA decarboxylase[39,40] and 9 to be a substrate for dopamine beta-hydroxylase[44] have been reported and were also discussed in the previous section.

In 1962, Porter and Titus[43] published a study of the in vivo metabolism of alpha-methyldopa in the rat. Utilizing thin-layer chromatography (tlc), countercurrent distribution, ultraviolet (uv) analysis, and ^{14}C-labeled substrates, they identified several urinary metabolites of S-alpha-methyldopa. These included the 3-O-methyl ether of alpha-

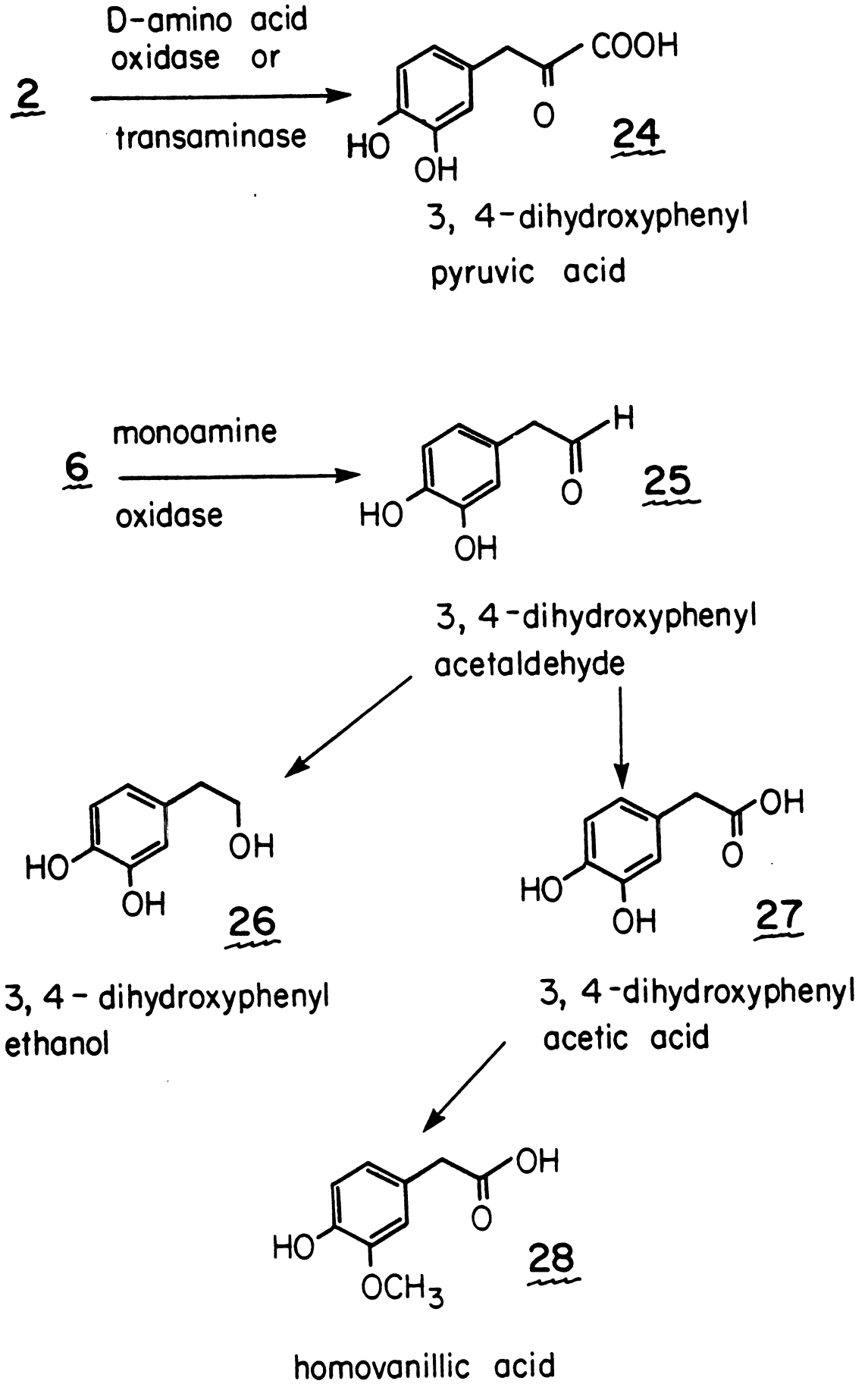


Figure 3. DOPA metabolism leading to excreted metabolites (Ref. 36,70).

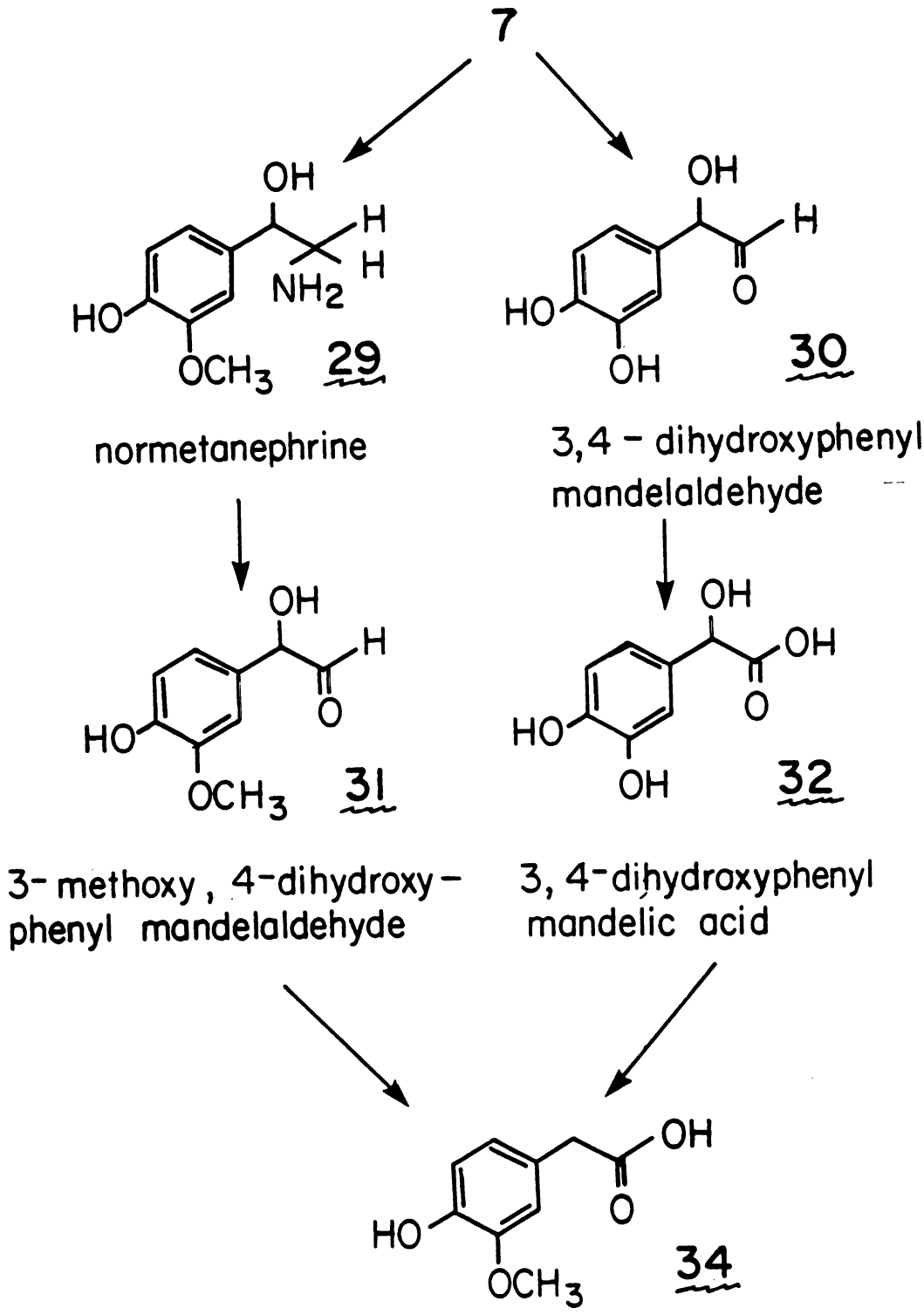


Figure 3, cont'd.

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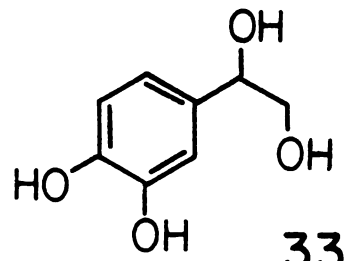

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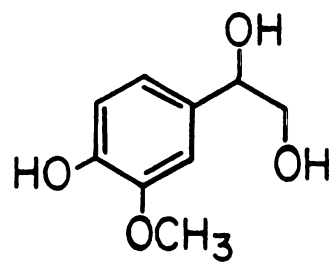
3,4-dihydroxyphenyl glycol



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3-methoxy, 4-hydroxyphenyl glycol

Figure 3, cont'd.

methyldopa 36, alpha-methyldopamine (9), its 3-O-methyl ether 37 , and the deaminated ketone 38 as shown in Figure 4. They also reported the 3-O-methyl ether of the ketone, but no later studies were able to verify this metabolite. After treatment with glucuronidase, alpha-methyldopamine (9) and its 3-O-methyl ether 37 were tentatively identified by tlc, suggesting the presence of glucuronide metabolites. Sulfate metabolites were mentioned, but not identified in the study.

Another study of urinary metabolites of 1 in rats was reported by Young and Edwards[81] in 1964. Using tlc, 1, 36, 9, and 37 were reported as metabolites. They too proposed glucuronide metabolite formation, on the basis of increased amounts of 36 and 37 obtained after treatment of samples with glucuronidase. Of more interest was their finding that when the R and S isomers were separately administered, five times as much S-alpha-methyldopa was absorbed as R-alpha-methyldopa based on urinary and faecal excretion. They suggested the possibility of an active transport system for the S isomer, and noted that such systems are known for naturally occurring amino acids. The extension of such a system to the blood brain barrier will be discussed in the *in vivo* metabolism studies section of this thesis.

A preliminary report[82] of alpha-methyldopa metabolism in man appeared in 1962. Alpha-methyldopamine (9) was identified in the urine of hospitalized patients receiving alpha-methyldopa. A more thorough study by Buhs *et al*[83] in 1964 accounted for 90% of an oral dose of 1 . Approximately 50% of the S isomer was absorbed, and 80-90% of the radioactivity from a ^{14}C-labeled substrate was eliminated in 48 hours. No data for the metabolism of the R isomer were reported.

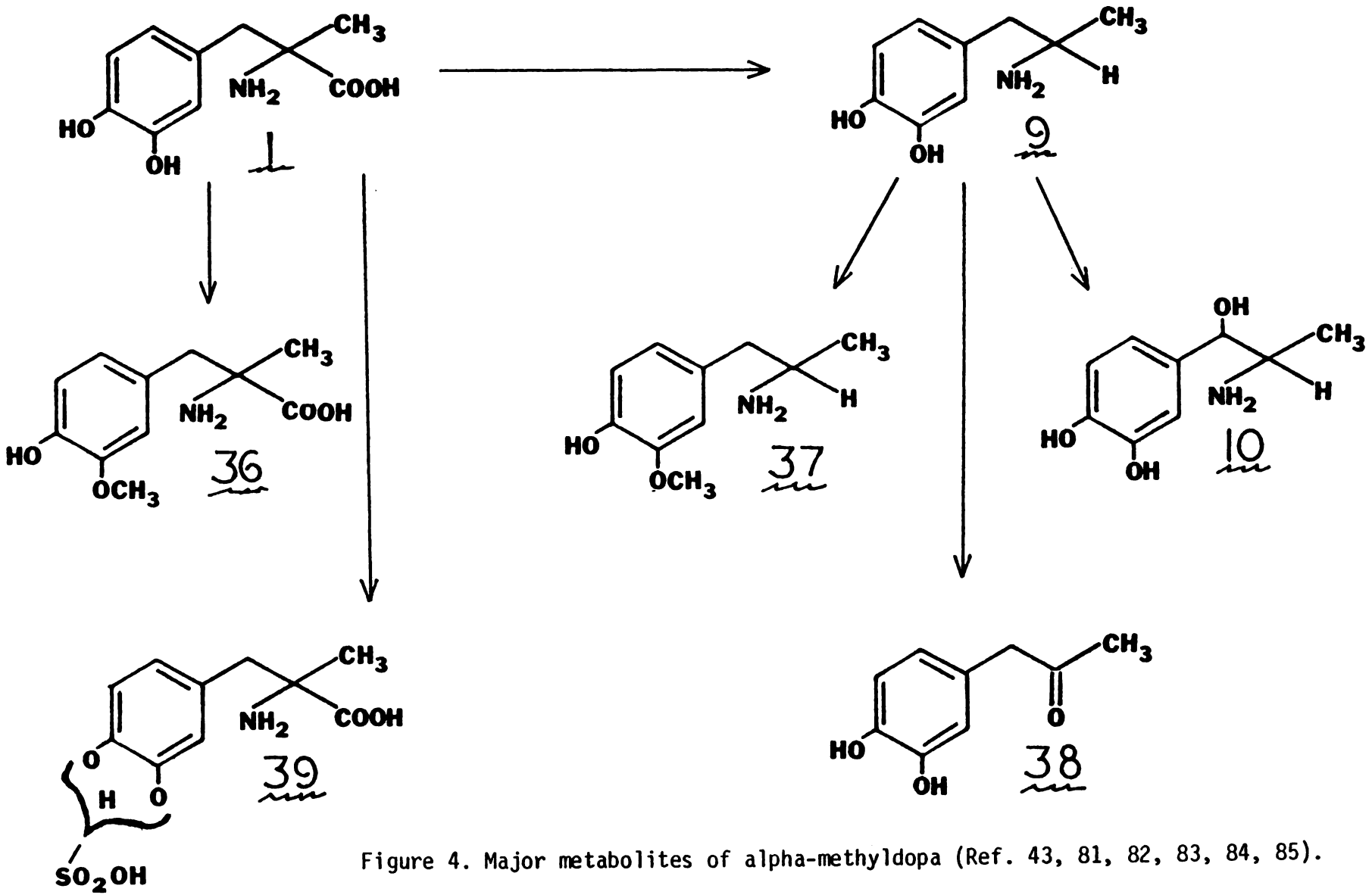


Figure 4. Major metabolites of alpha-methyldopa (Ref. 43, 81, 82, 83, 84, 85).

Ion exchange chromatography was utilized for the separation of urinary metabolites into basic, acidic, and neutral fractions. Conjugates were liberated by autoclaving in aqueous solution at pH 1 . Methods of analysis included the use of ^{14}C-labeled substratess, tlc, uv, nuclear magnetic resonance (nmr), infrared (ir), and elemental analysis.

Alpha-methyldopa (1, 24% of urinary products) and 3-O-methyl-alpha-methyldopa (36, 4% of urinary products) were characterized by nmr, uv, ir, and tlc analyses. The basic fraction (3-10% of urinary products) was acetylated (pyridine/acetic anhydride), and the tri-acetyl derivative of alpha-methyldopamine (9) identified by nmr and tlc. At least three other basic metabolites were isolated but not identified. The authors noted that one of these metabolites had apparantly been altered in the catechol portion of the molecule. Although not mentioned by the authors, a reasonable postulate for this amine is 3-O-methyl-alpha-methyldopamine (37). The neutral fraction revealed two spots on tlc analysis, neither of which were identified. The authors claimed that neither compound had the tlc characteristics of the ketone 38 or of the 3-O-methyl derivative earlier tentatively identified by Porter and Titus[43] in the rat.

The presence of the mono-O-sulfate of alpha-methyldopa (39, 64% of urinary products) was demonstrated with the aid of nmr, ir, and elemental analysis for sulfur. Thus, this paper[83] described the major metabolite of alpha-methyldopa metabolism in man, although the formation of the ketone 38, and the 3-O-methyl derivative of alpha-methyldopamine 37 could not be unambiguously established.

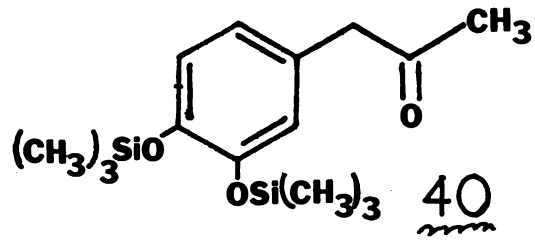
In a 1966 report by Prescott et al[84] utilizing ^{14}C-labeled 1, alpha-methyldopa (1, 40% of the radioactivity), the mono-O-sulfate

(39, 31% of the radioactivity), total neutrals (9% of the radioactivity), free bases (3% of the radioactivity), and conjugated bases (3% of the radioactivity) were described. The purpose of this metabolic study was an attempt to correlate the levels of metabolites with the time course of the antihypertensive activity of the drug. Their efforts were unsuccessful, as no correlation was found.

In 1972, Au et al[85] published another human metabolic study. As in the Buhs et al[83] study, approximately 50% of the S isomer administered orally was absorbed, and most of the radioactivity of the ^{14}C-labeled alpha-methyldopa was eliminated within 24 hours. In agreement with Young and Edward's animal work,[81] it was found that the R isomer was much less readily absorbed after oral administration. Indeed, the absorption of the R isomer was only about 10% that of the active S isomer as determined by urinary and faecal radioactivity data.

The absorption and elimination data were obtained by administering a ^{14}C-labeled substrate, and determining the radioactivity. Amine metabolites were isolated by ion exchange chromatography. Identification of metabolites was achieved by tlc with various solvent systems in conjunction with either radioactivity or color reactions, and by gas liquid phase chromatography/mass spectrometry (gc/ms). Conjugates were liberated by boiling in aqueous acid.

Free and conjugated alpha-methyldopa(1,39-25% of the dose), free and conjugated 3-O-methyl-alpha-methyldopa (36, 4% of the dose), and total amines including alpha-methyldopamine (9) and its 3-O-methyl ether 37 were identified by tlc analysis after administration of the S isomer. The ketone 38 (3-5% of the dose) was identified by gc/ms as its trimethylsilyl derivative 40 . The ketone had not previously been identi-



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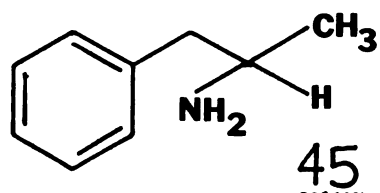
fied as a human metabolite. Metabolism of the R isomer resulted only in unchanged and conjugated parent drug (9-14% of the dose), a small amount of the 3-O-methyl ether of alpha-methyldopamine 36 (1-2% of the dose), and, surprisingly, a trace of the ketone 40 .

This study was of further interest in that the authors specifically searched for several other logical metabolites of 1 . Paper chromatography and tlc coupled with the development of characteristic color reactions were utilized for the determination of alpha-methylnorepinephrine (10), alpha-methylnormetanephrine (41), 3,4-dihydroxybenzoic acid (42), 3-methoxy-4-hydroxybenzoic acid (43), and the previously mentioned 3-methoxy-4-hydroxyphenylacetone (44), as the pure compounds (Figure 5). None of these compounds were detected in human urine. The benzoic acids 42 and 43 can be considered as possible metabolites due to the fact that formation of benzoic acid and derivatives represents a major metabolic pathway in the metabolism of the hallucinogenic amine amphetamine (45).[86]



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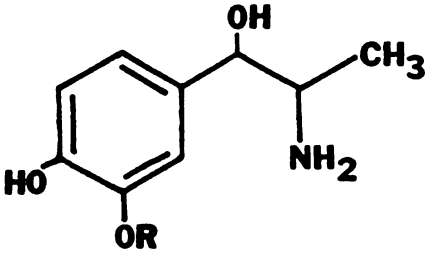

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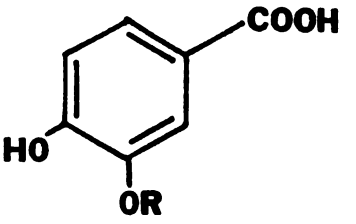
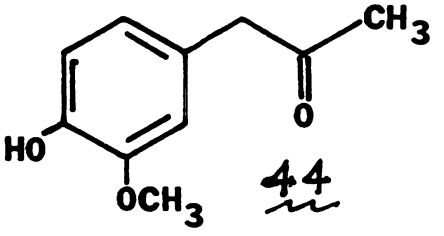
41 R=CH3



42 R=H
43 R=CH3



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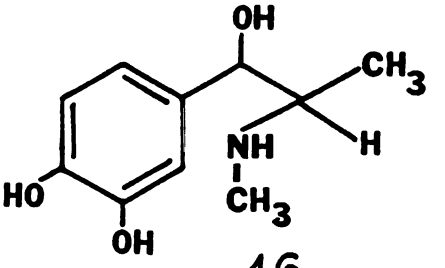

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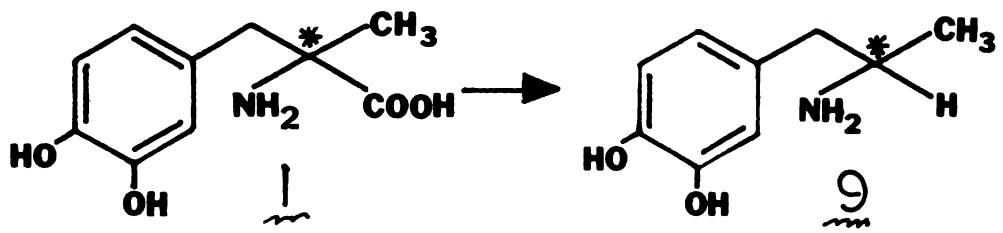
Figure 5. Metabolites which could not be identified in human urine by paper chromatography or tlc in a major study (Ref 85).

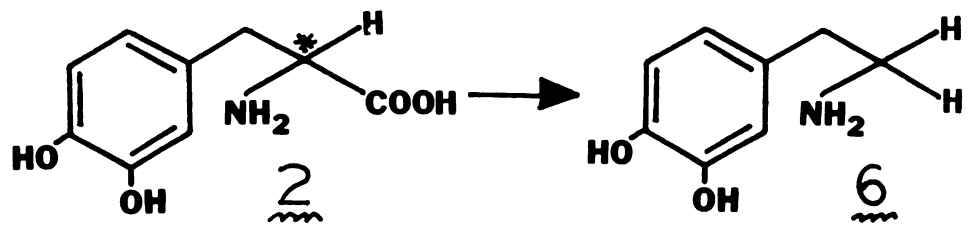
There have been several reports of the urinary excretion of alpha-methylnorepinephrine (10) in humans. In a tlc diagnostic test for 3-methoxy derivatives of catecholamines, patients receiving alpha-methyldopa were reported to excrete a urinary metabolite with the expected properties of alpha-methylnormetanephrine (41).[87] However, no detailed experimental data were reported. Lindmar et al[88] reported increased urinary levels of 10 in patients receiving alpha-methyldopa after physical exercise. But again, no experimental data were reported. They did, however, cite a study by Muscholl and Rahn[89] which identified 10 by means of ion-exchange chromatography, tlc and biological assays. No analytical details were reported. It would seem that the identification of 10 as a urinary metabolite is tenuous at best.

Alpha-methylepinephrine (46) was mentioned in a discussion section as a metabolite of 1 in Muscholl's review on adrenergic blockade.[34] The citation[90] was to a short report by Muscholl himself published in 1965 in which he identified the compound by tlc in urine. No experimental details were provided in the account. An interesting sidelight is the fact that in his review[34] Muscholl reported the compound to cause a fall in blood pressure "in various laboratory animals", unlike any of the other metabolites of 1 (see earlier pharmacology discussion).

As the pharmacology and metabolic literature make apparent, stereochemistry plays an important role in alpha-methyldopa metabolism. Several of the enzymatic conversions are potentially stereoselective and/or stereospecific. Additionally, the decarboxylation of 1 leads to the formation of a chiral product, alpha-methyldopamine, unlike the product of DOPA decarboxylation, dopamine. This asymmetric center presumably will be maintained in any further biotransformations of 1

which do not either replace the alpha-methyl or alpha-amino group with hydrogen, or convert the alpha-carbon to an sp^2 state of hybridization, such as in formation of the ketone 38.

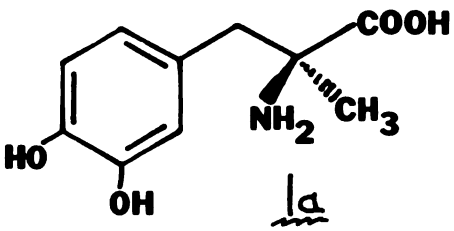




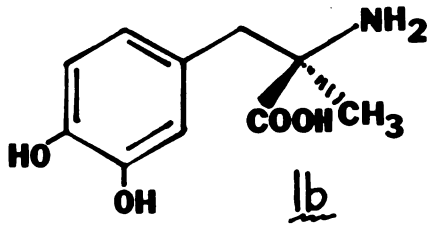
A consideration of primary importance therefore is the stereochemistry of the decarboxylation of alpha-methyldopa. As mentioned previously (p.40), DOPA decarboxylase has been reported to decarboxylate only the S (natural) isomer of DOPA (2a).[72] It is this study which is most often quoted when reporting that the enzyme only decarboxylates S amino acids. While no experimental details were included with regard to this specific point, the authors noted that, whenever both the R and S isomers of the amino acids were tested, only the S isomers were

decarboxylated. The original work done on the enzyme by Holtz[91] in the 1930's demonstrated that the incubation of S-DOPA (but not R-DOPA) resulted in pressor activity of the incubates when tested in animal preparations. The R isomer was inactive. Furthermore, it was not possible to isolate dopamine as its tribenzoyl derivative from R-DOPA incubations as was the case with the S isomer.

With regard to alpha-methyldopa, the metabolic studies just reviewed suggest that the drug is not decarboxylated when present as its R isomer 1b. None of the workers who studied 1a and 1b separately



1a



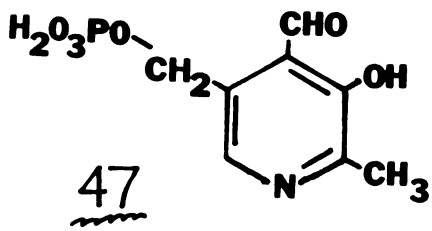
1b

found any amine metabolites after administration of the R isomer. The only indication of any side chain metabolism of 1b was the trace amount of ketone 38 identified by Au et al.[85] In a study directed only at the question of this decarboxylation in humans, Sjoerdsma et al[92] could find no trace of alpha-methyldopamine after administration of 1b.

Several of the pharmacology studies discussed earlier supplied further evidence against the decarboxylation of 1b. Porter et al[35] found no accumulation of serotonin (5) in the kidneys of mice after administration of 1b, nor any depletion of norepinephrine (7) in the brain after 1b. Hess et al[21] also found no amine depletion or decarboxylase inhibition after administration of 1b. In human studies,

Gillespie et al[12] found no fall in blood pressure, and much more tyramine excretion (but not as much as control) after administration of the R isomer than after administration of the S isomer.

The enzyme itself, DOPA decarboxylase, is a member of a large group of enzymes (including other decarboxylases and transaminases) which utilyze pyridoxal derivatives (B_6 vitamins) as cofactors.[93,94,95] The actual cofactor for DOPA decarboxylase has been shown to be pyridoxal-5-phosphate (47).[96,97,98] A brief review of studies with the



47

enzyme will be of interest with regard to the stereochemistry of not only the substrate (alpha-methyldopa), but also of the product (alpha-methyldopamine), which, as mentioned before, retains a center of symmetry.

The general mechanism by which decarboxylation takes place has been determined as shown in Figure 6, but the details are not clear at this time. Furthermore, none of the intermediates depicted in Figure 6 has been isolated. The existence of the bound imine form of the enzyme-cofactor complex A has been suggested by the isolation of a pyridoxyllysine fragment following sodium borohydride ($NaBH_4$) reduction and acid hydrolysis of an enzyme preparation.[95,99] UV absorption studies on the purified enzyme are also consistent with the spectra for imines (Schiff's bases) with an absorbance maximum at 415 nm.[100]

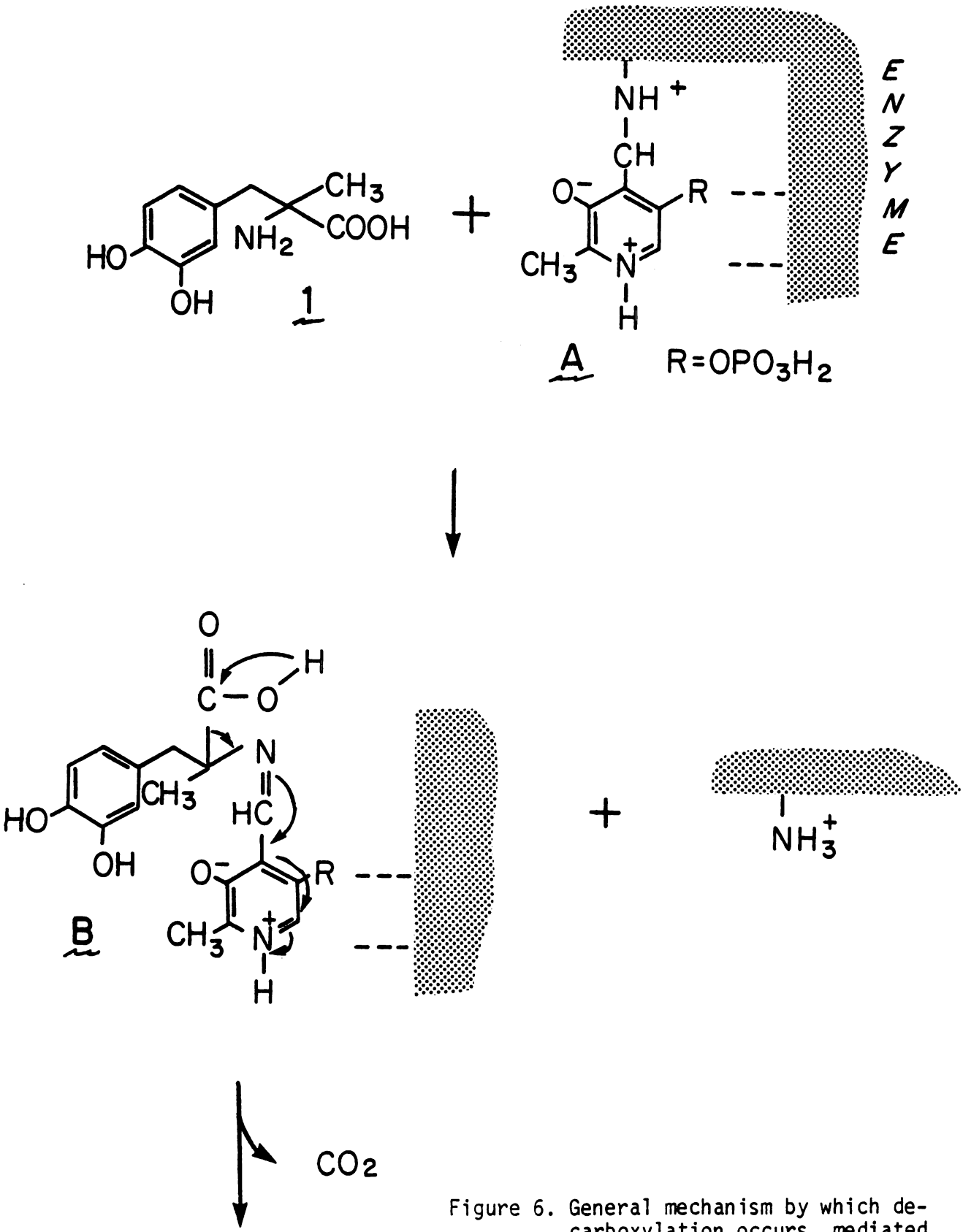


Figure 6. General mechanism by which decarboxylation occurs, mediated by DOPA decarboxylase (Ref. 93, 94, 95).

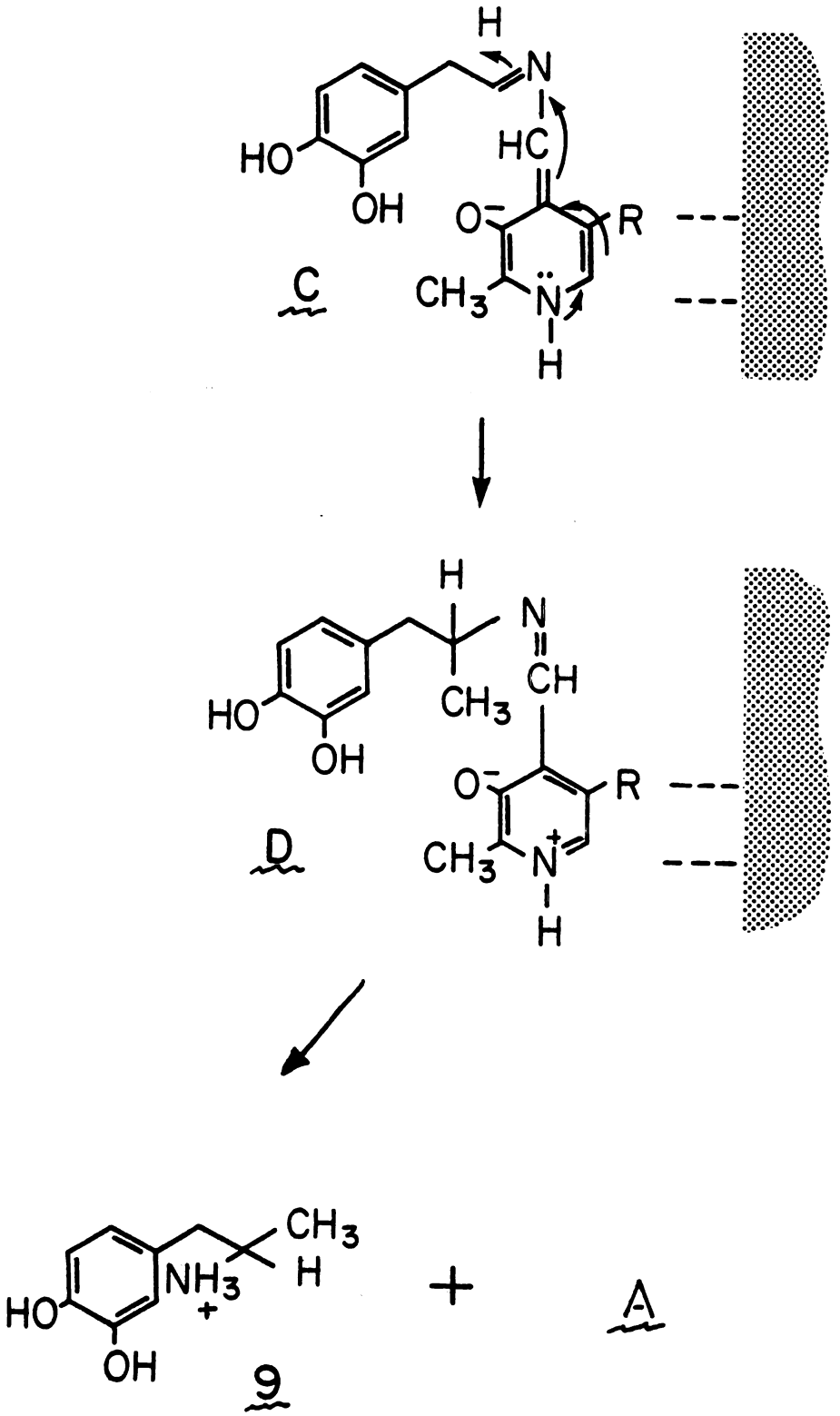


Figure 6, cont'd..

The proposed mechanism for decarboxylation involves several fundamental steps. The amino acid forms of imine with the enzyme-bound cofactor via a transaldimination, followed by tautomerization with concomitant decarboxylation to give C. A second tautomerization accompanied by the insertion of hydrogen yields D, which undergoes hydrolysis of the imine bond to release the decarboxylated amine.

The question of the stereochemistry of the substrate has been discussed by Snell;[93] but his comments were mostly of an empirical nature. He noted that the S isomers must be able to orient themselves correctly for binding in a manner not possible for the R isomers. We could find no reports of kinetic studies involving the enantiomers which might indicate if one enantiomer inhibited binding of the other. Snell also noted that the planarity introduced via the conjugated system (and the tautomerizations) would tend to introduce even more stereochemical control after binding takes place.

The other pyridoxal mediated reactions involving amino acids are similar in that they all involve labilization of an alpha-carbon substituent (COOH for decarboxylation, NH_2 for deamination). Thus, there are stereochemical factors involved not only in the substrate, but also in the particular reaction being catalyzed, and, therefore, in the production of the reaction product. The decarboxylation was shown to proceed by a direct hydrogen insertion by Belleau and Burba[101] who used D_2O in the *in vitro* decarboxylation of S- tyrosine. Further, Marshall and Castagnoli[102] have isolated from human urine the decarboxylation product of alpha-methyldopa and established its absolute configuration as (S)-(+)-i 9a.

The conclusions of the pharmacological, metabolic, and enzymatic studies seem to be that the enzyme is stereoselective in its selection of substrates, and stereospecific for production of amine reaction products. The enzyme work only addressed itself to the question of the particular enzyme DOPA decarboxylase. The pharmacological data, however, suggested that in the organism as a whole, only the S isomer was active in decarboxylase inhibition, amine depletion, and antihypertensive activity. The metabolic studies also indicated that only the S isomer was decarboxylated *in vivo*. However, as was stated in the introduction, no studies have been directed at the stereochemistry of the decarboxylation of alpha-methyldopa in the brain. Production of small amounts of amines from the R isomer might not be detected in urinary metabolites, nor in gross pharmacological effects such as decarboxylase inhibition or endogenous amine depletion. We were therefore interested in several reports suggesting the presence of other decarboxylase enzymes. In 1962 an enzyme preparation isolated from ox adrenal medulla was reported to be unable to decarboxylate S-tyrosine, S-tryptophan, and S-histidine.[103] In 1968 the decarboxylase activities for histidine and DOPA were shown to be separable in rat stomach by regional activity, activation by feeding or fasting, and the effects of gastrin or insulin.[104] Most interesting for our studies was a recent report[105] on the decarboxylation of DOPA and 5-hydroxytryptophan in rat brain. Using very sensitive microradiometric procedures for trapping $^{14}CO_2$, the activity towards the two substrates was separable by pH, cofactor, and most importantly, regional distribution in the brain. It should be noted that earlier studies on the purification of DOPA decarboxylase, including the work of Lovenberg *et al*[72] and two

more recent studies,[106,107] were unable to report the separation of activity towards any of the amino acid substrates, even when using sensitive immunological assays.[106] The possible existence of decarboxylase enzymes other than DOPA decarboxylase in the brain strengthened our interest in examining the decarboxylation of alpha-methyldopa as a function of stereochemistry in the brain.

Another important stereochemical consideration of alpha-methyldopa metabolism is the formation of alpha-methylnorepinephrine (10). The molecule contains a second center of asymmetry at the beta (or 3) carbon atom, and thus may exist as two diastereomeric pairs (p. 26). The erythro pair (10a,10b) is known as Nordefrin, Corbasil, and Corbefrin, the threo pair (10c,10d) as Ψ-Corbasil and threocorbefrin.

The configuration of metabolically formed 10 has, for the most part been assumed to be (-)-erythro (10a) or 1R:2S. In fact, it is frequently stated to be so without even a literature citation. A summary of the available information on this subject may be found in the "Catecholamine" volume of the Handbook of Experimental Pharmacology.[108]

When articles do cite a reference for the configuration of metabolically formed 10 as being 1R:2S, it is most often to an article by Lindmar and Muscholl[109] published in 1965. Careful reading will reveal that the authors do not state that the configuration is actually known, but that it may be surmised on the basis of two facts. First, as discussed earlier, the decarboxylation of alpha-methyldopa has been shown to produce alpha-methyldopamine of the S configuration (9a), and second, that the production of norepinephrine has been shown to yield the R isomer. These statements were not referenced in the article. The beta hydroxylation of 'S'-(+)-amphetamine has also been shown to yield the R enantiomer.[110]

In his review,[108] Muscholl noted that biological assays have been used to postulate the (-)-erythro isomer of alpha-methylnorepinephrine (10a) on the basis of comparative strengths of the various isomers as pressor agents. He summarized data suggesting that the threo pair was one-thousandth as active as the (-)-erythro isomer.

The best evidence for the erythro configuration of metabolically formed 10 was published by Carlsson et al,[111] and was based on the column separation of the erythro and threo pairs by the method of Hallhagen and Waldeck[52] discussed earlier (p.25). As was noted there, the method appeared to reliably separate the pairs. The Carlsson et al[111] paper, however, equated erythro with (-)-erythro. While the (+)-erythro isomer seems an unlikely metabolite, the actual configuration of the metabolite has not been unambiguously established.

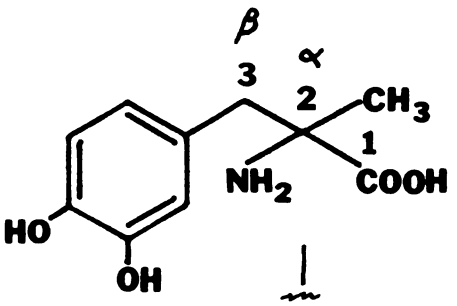
A recent study by Waldeck[112] has added another bit of information which suggests the (-)-erythro isomer (10a). It was shown that only S-(+)-alpha-methyldopamine (9a) formed alpha-methylnorepinephrine in mice. Analytical procedures for the identification of 10 included tlc, ion exchange chromatography and fluorimetric assay. No details were reported.

A review of the metabolism of alpha-methyldopa has indicated that while many of the metabolites of 1 have been identified, there are still some unanswered questions. Among these is the important question of the stereochemistry of the decarboxylation of alpha-methyldopa in the brain.

PART II

^{13}C-ENRICHED SYNTHESIS OF ALPHA-METHYLDOPA

To study the metabolism of alpha-methyldopa utilizing cims analytical procedures, the synthesis of labeled 1 for use as substrate and internal standard was undertaken. For reasons which will be explained below, we selected ^{13}C as the label, and the benzylic (beta or C-3) position as the site of the label.



1

Alpha-methyldopa, 3-(3,4-dihydroxyphenyl)-2-methylalanine), was first prepared by Stein et al[113] in 1955 as part of an investigation into the chemotherapeutic potential of amino acid analogs. The synthesis (Figure 7) proceeded from the phenylacetonitrile 48, with the phenylacetone 50 as the key intermediate. From 50, two routes were investigated, via either the hydantoin 51 or the aminonitrile 53. The authors stated that the hydantoin route proved superior to the alternate method, with no further explanation.

Catechols are sensitive to air oxidation, especially at basic pH,[114] and therefore the aryl oxygen functionalities must be protected during synthetic sequences such as shown in Figure 7. Stein et al[113] used the methoxy group for this purpose. Conditions for the removal of the protecting group were quite harsh (refluxing 47.5% hydrobromic acid for 55 hours).

Preparation of amino acids via aminonitriles (Strecker Synthesis) is very common.[115] Such methods have been employed in the synthesis of amino acids related to 1 such as 3-alkyltyrosine derivatives,[116] DOPA,[117] and tyrosine.[118] The latter two preparations[117,118]

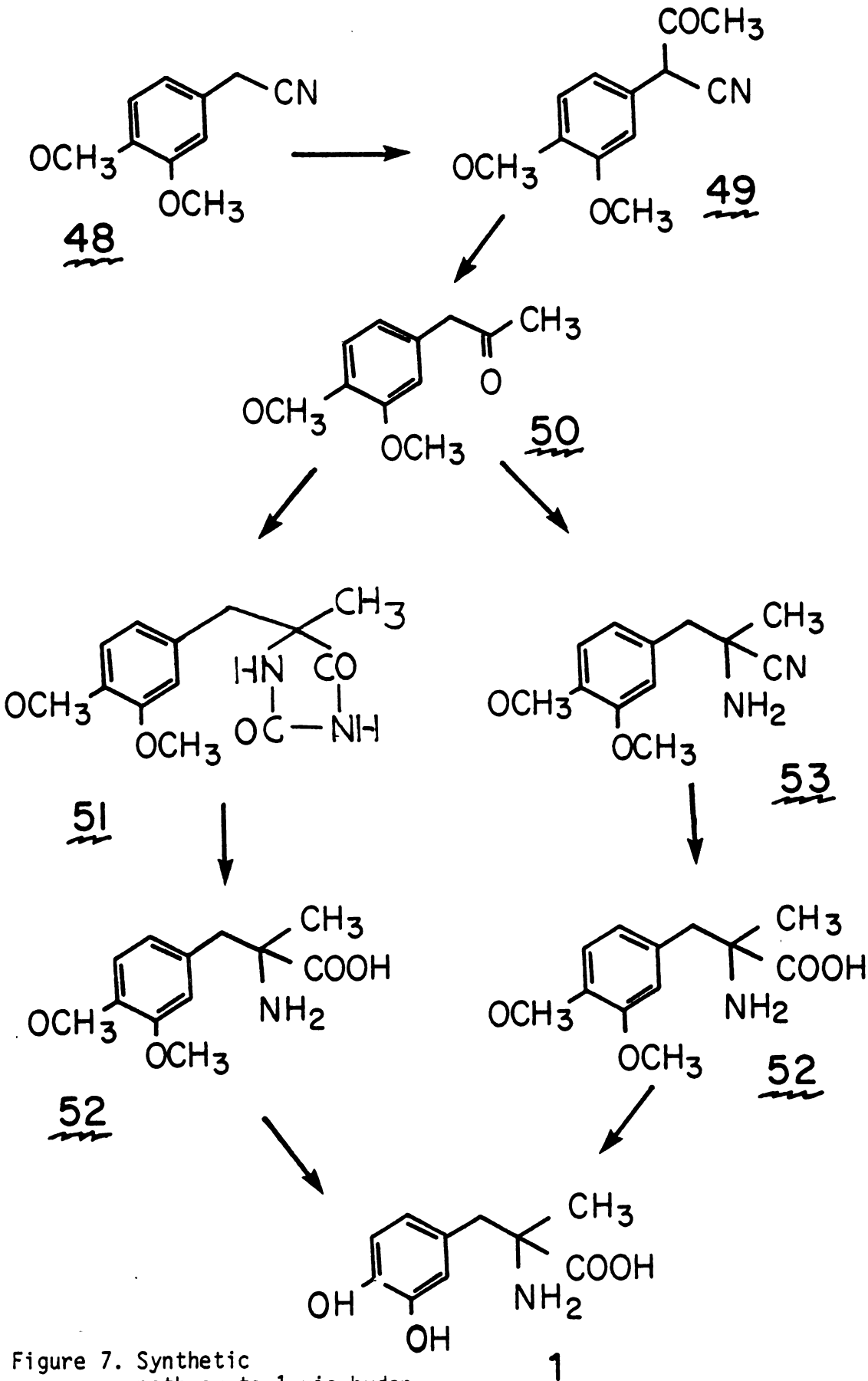
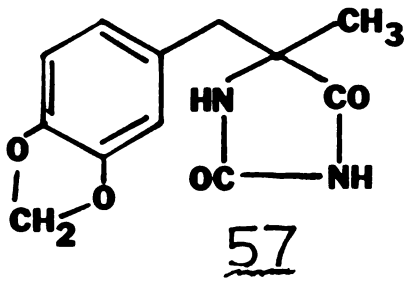


Figure 7. Synthetic pathway to 1 via hydantoin 51 or aminonitrile 53 (Ref. 113).

were also used to prepare the corresponding ^{14}C-labeled compounds. Several aminonitrile intermediates such as 53 have been investigated with regard to the synthesis of optically active 1.[119] This work will be reviewed in the discussion of the resolution of alpha-methyldopa.

The synthesis of S-alpha-methyldopa (1a) from 3,4-dimethoxyphenyllithium (55) by condensation with 56 was reported in a Dutch patent(Figure 8).[120] Experimental details were absent, and the preparation of 56 was not described. Difficulties with condensation of 55 and 56 might be anticipated due to the presence of the primary amine and carbonyl functions in the side chain.

In 1969, a Japanese patent reported the preparation of the methylenedioxyhydantoin intermediate 57 in the synthesis of 1.[121]



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Conversion of 57 to alpha-methyldopa required heating under reflux in 20% hydrochloric acid for 120 hours. No mention was made of the relative ease of removal of the protecting group versus hydrolysis of the hydantoin.

In contrast to the harsh conditions encountered for hydrolysis of methoxy protecting groups[113] and the conditions employed to obtain 1 from the hydantoin 57, Ong et al[122] reported the synthesis of 6-hydroxydopa via benzyloxy intermediates with mild reductive removal of the benzyl moiety. The benzyloxy precursor of the amino acid was reduced

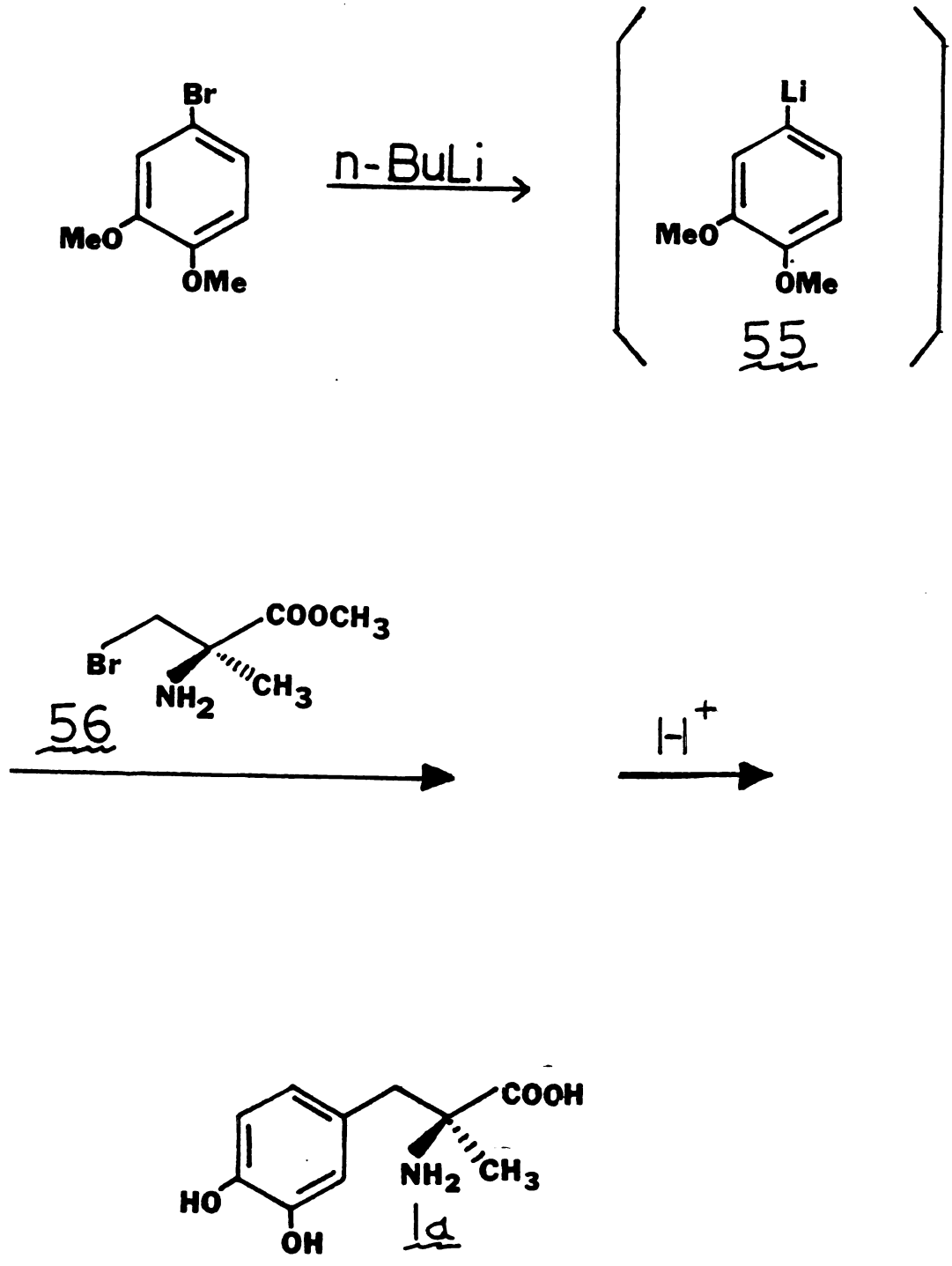


Figure 8. Synthesis of optically active 1a (Ref. 120).

with palladium on charcoal/hydrogen in weakly acidic ethanol. These conditions together with experience gained in our laboratory with the synthesis of alpha-methyldopamine[102] led us to select the benzyl protecting group for the aromatic oxygen functionalities.

The choice of a stable isotope and its placement in the molecule were prerequisites to the selection of the synthetic pathway for preparation of labeled 1. These decisions were governed by our anticipated requirements that the molecule serve as an in vitro and in vivo substrate, and also as an internal standard for quantitative studies.

We were concerned with avoiding any possibility of losing the label after incorporation into the molecule. The synthetic pathway leading to alpha-methyldopa was certain to include both strongly acidic and strongly basic conditions, regardless of the particular route selected. This dictated against a chemically labile label. The details of the analytical procedures employed in our metabolic work will be discussed in the analytical section. It should be noted however, that the derivatization procedures involved treatment with moderately strong aqueous acid or acidic ethanol. Additionally, the label had to be situated so that bond cleavage during metabolism would result in the label remaining with the major fragment.

Four stable isotopes are commonly employed in metabolic studies, ^{15}N, ^{18}O, ^{13}C, and ^{2}H.[123] Examples of ms applications of stable isotopes in metabolic studies will be discussed in the analytical section. We did not seriously consider ^{15}N primarily because of the likely formation of deaminated metabolites in the metabolism of 1. Incorporation of ^{18}O into the phenolic oxygen position would have provided an excellent label. However, synthetic methods for such incorporation could not be found,

and labeled ^{18}O precursors are quite expensive.

Deuterium has several desirable characteristics as a label for metabolic studies. More than one deuterium may often be easily introduced into the molecule. The resultant compound will then have a correspondingly higher molecular weight than the unlabeled molecule, a useful characteristic in ms analysis. It is often relatively inexpensive to synthesize deuterium labeled molecules, and there are a wide variety of simple labeled starting materials available.

Deuterium labeling suffers from several disadvantages in metabolic applications. Strongly acidic or basic conditions in the synthetic pathway precludes any procedure which would introduce deuterium under less vigorous conditions than those encountered in subsequent synthetic steps, since back-exhange would then be expected to occur in the molecule. We were particularly concerned about deuterium labeling of the aromatic ring protons (see below) and, as will be noted in the analytical and metabolism discussions, our concern was well founded. In addition to exchangeability, methods which introduce deuterium in a random manner could result in a non-uniform label with varying deuteirum composition from sample to sample. There might also be molecules within one sample containing different deuterium enrichments.

Deuterium labeling also gave rise to concern about the location of the label. Since many biotransformations involve the removal of a hydrogen atom (such as oxygen insertion reactions), isotope effects are sometimes observed in metabolic reactions.[124,125] As with any label, there was also concern with regard to maintaing the label on the major fragment after metabolic reactions.

While we selected ^{13}C as our label (see below), we did consider

several methods for the preparation of deuterium enriched alpha-methyldopa. Some of these procedures were followed for the preparation of deuterium labeled internal standards which were employed in our studies.

The phenyl-2-propanone 50 was an intermediate in the Stein et al[113] synthesis of 1 (Figure 7). Unsubstituted phenyl-2-propanone has been enriched with deuterium both at the benzylic and terminal carbon atoms.[126] Exchange of the two benzylic protons was accomplished with pyridine and D_2O at room temperature for 4 hours. Exchange of all five protons was accomplished with the same system after heating at reflux for 48 hours. Finally, the d_5 molecule was back-exchanged in aqueous pyridine at room temperature for 4 hours to yield the d_3 product. We anticipated a phenyl-2-propanone intermediate in our pathway to 1, and thus could have utilized such an exchange. It should be noted that the ease with which the benzylic protons exchanged (and back-exchanged) would probably not be a problem after conversion of the carbonyl carbon to an sp^3 state of hybridization (as in a hydantoin or aminonitrile).

Exchange of deuterium into the aromatic ring has been accomplished with DOPA[127] and tyrosine.[128] Furthermore, these procedures have been used in this laboratory to prepare deuterium labeled DOPA, alpha-methyldopa, and amine metabolites.[129] The nmr and cims of these standards indicated that the exchange of the aromatic protons was complete. These compounds have been used for internal standards for in vivo studies. As mentioned above, problems arising with back-exchange will be discussed in the analytical and metabolism sections.

We also considered the specific introduction of deuterium into the side chain. The synthetic pathway to 1 (Figure 7) described by Stein et al[113] included the conversion of the nitrile 48 to the ketone 50

via the intermediate 49. The use of labeled ethyl acetate in the conversion of the nitrile 48 to 49 could provide the d_3 ketone, labeled in the terminal methyl group. However, the previously described incorporation of deuterium via pyridine and D_2O[126] also gave the d_3 compound, and would appear to be a simpler procedure.

The final label considered and selected for our work was ^{13}C. Incorporation of ^{13}C into the carbon skeleton assures that there will be no back-exchange of the label even under the most vigorous reaction conditions. Isotope effects involving ^{13}C would be expected to be insignificant, even when the reaction site involved the labeled carbon atom. Finally, the problems associated with random incorporation of labels (such as deuterium) would be avoided. We therefore selected ^{13}C for incorporation into alpha-methyldopa for use in metabolism studies.

Selection of the position in the molecule for introduction of the label was based on synthetic opportunities available and expected metabolic cleavage pathways of aromatic amino acids such as DOPA and alpha-methyldopa, and similar pathways of 1-phenyl-2-aminopropanes. While metabolic carbon-carbon cleavage to benzoic acid derivatives has not been observed with 1, benzoic acid is a major product of amphetamine metabolism.[86] In order to optimize the identification of alpha-methyl dopa metabolites, including potential products of carbon-carbon bond cleavage, we selected the benzylic position of the molecule as the location of the ^{13}C label.

The specific route to the synthesis of beta or C-3 ^{13}C-enriched alpha-methyldopa(1-^{13}C) which we followed is shown in Figure 9.[130] The preparation of 50% and 89% enriched compounds was achieved following this route. The discussion which follows refers to the 50%

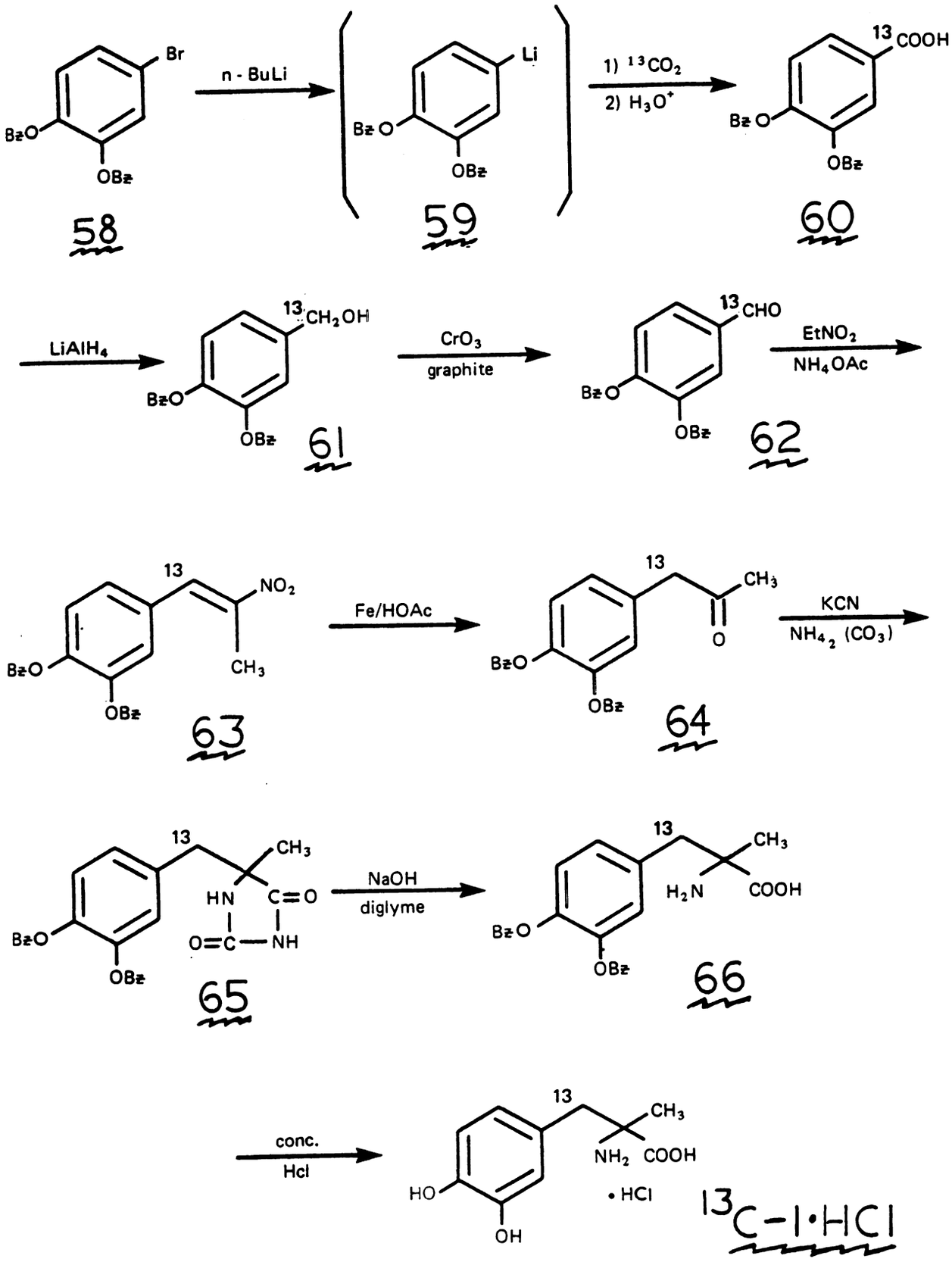


Figure 9. Synthetic pathway to ^{13}C-enriched alpha-methyldopa (Ref. 130).

enriched compound except where noted, although descriptions apply equally well to the 89% ^{13}C synthesis.

Introduction of the benzylic label was achieved by a carbonation procedure described by Neish.[131] While formylation is a common method of introducting benzylic labels, and would introduce the benzylic carbon at the desired oxidation state [132,133] for subsequent construction of the side chain, we selected the carbonation procedure based on the availability of labeled barium carbonate ($Ba^{13}CO_3$). The lithio intermediate 59 was prepared from 3,4-dibenzyloxybromobenzene (58) and n-butyllithium (n-BuLi). The lithio intermediate 59 was carbonated *in vacuo* after generation of $^{13}CO_2$ with sulfuric acid and 65 atom percent $Ba^{13}CO_3$. Dilution of the 65% ^{13}C-enriched compounds to a 50:50 ^{13}C:^{12}C mixture was accomplished at the hydantoin(65) stage, and will be discussed at that point in the pathway. The carbonation led to the formation of a crude yellow solid which was recrystallized from ethyl acetate to provide the pure acid 60 in 50% yield. This was the lowest yield step in the synthetic sequence.

Characterization of the ^{13}C-enriched acid 60 was achieved by melting point (mp), pmr, and cims. The cims spectrum (Figure 10) displayed the parent ions (MH_2^+ = 336,335) in the ratio 89:50. After subtracting the natural abundance of ^{13}C in the molecule (1.1%/carbon = 23%), we obtained a ratio of 78:50 or about 60% enrichment. This was 5% lower than the stated enrichment of the $Ba^{13}CO_3$. However, since we were planning to prepare the 50:50 mixture for cims metabolite analysis, this discrepancy did not concern us. The cims spectrum displayed doublets at m/e 318,317 corresponding to the loss of H_2O, and a singlet at m/e 291 corresponding to the loss of CO_2 and concommitant loss of the

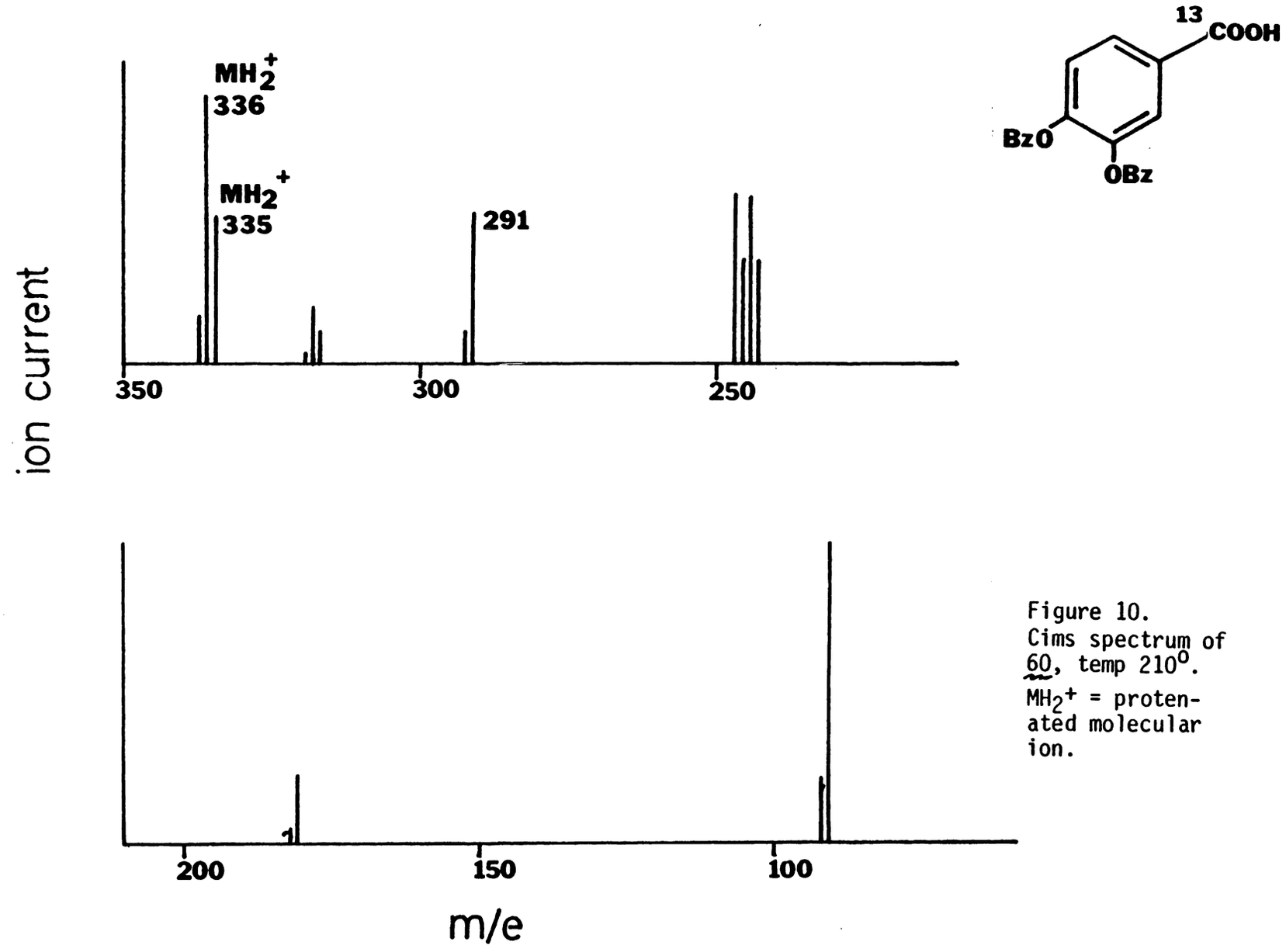
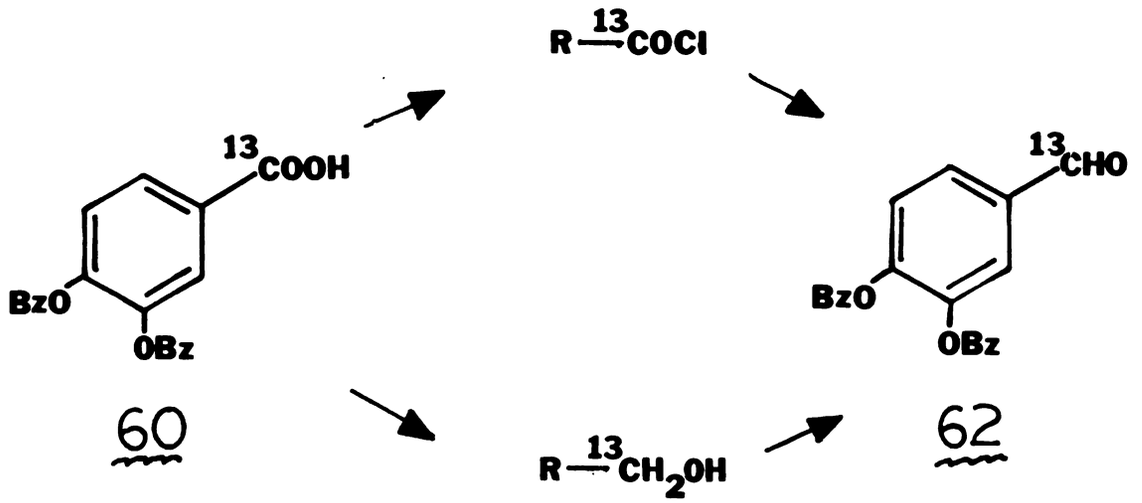


Figure 10.
Cims spectrum of 60, temp 210°.
MH_2^+ = protenated molecular ion.

^{13}C label. Doublets were also observed at MH_2^+-90 (m/e 228,227) and at MH_2^+-92 (m/e 226,225). These ions most likely arise from alternate fragmentations of the benzyl moiety and appear in most of the spectra of the compounds in this dibenzyloxy series. The nmr spectrum (DMSO-d_6) displayed only the aromatic proton signals at 7.82-7.08 ppm and the benzylic proton signals at 5.25 and 5.22 ppm.

Having selected carbonation rather than formylation as the method for introduction of the label, it was necessary to convert the carboxyl function to an aldehyde in order to introduce the remaining side chain atoms. Conversion of the benzoic acid 60 to the key intermediate aldehyde 62 could be accomplished by two general methods.



The acid chloride route, involving a Rosenmund reduction, has been utilized for preparation of unlabeled 3,4-dibenzyloxybenzaldehyde (^{12}C-62) in 70% yield by Neish.[131] The same method was utilized in our laboratory by Kent Marshall[102] in the preparation of alpha-methyldopamine. However, the procedure proved to be fairly difficult, and gave varying yields. With the hope of improving the overall yield, and developing a

more satisfactory route to the benzaldehyde, we investigated an alternative approach involving reduction of the benzoic acid 60 to the benzyl alcohol 61, followed by selective oxidation to the benzaldehyde 62.

Reduction of the model ^{12}C acid ^{12}C-60 was accomplished with lithium aluminum hydride ($LiAlH_4$) in dry tetrahydrofuran (THF). The unlabeled crude alcohol ^{12}C-61 was recrystallized from benzene/hexane, and characterized by mp, nmr, ir, uv, and elemental analysis. Further studies demonstrated that the crude product had essentially identical properties to the analytical sample, and also gave satisfactory cims analysis. The crude product was found to give excellent yields in the subsequent oxidation to the benzaldehyde.

We therefore reduced the labeled acid 60 with $LiAlH_4$ in dry THF and obtained a 90% yield of the crude alcohol 61. The product was characterized by mp, nmr, and cims analysis. The nmr spectrum ($CDCl_3$, Figure 11) displayed the benzylic proton signal as a singlet (Ar-$^{12}\underline{CH}_2$-OH) and as a doublet (Ar-$^{13}\underline{CH}_2$-OH, $\underline{J}_{^{13}C-H}$ = 144 Hz) centered at 4.53 ppm. The ratio of the integrated areas of the doublet to that of the singlet gave a ^{13}C:^{12}C ratio of 64:36.

The cims spectrum revealed an unusual fragmentation pattern. The parent ions were observed (MH_2^+ = 322, 321) in a ratio of 12:23; two fragment ions (m/e 320, 319) appeared in a ratio of 23:9. This portion of the cims spectrum is illustrated in Figure 12a. We attributed this pattern to species corresponding to the following ions: ^{13}C-MH_2^+, ^{12}C-MH_2^+, ^{13}C-MH^+, ^{12}C-MH^+, ^{13}C-(MH_2^+-H_2), and ^{12}C-(MH_2^+-H_2). The ion structures of these fragments remain unassigned. We then obtained the cims spectrum of the unlabeled compound and found ion intensities corresponding to MH_2^+, MH^+, and MH_2^+-H_2 (m/e 321, 320, and 319) as shown in Figure 12b. The ratios were consistent with those obtained in the

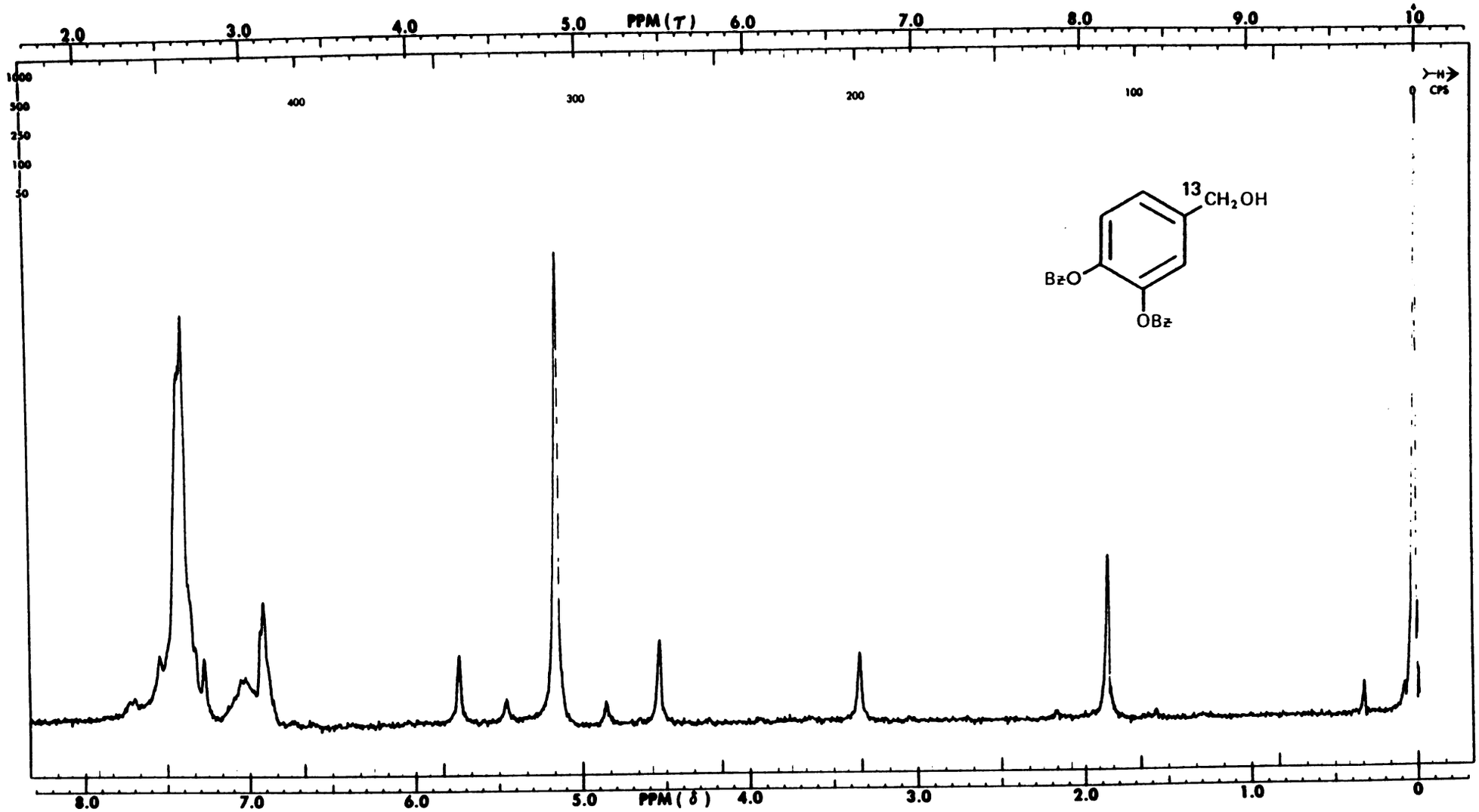


Figure 11. Nmr spectrum ($CDCl_3$) of the alcohol 61.

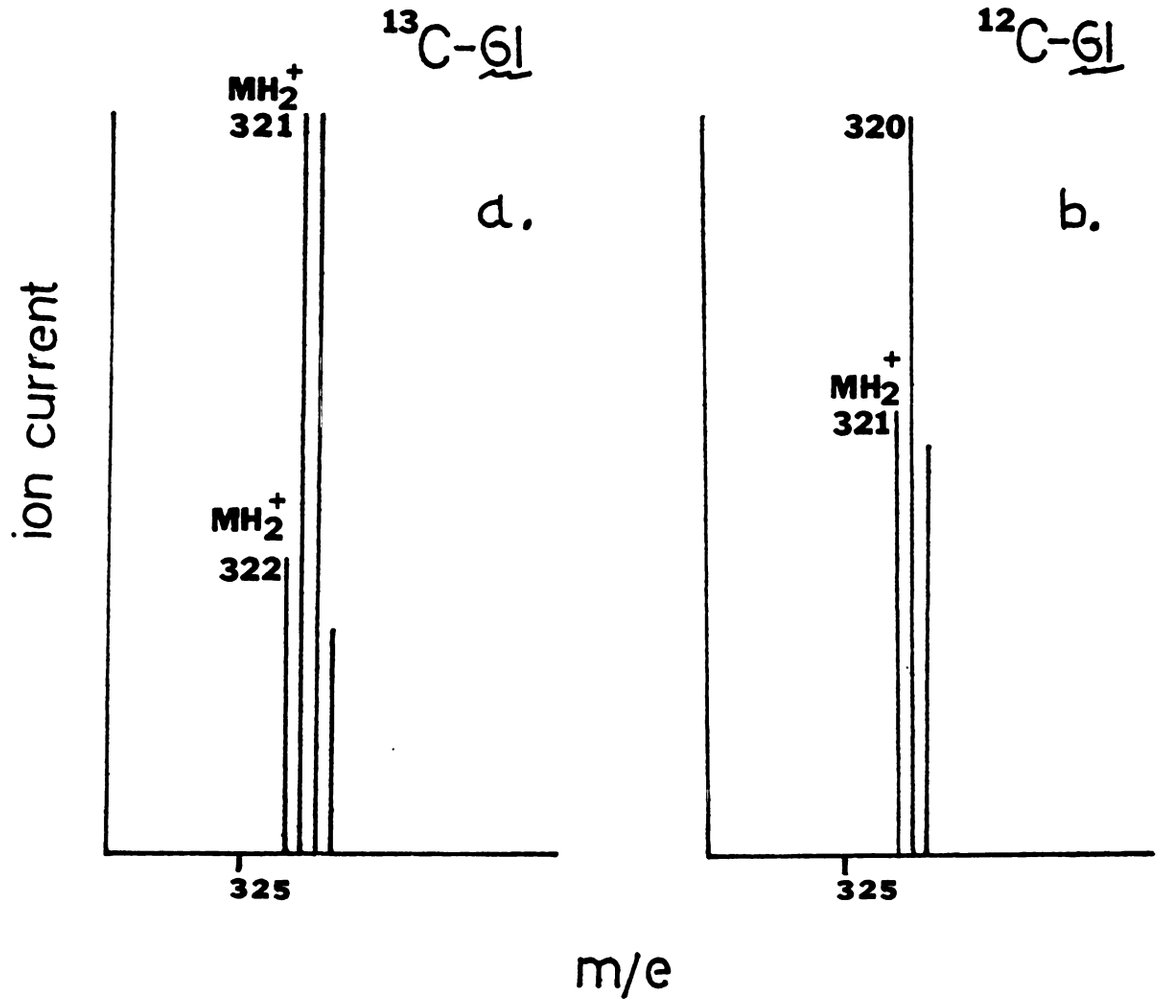


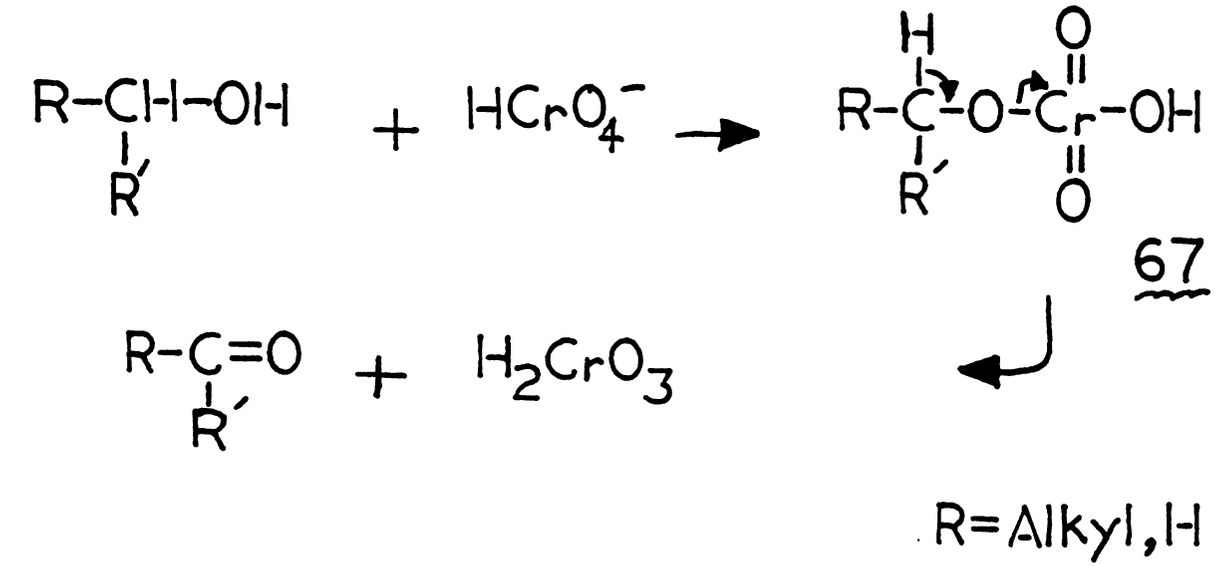
Figure 12. (a) Parent ion portion of the cims spectrum (temp 210°) of the ^{13}C-labeled alcohol 61. (b) Same portion for the unlabeled alcohol ^{12}C-61.

^{13}C-enriched compound.

Selective oxidation of the benzyl alcohol was first studied with the unenriched model alcohol ^{12}C-61. We first attempted the oxidation with Jones Reagent,[134] a mixture of chromium trioxide, sulfuric acid, and water. Reviews on chromium oxide oxidations[135,136,137] do not discuss the use of this reagent for selective oxidations to benzaldehydes except to suggest that over-oxidation to benzoic acids is common. Nevertheless, we had evidence that the reagent might be satisfactory for our particular molecule. Based on the fact that benzaldehydes are readily oxidized to benzoic acids by this system, Patrick Callery[138] attempted the oxidation of 2,5-dimethoxybenzaldehyde with Jones Reagent. The reaction failed, with starting material being recovered. In a subsequent experiment, 2,5-dimethoxybenzaldehyde was isolated after reaction of the precursor alcohol with Jones Reagent. Further reading of the review articles[135,136,137] suggested a mechanistic rationale for the observations of Callery, and indicated that similar results (i.e. oxidation of 3,4-dibenzyloxybenzylalcohol to the corresponding benzaldehyde) might be achieved.

According to the review articles, oxidations of primary or secondary alcohols proceeds via a chromate ester 67 as shown in Figure 13. In the oxidation step, concerted loss of H_2CrO_3 with proton abstraction leads to the aldehyde or ketone. The subsequent oxidation of aldehydes probably proceeds via the hydrated aldehyde which then may form a chromate ester 68.

The Hammett rho value for the oxidation of phenylmethylcarbinols has been reported to be approximately -1.0,[139,140] suggesting that electron donating groups such as benzyloxy should accelerate the



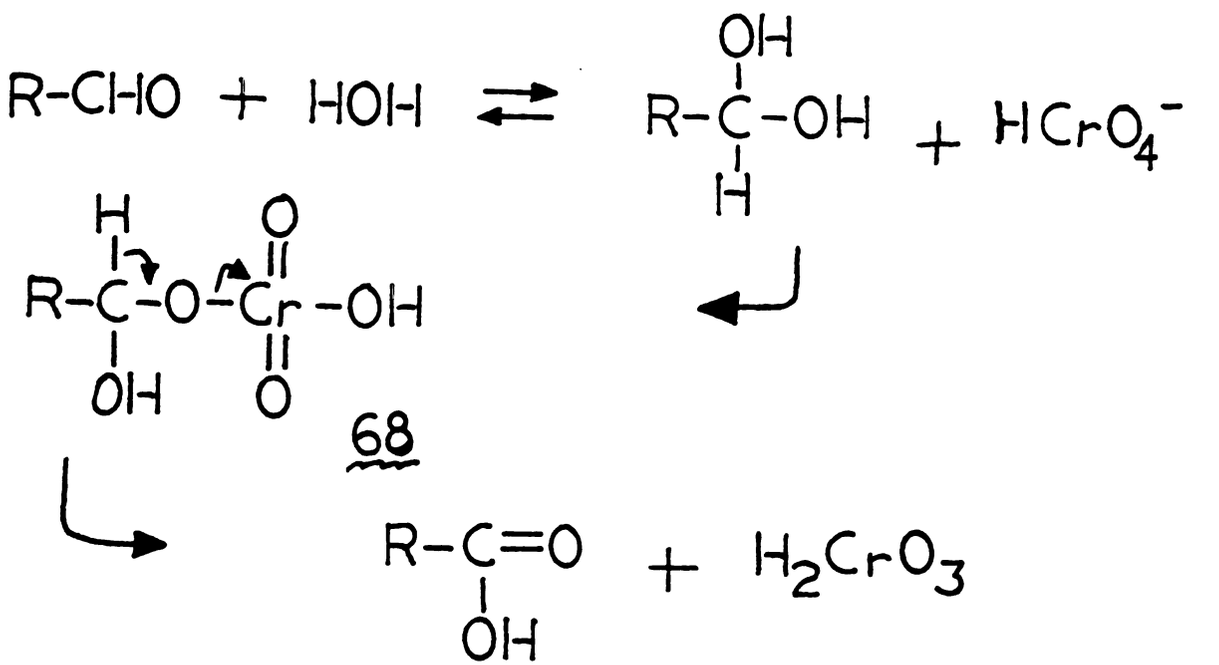


Figure 13. Oxidation of alcohols and aldehydes via chromate esters, by chromic acid (Ref. 135,136,137).

oxidation of the alcohol. Further, the Hammett rho value for the oxidation of benzaldehydes with chromic acid have been reported to be approximately +1.0,[141,142] suggesting that electron donating groups such as benzyloxy should hinder the reaction. This may be rationized by the consideration that the aldehyde must first undergo hydration before oxidation takes place. It therefore seemed logical that an electron rich benzyl alcohol such as Callery's 2,5-dimethoxybenzylalcohol would be selectively oxidized to the corresponding aldehyde, and that similar results might be obtained with our dibenzyloxy system. It should be noted that a very recent literature report has discussed the oxidation of several benzyl alcohols (including unsubstituted benzyl alcohol) with Jones Reagent.[143] The authors noted that most reviews do not suggest benzylic oxidation with Jones Reagent. However, they found that with reasonable care, such oxidations are possible.

We attempted the oxidation of the model alcohol ^{12}C-61 with Jones Reagent in acetone, and obtained a brown crude product. Attempts to purify the solid by recrystallization were unsuccessful. However, sublimation provided the aldehyde as a white crystalline solid in 73% yield. The nmr spectrum displayed a characteristic aldehyde proton signal at 9.83 ppm. In order to establish optimal reaction conditions before undertaking the oxidation of the ^{13}C-labeled compound, the reaction was repeated several times. Unfortunately, it was found that the crude product frequently decomposed during the sublimation. We felt the problem was due to the presence of trace amounts of chromium salts which were catalyzing further oxidation and decomposition. All attempts to rectify this problem (such as more moderate reaction temperatures and more efficient filtering of the

chromium salts) were unsuccessful. Because we could not feel secure about isolating the purified aldehyde, we examined another method of oxidation.

Chromic anhydride imbedded in graphite has been reported to selectively oxidize alcohols to aldehydes and ketones.[144] Benzyl alcohol, when heated to reflux with this reagent in toluene for 24 hours, was reported to give a 98% yield of benzaldehyde. The authors stated that the reagent was attractive in that the aldehyde was desorbed from the graphite lattice while the chromium salts remained imbedded, thus preventing secondary reactions. Further, since the reaction is run under anhydrous conditions, oxidation of the aldehyde would be avoided. Filtration of the reaction mixture is reported to give the pure product directly upon removal of solvent.

Studies with our unenriched benzyl alcohol demonstrated that consistently high yields (75-85%) could be obtained with this reagent. We therefore treated the ^{13}C-labeled alcohol 61 to the same conditions, and after sublimation, obtained the benzaldehyde 62 in 82% yield. The nmr spectrum ($CDCl_3$) displayed the aldehyde proton signal as a singlet (Ar-$^{12}C\underline{H}O$) and as a doublet (Ar-$^{13}C\underline{H}O$, $J_{^{13}C-H}$=174 Hz) at 9.83 ppm. The cims spectrum displayed the parent ions (MH_2^+=320,319) and two doublets corresponding to the alternate fragmentations for the loss of the benzyl moiety (m/e 230,229 and 228,227).

Conversion of the aldehyde 62 to the nitrostyrene 63 was first studied with unlabeled compound. As with the benzyl alcohol, these studies demonstrated that the crude product was of sufficient purity (mp, cims, nmr) to be used directly in the subsequent step. Thus, ^{13}C-labeled aldehyde 62 was allowed to react with ammonium acetate

and nitroethane to provide the crude nitrostyrene 63 in 85% yield. The nmr spectrum ($CDCl_3$, Figure 14) displayed the methine proton signal as a singlet (Ar-^{12}C<u>H</u>=C) and as a doublet (Ar-^{13}C<u>H</u>=C, $J_{^{13}C-H}$=159 Hz) centered at 7.99 ppm. Long range ^{13}C-proton coupling was also observed for the terminal methyl proton signal (<u>J</u>=3 Hz) and for the <u>ortho</u> proton signal in the aromatic ring (<u>J</u>=3 Hz). Such ^{13}C coupling through three bonds (^{13}C-C-C-H) is well known, and examples have been summarized in several nmr volumes.[145,146] The further splitting of the methyl proton signal is due to long range coupling of the methine proton (which is noticeably broad) through the double bond to the terminal methyl group, and was observed in the unlabeled molecule (<u>J</u>=1 Hz). This allylic coupling through one double bond and three single bonds is also well documented.[147,148]

Reductive hydrolysis of the unlabeled model nitrostyrene ^{12}<u>C-63</u> utilizing iron and hydrochloric acid to yield the ketone ^{12}<u>C-64</u> has been reported in the literature.[149] The nitrostyrene was heated under reflux with the iron/hydrochloric acid mixture in toluene. The crude product reported could not be satisfactorily purified, and was not characterized. In this laboratory, the reaction was attempted by Kent Marshall.[150] As with the literature preparation, all attempts at purification were unsuccessful. As will be discussed later, the benzyl protecting group can be cleaved in concentrated aqueous hydrochloric acid at room temperature. Therefore, it is possible that the strongly acidic and vigorous reaction conditions utilized in this procedure partially removed the benzyl groups, leading to the formation of a complex mixture.

We therefore investigated the use of milder reducing conditions employing glacial acetic acid and iron.[151] The resulting crude oil

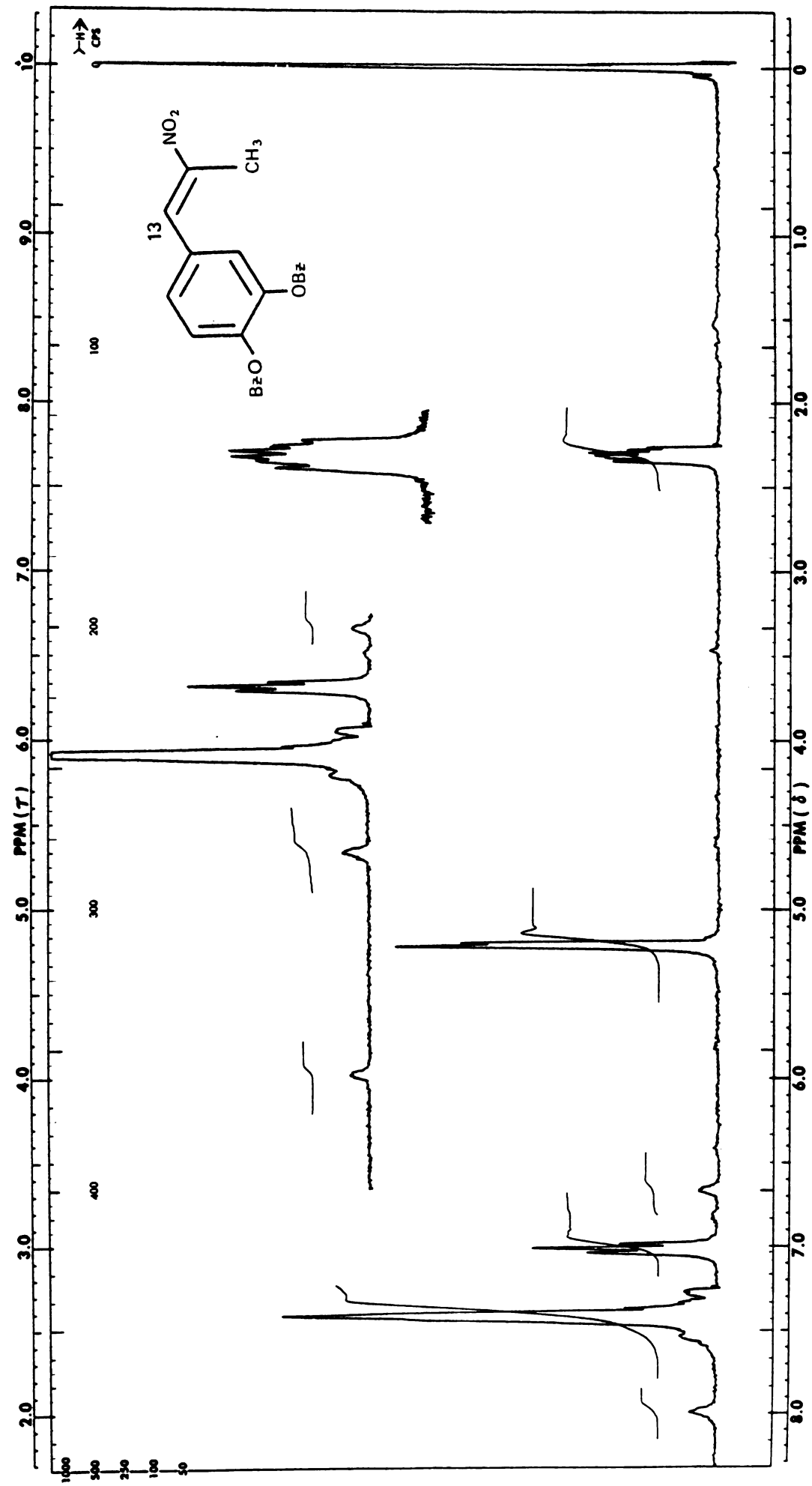
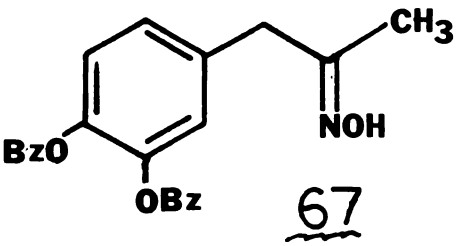


Figure 14. Nmr spectrum ($CDCl_3$) of the nitrostyrene 63.

was distilled to give a pale yellow oil, which was characterized by nmr, cims, and ir. We also treated the ketone with hydroxylamine to form the oxime 67, an oil which did not solidify. While further



67

purification of the oil was not attempted, the ir spectrum was obtained and compared to that of the ketone. It was found that the ketone spectrum contained the characteristic carbonyl (C=O) stretching frequency at 1713 cm^{-1}, while the oxime spectrum displayed no carbonyl band, but did contain a band corresponding to the imine (C=N) stretching frequency at 1680 cm^{-1}.

With conditions well established, the reductive hydrolysis was employed for the preparation of the ^{13}C ketone 64, which was obtained as a pale yellow oil in 86% yield after distillation. The product was characterized by nmr, cims, and elemental analysis. The nmr spectrum ($CDCl_3$, Figure 15) displayed the characteristic methyl ketone signal at 2.03 ppm. The benzylic proton signal was observed as a singlet (Ar-$^{12}CH_2$-CO) and as a doublet (Ar-$^{13}CH_2$-CO, $J_{^{13}C-H}$=128 Hz) centered at 3.53 ppm. The cims spectrum displayed the parent ion doublet(MH_2^+ =348,347) and the doublets associated with the two modes of debenzylation (MH_2^+-90, m/e 258,257 and MH_2^+-92, m/e 256,255).

Based on the work of Stein et al[113] and a general review of hydantoin chemistry[152] which indicated ready hydrolysis of hydantoins to amino acids, we selected to proceed to alpha-methyldopa via the

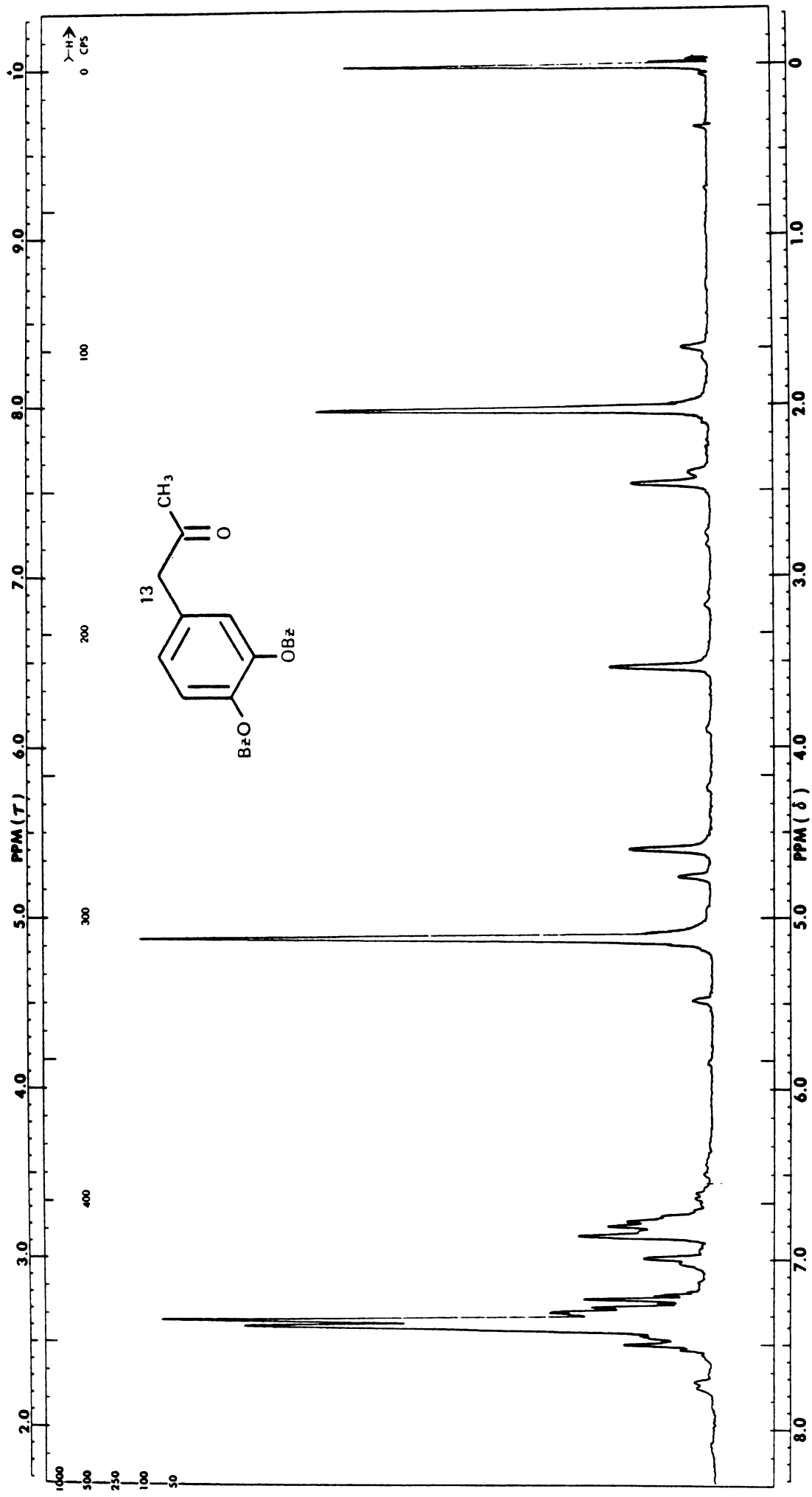


Figure 15. Nmr spectrum ($CDCl_3$) of the ketone 64.

hydantoin 65 intermediate rather than by an aminonitrile. The ^{13}C-labeled hydantoin 65 was readily prepared by reaction of the ketone 64 with potassium cyanide and ammonium carbonate in refluxing ethanol and water. The crude ^{13}C hydantoin was selected for dilution with unenriched hydantoin in order to prepare a 50% mixture of ^{13}C:^{12}C alpha-methyldopa for use in metabolic studies involving cims analysis. We selected this particular stage for dilution since the hydantoin yields a very clean white product which is readily recrystallized from absolute alcohol, and is therefore well suited for dilution. The estimated yield of the purified hydantoin was 82%.

The nmr spectrum of 65 ($CDCl_3$) as the 50:50 enrichment displayed the benzylic proton signal as an AB quartet (Ar-$C\underline{H}_2$, $\underline{J}_{AB}$=14.5 Hz) and as a doublet of that quartet (Ar-$^{13}C\underline{H}_2$, $\underline{J}_{^{13}C-H}$=128 Hz) with calculated chemical shifts of 2.72 and 2.91 ppm. The cims spectrum displayed the parent ions as a doublet with equally intense components (MH_2^+=418,417), and doublets for debenzylation at MH_2^+-90 (328,327) and MH_2^+-92 (326,325).

The conversion of 65 to the 3,4-dibenzyloxyamino acid 66 required hydrolysis of the hydantoin. The hydantoins can be hydrolyzed under basic or acidic conditions[152] with basic hydrolysis being more common. The literature[149] described a basic hydrolysis of unlabeled ^{12}C-65 with sodium hydroxide in diglyme and water. After 12 hours reflux, isolation of the amino acid by precipitation at ph 5.5 was reported to give 50-60% yields. We investigated the reaction and found that a 24 hour reaction period gave yields 5-10% higher than reported. This was verified by tlc analysis (chloroform/ether). These conditions were then applied to the hydrolysis of the ^{13}C-labeled hydantoin 65. Since the yields had been increased by the longer reaction time, we decided to allow the hydrolysis to proceed for 48 hours. Unfortunately, slow etching of the

glass flask by base led to fracture of the flask. A large portion of the ^{13}C material was lost. Repitition of the reaction for 36 hours only led to a 68% yield of 66. When the hydrolysis was repeated with the 89% ^{13}C-labeled compound, a teflon flask was employed. After 48 hours of hydrolysis, precipitation of the amino acid at pH 5.5 gave a 90% yield of crude 66. The product was characterized by mp and cims analysis. A portion of the cims spectrum of the 89% labeled material is shown in Figure 16. The parent ions (MH_2^+=393,392), and ions resulting from decarboxylation (m/e 347,346) are seen along with the debenzylation (m/e 303,302), all with the expected isotopic enrichment of 89%.

We also investigated other basic hydrolysis procedures. Most basic hydrolyses of hydantoins are based on the solubility of the reactant and the product amino acid in aqueous base.[149] Many of these proceudres employ barium hydroxide as the base. Addition of sulfuric acid after the reaction has been completed precipitates barium sulfate salts and removal of solvent after filtration will yield the amino acid directly. We found that various combinations of cosolvents (ethanol, glyme, ethylene glycol) did not provide the required solubility for the hydantoin, and especially for the amino acid product. We felt this was due to the presence of the large benzyloxy groups on the aromatic ring. After adoption of the tlc system (chloroform/ether) to monitor the reaction, we found that some hydrolysis procedures did give products. However, low yield and technical difficulties were not successfully overcome with these methods. With the availability of the teflon reaction vessel, these problems were not encountered.

We had originally planned to effect debenzylation of the amino acid by catalytic hydrogenation, as was done in the preparation of 6-hydroxydopa[122] and alpha-methyldopamine.[102] Kent Marshall found that

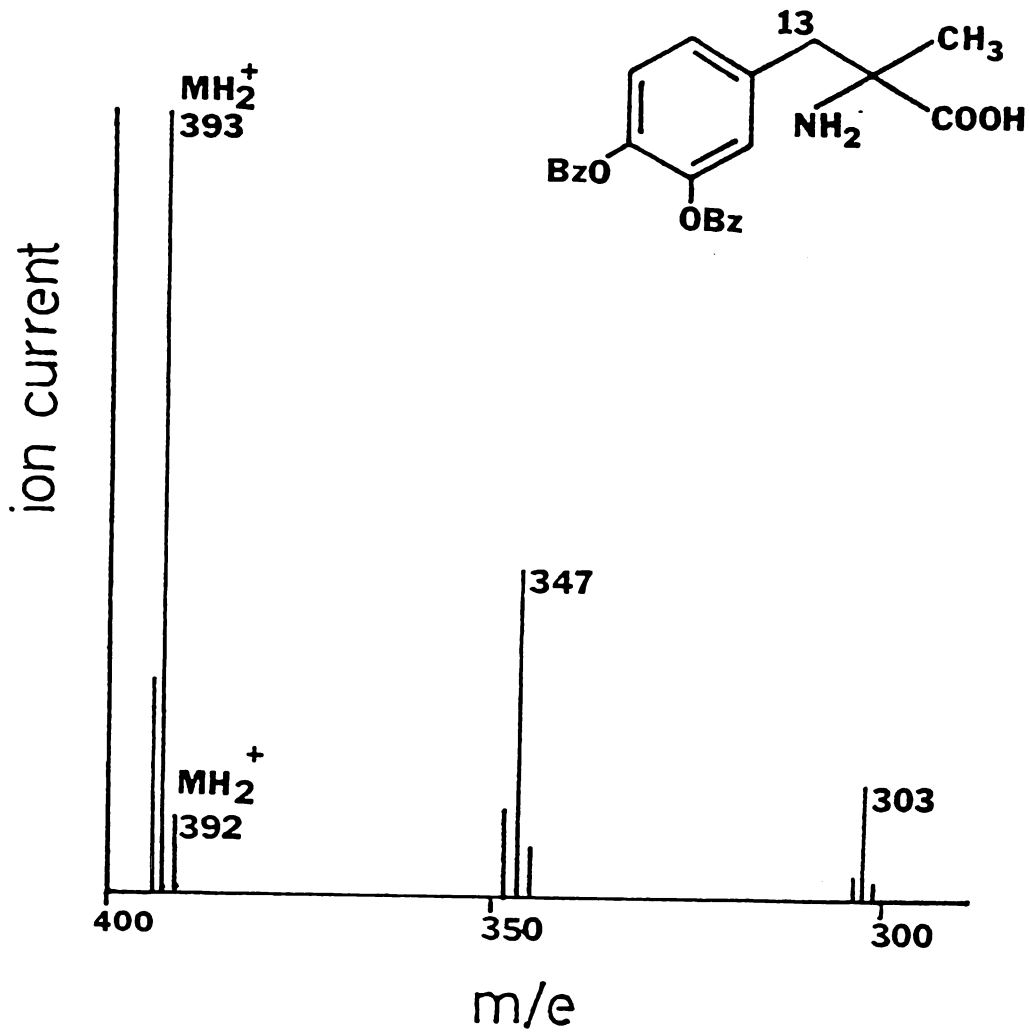


Figure 16. Portion of the cims spectrum (temp 285^0) of the benzyl amino acid 66, ^{13}C = 89%.

while the method was successful, the dual problems of solubility of the compound and ease of oxidation after debenzylation caused some loss of the product.[150] We then found a literature reference to the debenzylation of benzyloxy aromatic systems with concentrated hydrochloric acid at room temperature.[153] The ease with which the reaction proceeded, as compared to the acidic removal of the methoxy protecting group[113] led us to investigate the reaction. We found that the reaction went smoothly in a mixture of benzene and hydrochloric acid, giving quantitative yields with the unlabeled ^{12}C-66. A white solid was obtained after removal of solvents, unlike the grey material obtained after catalytic reduction.[150] The ^{13}C-labeled dibenzyloxyamino acid 66 was debenzylated in this manner to give a quantitative yield of ^{13}C-labeled alpha-methyldopa (^{13}C-1). The compound was characterized by mp, cims, and nmr. The nmr spectrum (D_2O, Figure 17) was identical to the spectrum obtained from the commercially prepared drug, with the exception of the ^{13}C coupling. The spectrum displayed the signal of the two benzylic protons as an AB quartet (Ar-C$\underline{H}_2$, $\underline{J}_{AB}$=15 Hz) and as a doublet of that quartet (Ar-^{13}C$\underline{H}_2$, $\underline{J}$=133 Hz) with calculated chemical shifts of 3.32 and 3.12 ppm. The cims spectrum (Figure 18) displayed the parent ions in the expected ratio (MH+ 213,212), decarboxylation (m/e 167,166), and alpha-beta cleavage to yield the benzylic fragment (m/e 124,123).

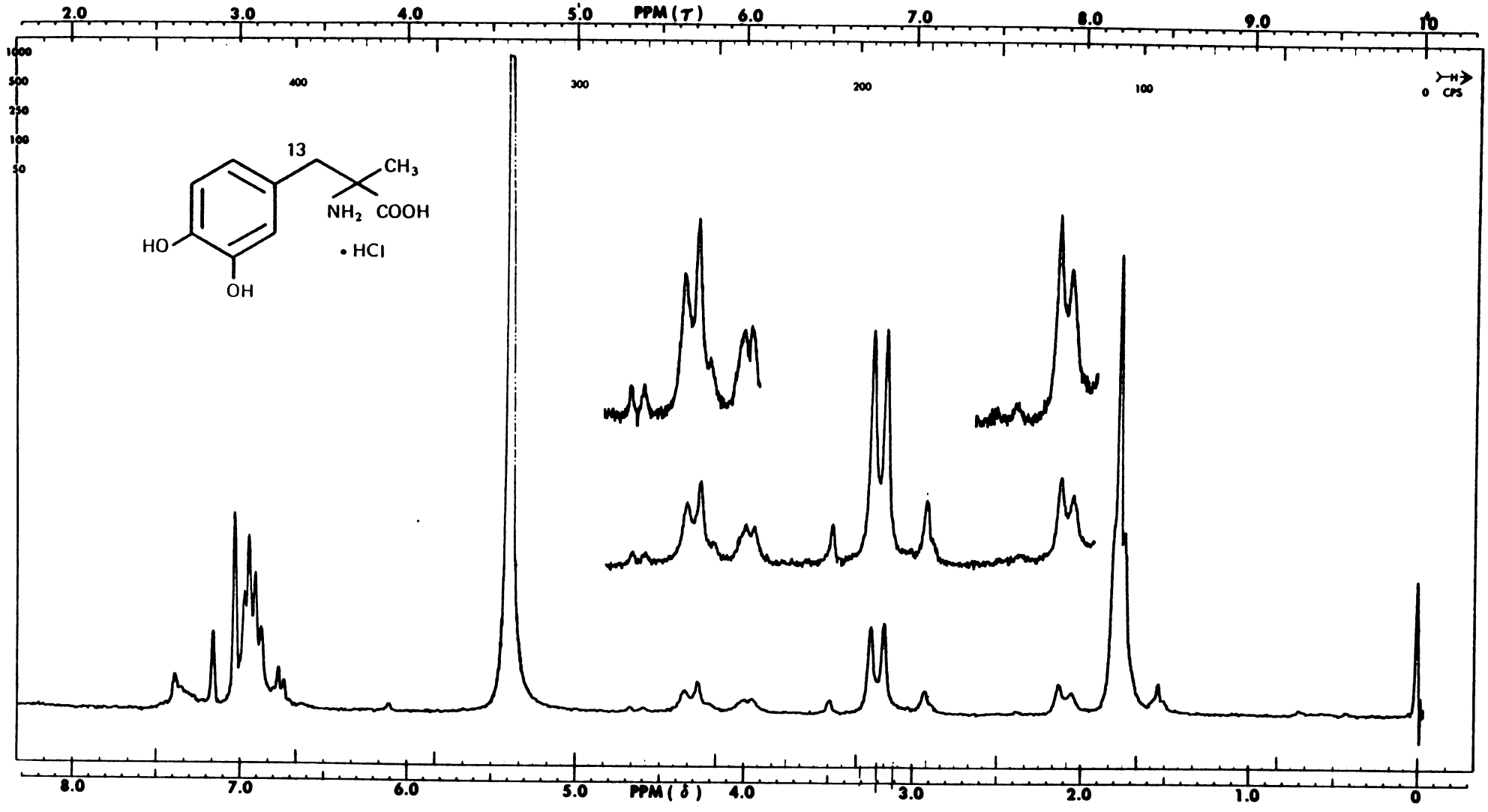


Figure 17. Nmr spectrum (D_2O) of ^{13}C-1·HCl.

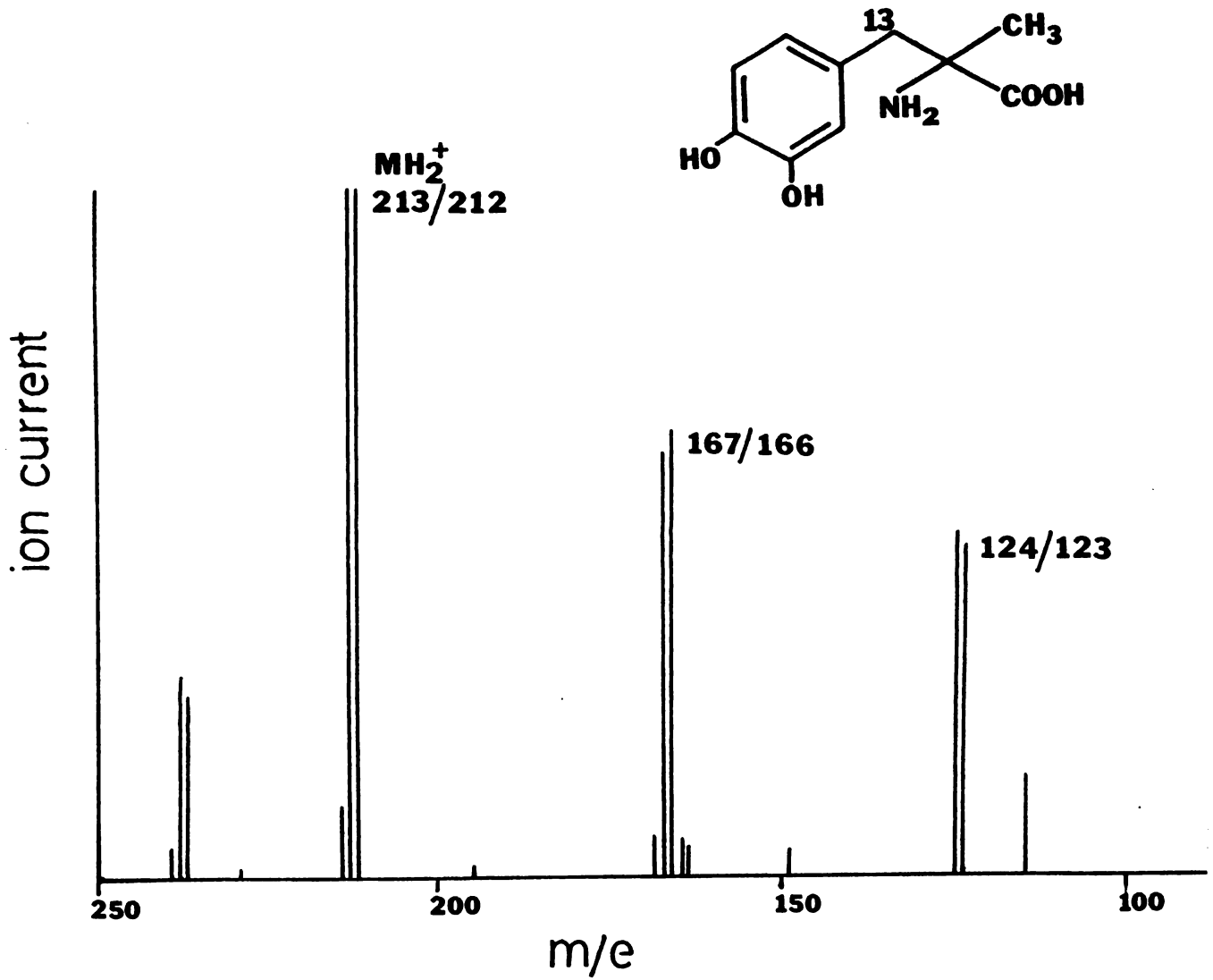
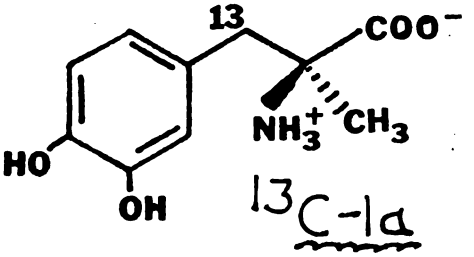


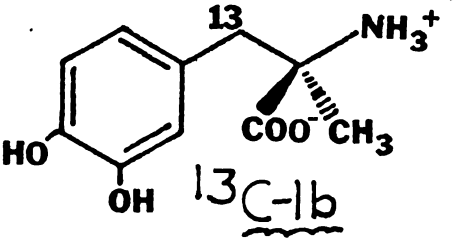
Figure 18. Cims spectrum of ^{13}C-1, temp 290^{o}.

RESOLUTION OF ALPHA-METHYLDOPA

In order to investigate the stereochemical parameters associated with the metabolism of alpha-methyldopa, it was necessary to resolve the ^{13}C-labeled drug into its S-(-) and R-(+) enantiomers ^{13}C-1a and ^{13}C-1b, respectively. The absolute configuration of 1a has been deter-



^{13}C-1a

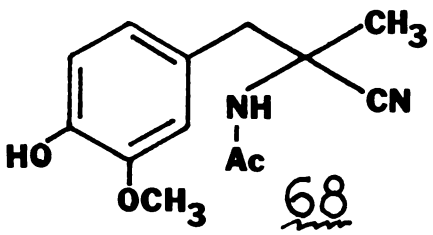


^{13}C-1b

mined by chemical correlation with S-(+)-isovaline.[154] The assignment was also determined by comparison of rotatory dispersion curves with those of S-tyrosine, S-phenylalanine, and R-DOPA.[155]

The enantiomers of alpha-methyldopa have been prepared by several methods. A rather specialized procedure for this resolution was by direct crystallization of either isomer through seeding of a saturated solution of the racemic compound with the appropriate isomer.[156] This patented method has been utilized for the resolution of very large quantities of alpha-methyldopa. The commercially important S-(-) isomer 1a has been prepared by condensation of the chiral intermediate 56 with the lithio intermediate 55, as mentioned in the synthetic discussion (p.65).[120]

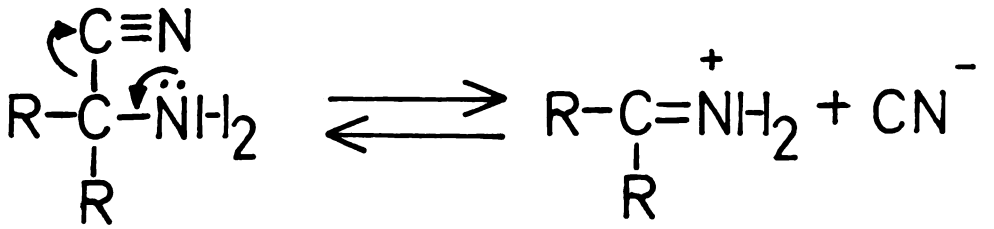
The resolution and racemization (in order to recycle the unwanted R isomer) of 1 was briefly mentioned in the synthetic section (p.65).[119] The key intermediate, which can be both resolved and racemized, is the 3-methoxy derivative of alpha-acetamido-alpha-vanillylpropionitrile 68. The intermediate was prepared either by selective



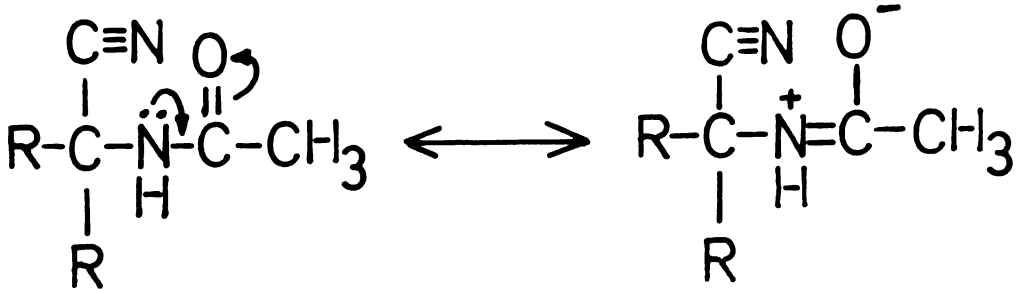
68

N-acetylation or by selective base hydrolysis of the O,N-diacetyl precursor. The authors found that racemic 68 could be resolved by seeding with either enantiomer, and thus the desired S isomer could be obtained from a racemic solution.

Racemization of the unwanted R isoemr was then studied. Alpha-aminonitriles will normally undergo facile racemization, due to the free electron pair on the nitrogen atom which facilitates the loss of the cyano group:



However, the electron pair of 68 is delocalized by the carbonyl group of the N-acetyl substituent:



Thus, the R isomer of 68 was optically stable under conditions compatible with the chemical stability of the molecule. Racemization was achieved, however, by treating the R isomer with sodium cyanide in anhydrous dimethylsulfoxide (DMSO). The racemized intermediate could then be recycled through the resolution procedure.

None of these procedures were suitable for our needs. The direct crystallization was not practical for the small amounts of material we planned to resolve. The synthetic procedures[119,121] were unapplicable as our synthetic scheme led to the amino acid itself. We were therefore limited to resolution procedures based on the amino acid or a compound readily prepared from it. We turned to the resolution of the triacetyl derivative of alpha-methyldopa 69 as described in the literature.[85,155] Au et al[85] reported the synthesis and resolution of small amounts of ^{14}C-labeled 1 for use in metabolism studies and therefore we were confident in following procedures discussed in their report.

The resolution sequence is shown in Figure 19. Alpha-methyldopa as its free amino acid (not as the hydrochloride shown in Figure 19) 1 was treated with acetic anhydride in pyridine to presumably yield the bis-acetoxyazlactone intermediate (not shown). This compound was not isolated or characterized in either literature report. Selective hydrolysis of the intermediate in dilute acid gave the triacetyl compound ^{12}C-69 either by direct crystallization[155] or by extraction with ethyl acetate.[85] The triacetyl compound was characterized by mp,[85,155] ir,[155] uv,[155] and elemental analysis,[155] but not by nmr.

Resolution of ^{12}C-69 was achieved with quinine in acetone. The insoluble quinine salt of the triacetyl compound proved to be the salt ^{12}C-70a of the S enantiomer of the triacetyl compound (^{12}C-69a).

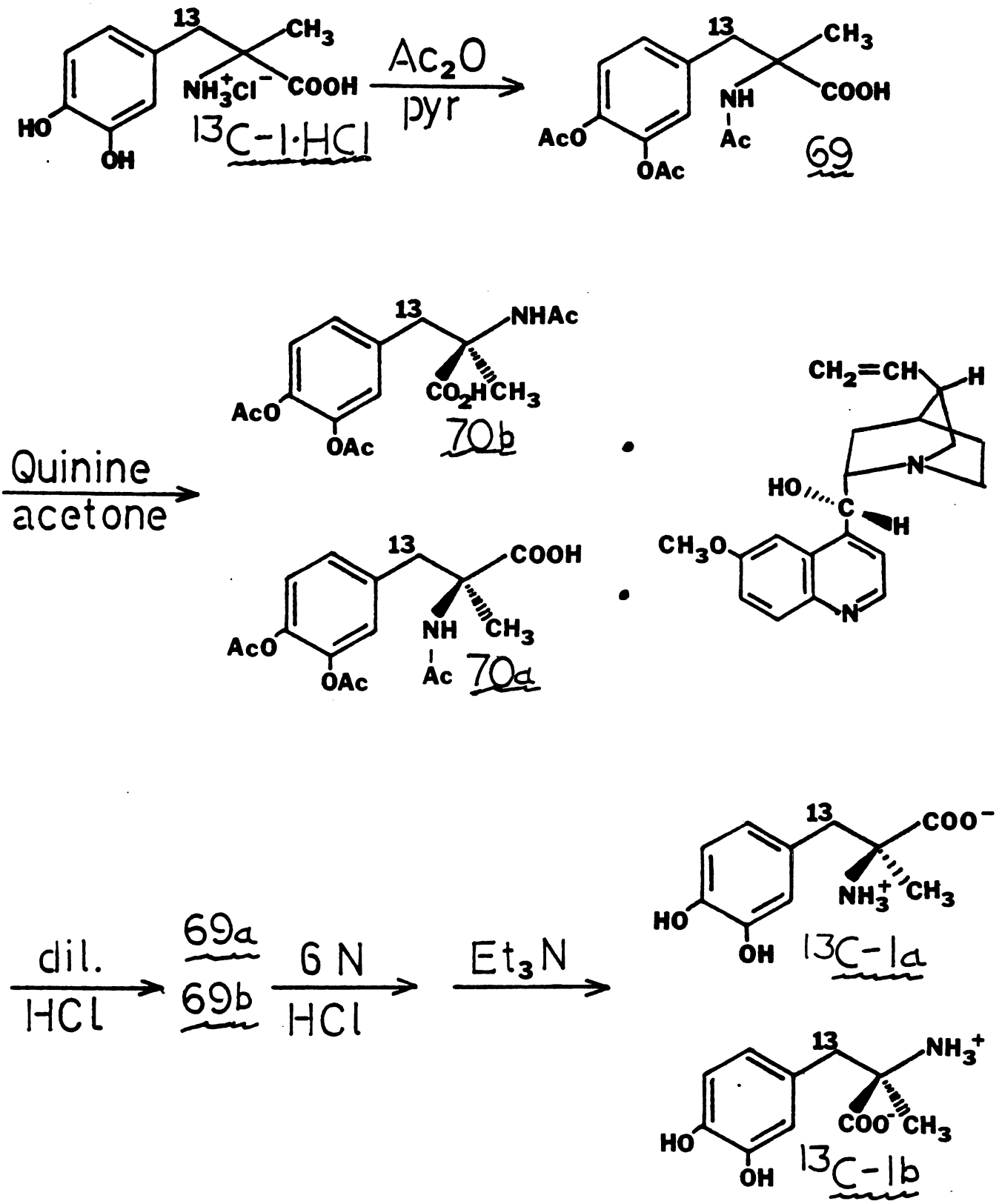
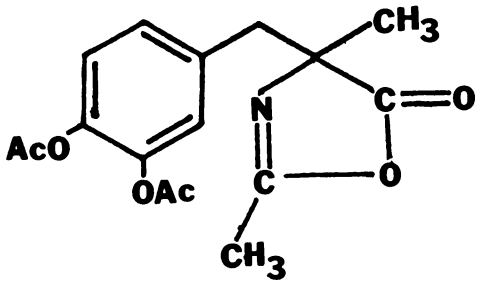


Figure 19. Resolution sequence for ^{13}C-enriched alpha-methyldopa (Ref. 85, 155).

The salt was filtered and rinsed with cold acetone, but not further purified. The compound was characterized only by mp.[85,155] The crude, resolved quinine salt ^{12}C-70a was subjected to acidic hydrolysis, and the the liberated triacetyl compound ^{12}C-69a obtained either by direct crystallization[155] or by extraction with ethyl acetate.[85] The resolved compound was then treated with 6N hydrochloric acid to give the hydrochloride salt of the amino acid, which was liberated with ammonia to yield the free amino acid of resolved alpha-methyldopa 1a. Optical rotation was used to determine optical purity.

We began our resolution studies with unlabeled 1 in order to establish optimal reaction conditons for the resolution procedure. As our synthetic pathway yields the hydrochloride salt of 1, we first attempted to liberate the free amino acid to study the acetylation procedure. Although (as we discovered later) treatment of a nitrogen purged aqueous solution of the hydrochloride salt of either enantiomer with triethylamine provided the liberated alpha-methyldopa as a grey-white solid, repeated attempts to precipitate the racemic mixture in this manner failed. Only very small quantities of grey-black solid were obtained. Consequently, in order to avoid losses of racemic ^{13}C-1, we turned our efforts to working out acetylation conditions applicable to the hydrochloride salt of alpha-methyldopa.

We first employed conditions described in the literature[85,155] for the acetylation of the free amino acid. Reaction of the hydrochloride salt of ^{12}C-1 in pyridine containing excess acetic anhydride for 48 hours at room temperature[85] or heating the same mixture on a steam bath for three hours[155] gave a brown oil which could not be purified. Acidic hydrolysis of the brown oil (presumably the azlactone ^{12}C-71) did not yield the expected solid triacetyl derivative.



^{12}C-71

Cims analysis (Figure 20a) revealed the presence of species with apparant molecular weights corresponding to a monoacetyl derivative of 1 (m/e 254) and a diacetyl derivative (m/e 296) suggesting an incomplete acetylation reaction. This result was confirmed by comparison of this spectrum with one obtained from a subsequently prepared product of acetylation using the free amino acid as the starting material, rather than the hydrochloride salt (Figure 20b).

The spectrum of the triacetyl compound displayed a large ion intensity corresponding to the parent ion (MH_2^+=338), with only weak ion intensities at m/e 254 and 296. It was then determined that reasonable yields of ^{12}C-69 could be obtained by refluxing pyridine and acetic anhydride with the hydrochloride salt of 1 for 15 hours under an atmosphere of nitrogen. We neither isolated nor characterized the azlactone intermediate 71. We did obtain the crude product of hydrolysis ^{12}C-69 by extracting the acidic hydrolysis solution with ethyl acetate. The nmr spectrum (pyr-d_5, Figure 21) was characterized by singlets for the O-acetyl proton signals at 2.23 and 2.20 ppm, and by a singlet

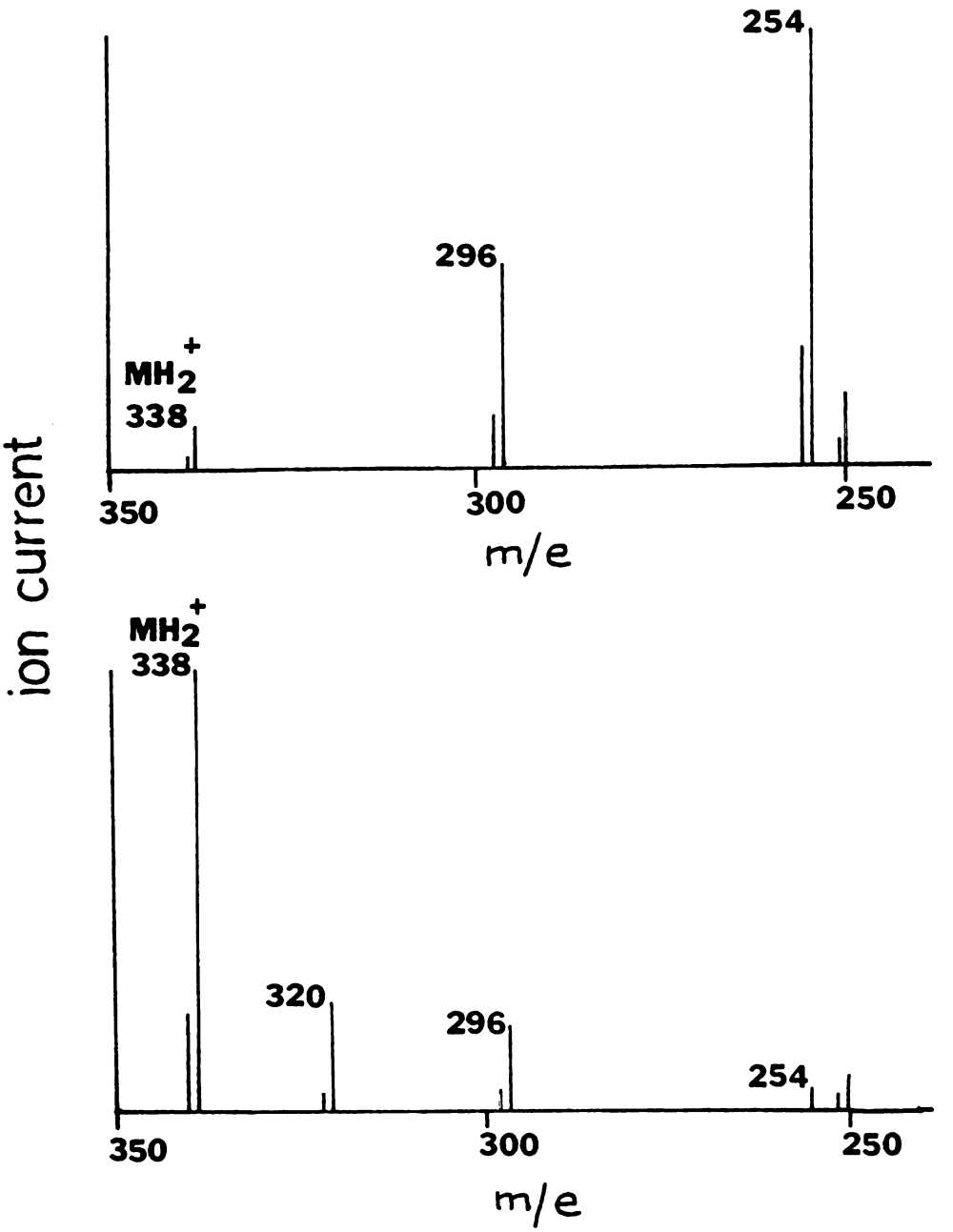


Figure 20. (a) Cims spectrum (240°) of an incomplete acetylation of alpha-methyldopa hydrochloride. (b) Spectrum of acetylation reaction of alpha-methyldopa as the zwitterion.

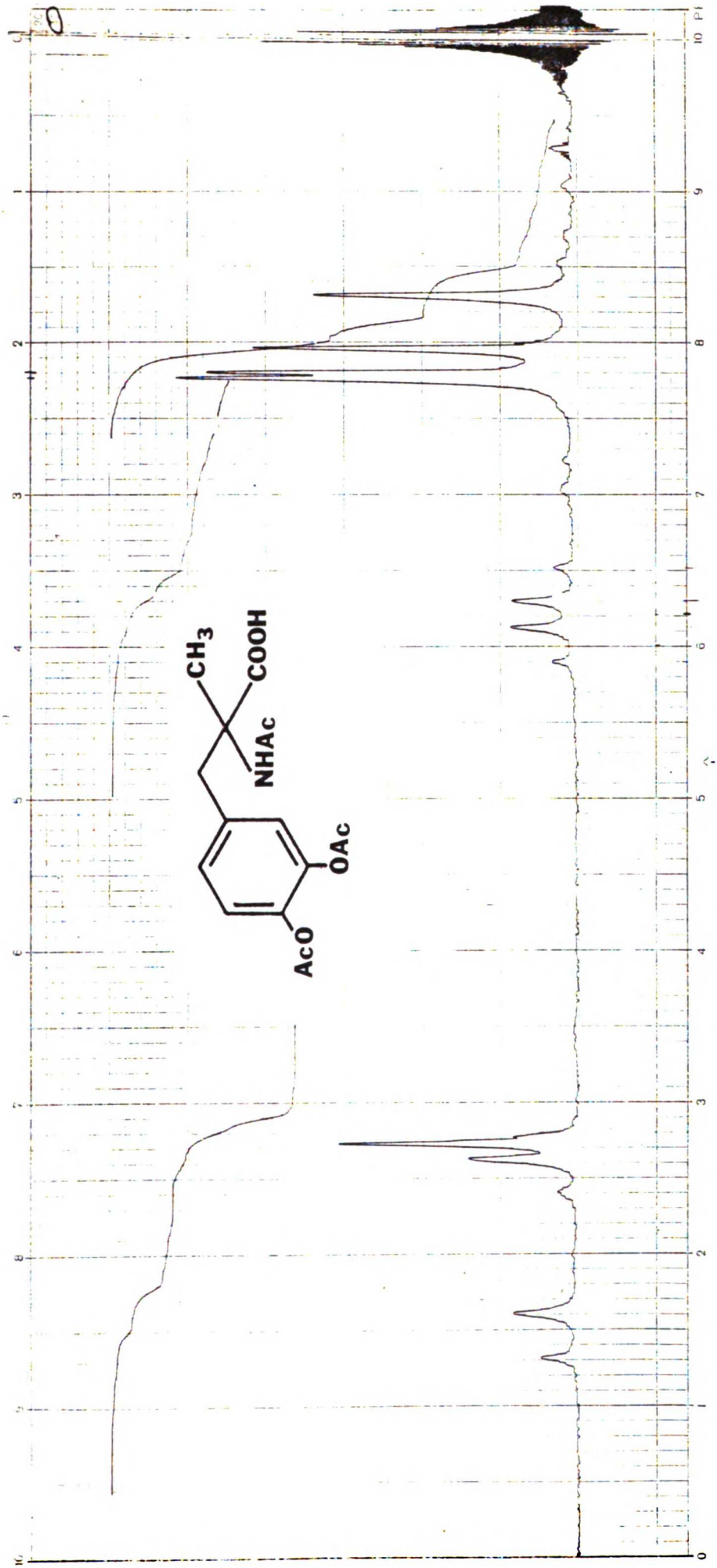


Figure 21. Nmr spectrum (pyr-d_5) of the triacetyl compound ^{12}C-69.

for the N-acetyl proton signal at 2.04 ppm. The compound was further characterized by cims (almost identical to Figure 20b) and mp. The cims spectrum was characterized by the parent ion (MH_2^+=338), loss of water (m/e 320), and decarboxylation (m/e 292). Small amounts of the monoacetyl (m/e 254) and diacetyl (m/e 296) ion intensities were observed. It is not possible to say if these are small amounts of the incompletely acetylated material, or fragmentation products.

Formation of unlabeled quinine salt ^{12}C-70a was readily accomplished at room temperature with a 10% excess of quinine in acetone. The salt obtained melted over a broad (and low) range. While we recognized that the melting point, 140-150° (lit.[155] mp 164-166°), could be indicative of optical impurity, we continued our studies without further purification, since the literature reports[85,155] indicated that no further purification was necessary.

The resolved S-triacetyl compound ^{12}C-69a was obtained by extraction with ethyl acetate of the dilute acid solution of the quinine salt. The nmr spectrum (pyr-d_5) was identical to that of the racemic compound (Figure 21). Hydrolysis of ^{12}C-69a in 6N hydrochloric acid afforded the hydrochloride salt 1a·HCl of unlabeled alpha-methyldopa. The salt was extremely hygroscopic, and was therefore not characterized. Rather, it was treated with triethylamine to yield the desired S-(-)-alpha-methyldopa (1a). The compound was characterized by mp, cims, and the optical purity (see below) estimated by optical rotation measurements.

We first obtained optical rotation data with unlabeled 1a, both resolved in our hands, and commercially resolved drug. As can be seen

in Table III, there was a very wide range of specific rotation values between the samples and the literature value.[155] The large variation is at least partially due to the low specific rotation values for 1a. Our experimentally determined values were as low as -0.003° , which magnifies sample handling (weighing, diluting, etc.) errors. We then turned to the resolved triacetyl derivative ^{12}C-69a which as indicated in Table III has a much larger specific rotation value. The measurements indicated 95% optical purity of the S-triacetyl compound ^{12}C-69a. This was deemed a satisfactory degree of resolution , and we proceeded with the resolution of ^{13}C-1.

We resolved only the 89% ^{13}C-enriched alpha-methyldopa, for use in brain metabolism studies, the 50% enriched material being utilized for in vitro studies (see metabolism section). The acetylation of the ^{13}C-alpha-methyldopa hydrochloride (^{13}C-1·HCl) was accomplished as described for the model compound, with one modification. The crude product of the hydrolysis was pale brown in color. The brown solid was dissolved in a minimum amount of acetone at room temperature, and the solution slightly cooled. Within a few minutes, a white crystalline solid separated from the brown solution. Collection of this solid provided the ^{13}C-labeled triacetyl compound 69 in 55% yield. The racemic triacetyl compound 69 was characterized by mp and cims. The cims spectrum (Figure 22) displayed the parent ion (MH_2^+= 339) in the expected 89% abundance. Additional ions observed corresponded to loss of water (m/e 321), loss of acetyl (m/e 297, possibly due to diacetyl impurity), and decarboxylation (m/e 293).

Quinine salt formation was accomplished with a 17% excess of quinine in acetone. The ^{13}C quinine salt 70a was obtained in a 50% yield

Table III

I. Specific rotation of alpha-methyldopa (^{12}C and ^{13}C) in 0.1N HCl (c = 1, 436 nm, 25°).

Compound	α observed	$\alpha^{25^\circ}_{436}$
Commerical S-alpha-methyldopa	-0.044°	-4.3°
Resolved ^{12}C-alpha-methyldopa	-0.086	-8.0
Literature value[155]	----	-5.5

II. Specific rotation of triacetyl alpha-methyldopa (^{12}C and ^{13}C) in 96% EtOH (c = 2, 589 nm, 25°).

Compound	α observed	$\alpha^{25^\circ}_{589}$
Resolved ^{12}C-triacetyl alpha-methyldopa	-1.381°	-68.7°
Resolved ^{13}C-S-triacetyl alpha-methyldopa (1st resolution)	-1.300	-64.8
Resolved ^{13}C compound (2nd resolution)	-1.382	-68.7
Literature value[155]	---	-74.5

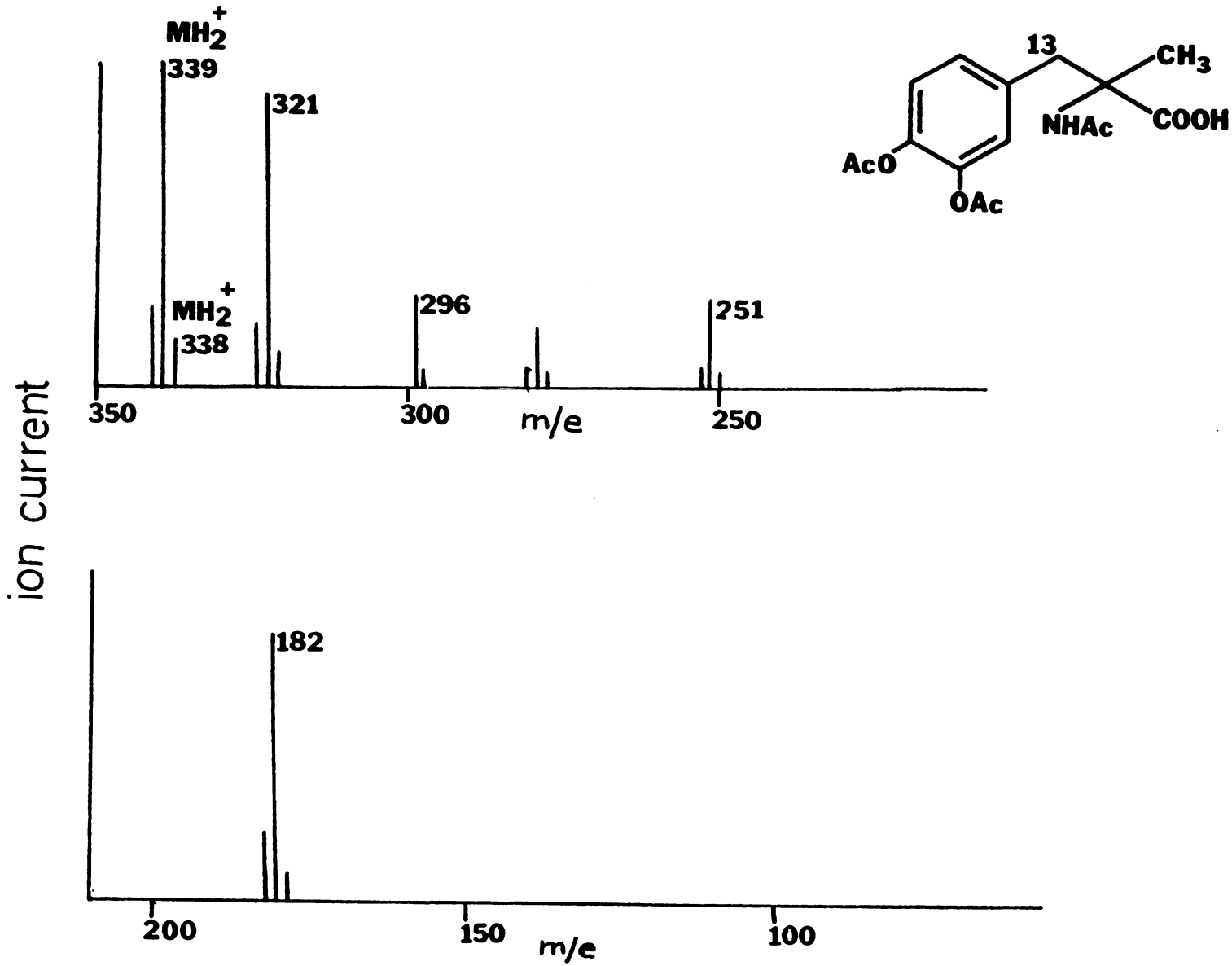


Figure 22. Cims spectrum (280°) of the triacetyl compound 69.

of theory, and characterized only by mp, 145-150° (lit.[155] mp 164-166°). Again, we were concerned that the low melting point indicated contamination of our product with the diastereomeric quinine salt 70b, the salt of the R isomer of the triacetyl compound. Since we required a high degree of enantiomeric purity for our metabolic work, we submitted the liberated and resolved ^{13}C-triacetyl compound to the quinine salt resolution step again. There was, however, no improvement in the melting point of the salt.

The S-triacetyl compound 69a was liberated in dilute acid and extracted with ethyl acetate. The compound was characterized by mp. The R isomer 70b was also isolated from the solids obtained upon removal of solvents from the mother liquor of the quinine salt resolution. The cims spectrum was almost identical to that of the racemic compound (Figure 22), and the nmr spectrum (pyr-d_5, Figure 23) identical to that of the unlabeled compound (Figure 21), but with ^{13}C coupling.

The optical purity of the ^{13}C-labeled S-triacetyl compound 69a was estimated by optical rotation as seen in Table III. Based on these values, the second quinine salt resolution improved the enantiomeric purity by perhaps 1-2%. The triacetyl compound 69a was hydrolyzed in 6N hydrochloric acid, and the hydrochloride salt treated with triethylamine to yield the free amino acid ^{13}C-1a, mp 295-300° with dec (lit.[155] mp 290-300° with dec).

While our results suggested that the optical purity of our resolved amino acid was equal to that reported,[85,155] we were not completely satisfied for the following reasons. The broad melting point of the quinine salt suggested the presence of the unwanted diastereomer. The optical rotation measurements, while being quite useful for an approxi-

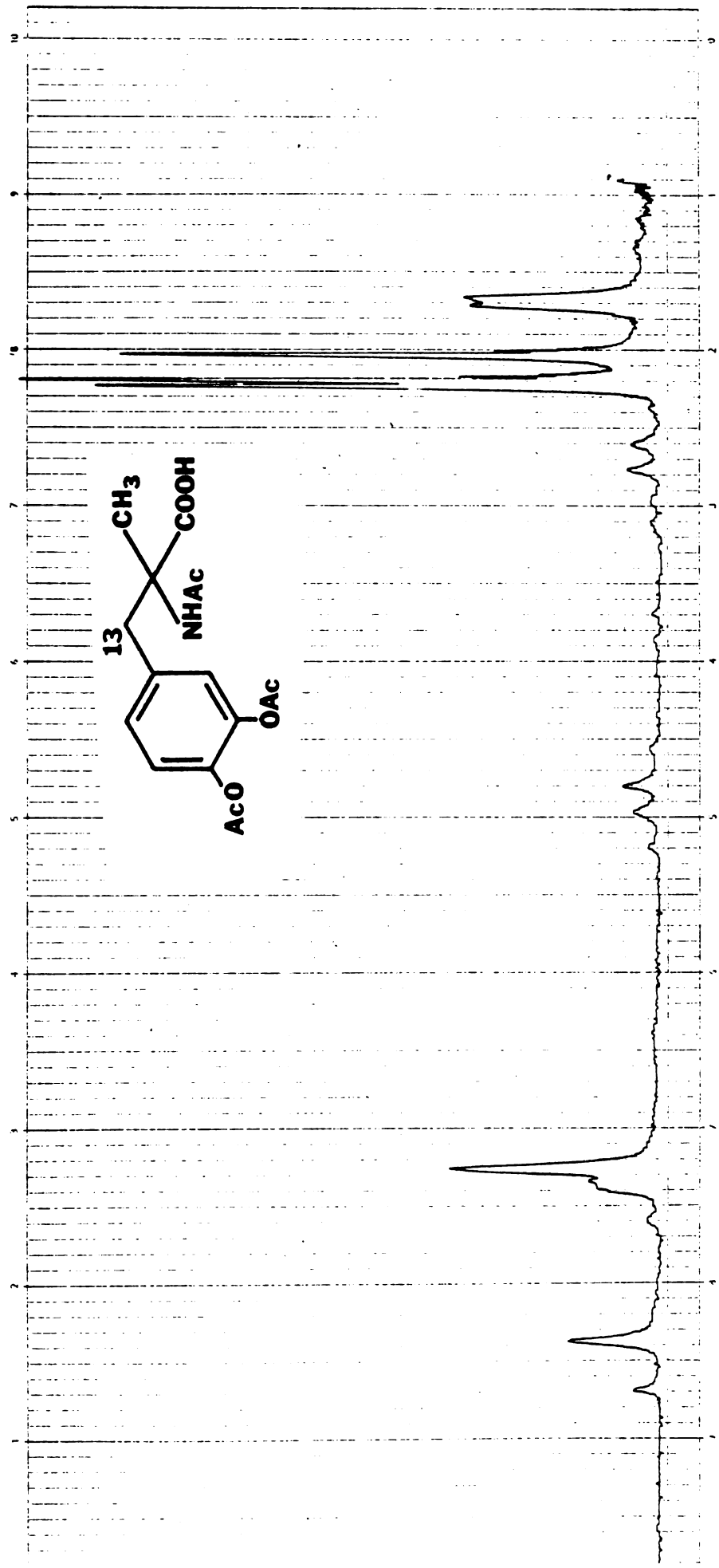


Figure 23. Nmr spectrum (pyr-d_5) of the ^{13}C-triacetyl compound 69.

mate determination of enantiomeric composition, are not precise. The method is suseptible to errors introduced by manipulations, such as in weighing and dilution. Finally, since the focal point of our studies was the stereochemistry of alpha-methyldopa metabolism in the brain, even a 5-10% optical impurity would compromise the accuracy of our metabolic data.

With these thoughts in mind, we sought an alternative procedure to establish the enantiomeric purity of the alpha-methyldopa, one which did not depend upon the accuracy of weighing, dilution, and other manipulations. In particular, we were interested in methods which would allow the detection of one isomer in the presence of the other isomer, rather than measuring a parameter such as optical rotation which describes the optical mixture in terms of an overall physical characteristic. This reasoning led to the consideration of glpc assays in which with requisite resolution, the presence of a large amount of one isomer need not jeoprodize the detection of a small amount of the other enantiomer.

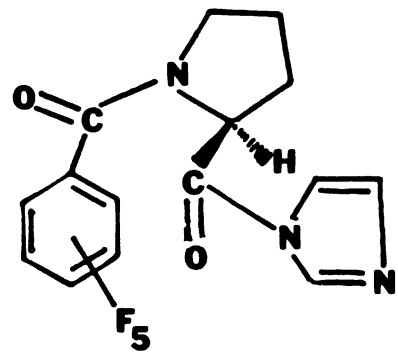
Glpc assays have been used to separate the enantiomers of amines and amino acids by several general methods. The most common is to convert the asymmetric substrate with a chiral reagent to the corresponding diastereomers. With a racemic mixture, the resulting diastereomeric pair may be separated on columns packed with common liquid phases. One commonly used reagent is N-trifluoroacetyl-S-prolyl chloride.[157, 158,159] This chiral agent has been used to resolve amphetamine and several other 1-phenyl-2-aminopropanes. However, this reagent has been reported[158] to racemize to some extent (5-15%) and is therefore of limited utility for quantitative work. Other reagents reported for

enantiomeric separation include alpha-methoxy-alpha-methylpentafluorophenylacetyl chloride[160] and N-trifluoroacetyl-S-phenylalanine[157].

Another general method for the glpc separation of enantiomers is with optically active column materials. Koening and Nicholson[161] recently reported the use of optically active peptides for the separation of amino acid enantiomers. The technique employs glass capillary columns coated with the active peptide material. Of the amino acid separations they reported, only two were aromatic, those being phenylalanine and tyrosine. This is a rather specialized method requiring the glass capillary glpc system, and the synthesis and bonding of the active peptides.

We have had considerable experience in our laboratory with the use of the chiral resolving reagent N-pentafluorobenzoyl-S-prolyl-1-imidazolide (72, PFBPI).[162] It has been successfully used for stereochem-

72



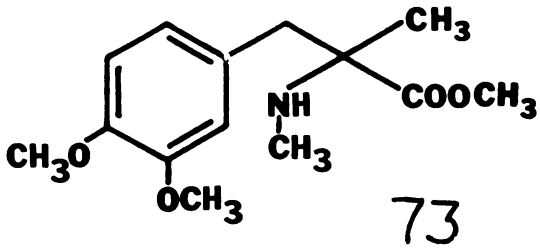
ical studies with the psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane[163] and for the determination of the absolute configuration of metabolically formed alpha-methyldopamine.[102] We therefore attempted to develop an assay to estimate the enantiomeric purity of alpha-methyldopa based on the PFBPI reagent.

Before we could derivatize alpha-methyldopa with PFBPI, it was necessary to protect the phenolic and carboxylic functions, both to

prevent side reactions with PFBPI and to improve the volatility and stability of the compounds. We selected diazomethane, which has been reported to react readily with carboxylic acids and phenols, but to react with alcohols and amines only in the presence of a catalyst such as aluminum chloride ($AlCl_3$).[164,165] Walson et al[166] found however, that the derivatization of alpha-methyldopa with diazomethane produced significant amounts of N-methylated products. DOPA was also successfully derivatized with diazomethane, but with some N-methylation.[167] Studies in our laboratory have shown that reaction of PFBPI with secondary amines such as nornicotine and ketamine is very slow compared to primary amines. Therefore, we anticipated no problems with PFBPI derivatization of the N-methylated side products of alpha-methyldopa methylation. Diazomethane is evidently not extensively employed as a derivatizing reagent for amino acids. A recent review of synthetic methods[168] as well as Fieser and Fieser's "Reagents for Organic Synthesis"[169] reported no reactions with amino acids in discussions of diazomethane chemistry.

We proceeded with the methylation of 1 (as the free amino acid) employing an excess of diazomethane at room temperature in methanol. In an attempt to follow the methylation reaction, aliquots of the reaction mixture were removed after varying times, from 2-10 days, and cims spectra obtained from the aliquots. While it is not possible to determine relative amounts of the various methylation products present (due to varying volatilities), we did verify earlier reports of N-methylation. We observed significant ion intensity corresponding to the mono N-methylation product 73 (m/e 268), and a very small intensity corresponding to the N,N-dimethylation product

74 (m/e 282). The major ion after 10 days was found at m/e 254, corresponding presumably to the desired 0,0,0-trimethylalpha-methyldopa (75).



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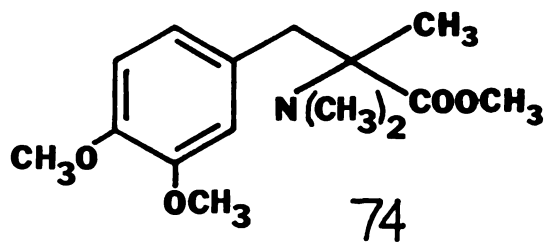

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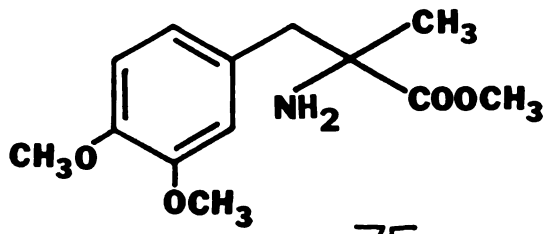

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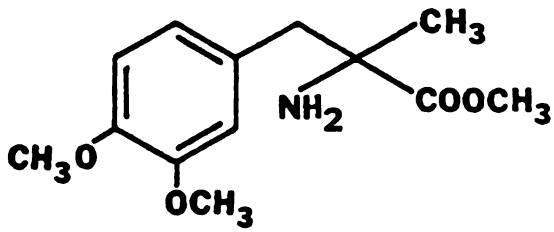
As part of more thorough studies on the methylation of alpha-methyldopa (and glpc enantiomeric analysis of 1 and ^{13}C-1 which will be described below),[170] the products of a seven day methylation reaction mixture were analyzed by gc/ms. The gc/ms data have been summarized in Table IV. The compounds readily separated on an OV-17 column, and there are several fragments clearly diagnostic for the presence of N-methylated products. As can be seen, the approximate ratios are 5:1:0.2, suggesting that the derivatization with PFBPI should be readily accomplished.

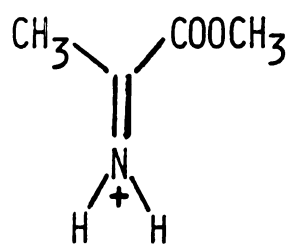
The methylation products (from 7-10 day methylations) were

Table IV

Gc/ms characteristics of the N-methylation products of alpha-methyldopa and diazomethane derivatization.#

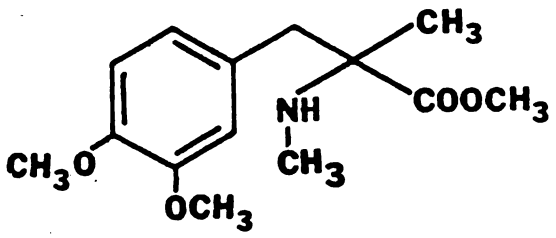


Retention Time	Relative Amount
3.0 MIN	100%

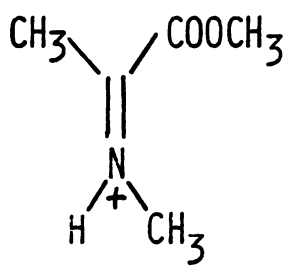
Ions	Significance	Intensity
253*	$M^{+\cdot}$	3%
194*	$M^{+\cdot}-COCH_3$	25%
152	$A(-CH_3^{+\cdot})$	60%
151	$A(-CH_2^{+})$	60%
102*		100%
42*	$CH_3-C\equiv\overset{+}{N}-H$	35%

* Diagnostic for N-methylation
# 3% OV-17 column, column temp 200°, source temp 235°

Table IV, cont'd

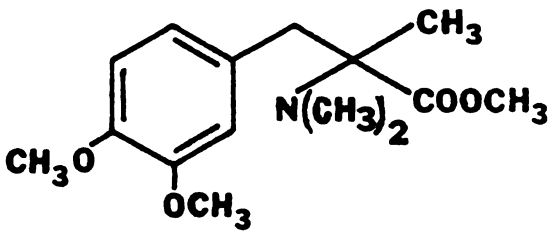


Retention Time	Relative Amount
3.5 MIN	~18%

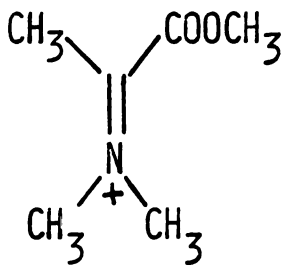
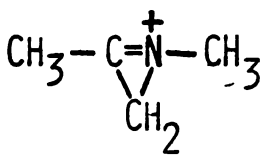
Ions	Significance	Intensity
267*	$M^{+\cdot}$	---
208*	$M^{+\cdot}-COOCH_3$	20%
152	$A(-CH_3^{+\cdot})$	10%
151	$A(-CH_2^{+})$	10%
116*		100%
56*	$CH_3-C\equiv\overset{+}{N}-CH_3$	45%

* Diagnostic for N-methylation

Table IV, cont'd



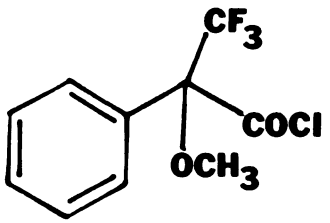
Retention Time	Relative Amount
4.7 MIN	2%

Ions	Significance	Intensity
281*	$M^{+\cdot}$	---
222*	$M^{+\cdot}-COOCH_3$	20%
151	$A(-CH_2^+)$	8%
130*		100%
70*		40%

* Diagnostic for N-methylation

extracted with dilute base, and reacted with a 2-3 molar excess of PFBPI in a minimum amount of dichloromethane. After two days at room temperature, glpc analysis of the mixture was performed with an SE-30 column. Glpc tracings of racemic mixtures revealed no patterns suggestive of a resolved diastereomeric pair. Studies with an OV-25 column were also unsuccessful. While cims analysis did show some ion intensity corresponding to the PFBPI derivative (m/e 545), further glpc efforts were unsuccessful. We really had no way of knowing whether the derivatization was proceeding to a sufficient extent, or if we were merely unable to separate the diastereomers under our glpc conditions. In either case, the results led us to explore another chiral reagent.

The acid chloride 76 of the levorotatory isomer of alpha-methoxy-alpha-trifluoromethylphenylacetic acid has been used to separate the



(−)76 (MTP-Cl)

enantiomers of amphetamine and related 1-phenyl-2-amino-propanes.[171] More importantly, the same assay had been successfully employed to separate the enantiomers of the aromatic amino acid phenylalanine. We therefore investigated the use of 76 in the analysis of the enantiomers of 1.[170] The diazomethane methylation procedure was modified by shortening the reaction time to seven days and allowing the reaction to proceed in the dark, at room temperature. After removal of solvent, the methylation mixture (without base extraction) was allowed to react with

the acid chloride 76 in pyridine and dichloroethane. This reagent has also been shown to react only very slowly with secondary amines.[171] The glpc tracings for racemic and R-alpha-methyldopa after the derivatization procedures are illustrated in Figures 24 and 25, respectively. The results for ^{13}C-1a, the S enantiomer of the labeled alpha-methyldopa, are illustrated in Figure 26. As evidenced by these glpc tracings, we felt confident in stating that we could observe a 2% optical impurity by inspection of the glpc data. Gc/ms analysis verified the presence of the derivatized molecules in the glpc peaks, and will be published elsewhere.[170]

With this assay, we estimated that the resolution of the 89% ^{13}C-labeled alpha-methyldopa had proceeded to at least 98% optical purity. This level of enantiomeric purity was acceptable for our metabolic studies of the *in vivo* metabolism of alpha-methyldopa in the rat brain.

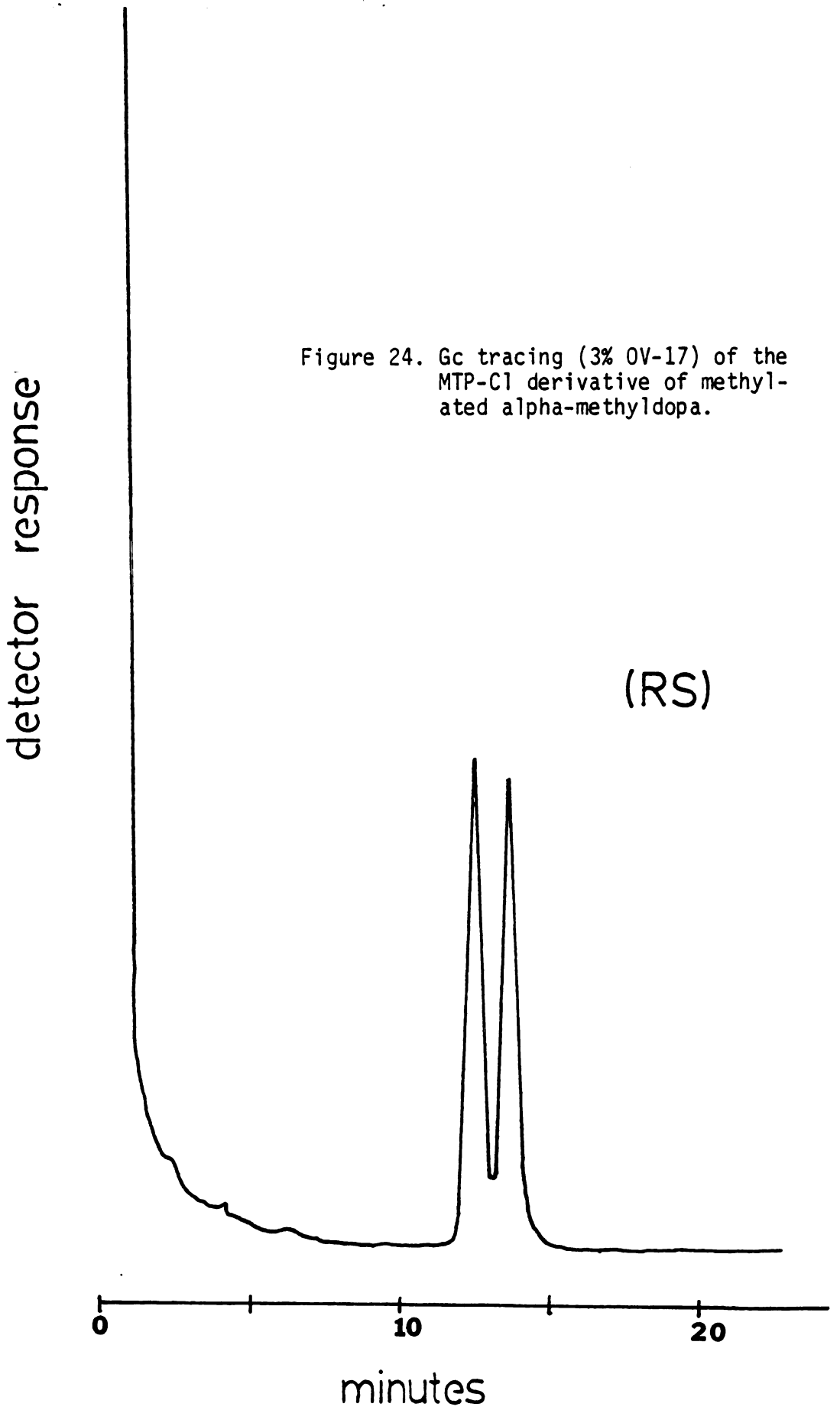


Figure 24. Gc tracing (3% OV-17) of the MTP-Cl derivative of methylated alpha-methyldopa.

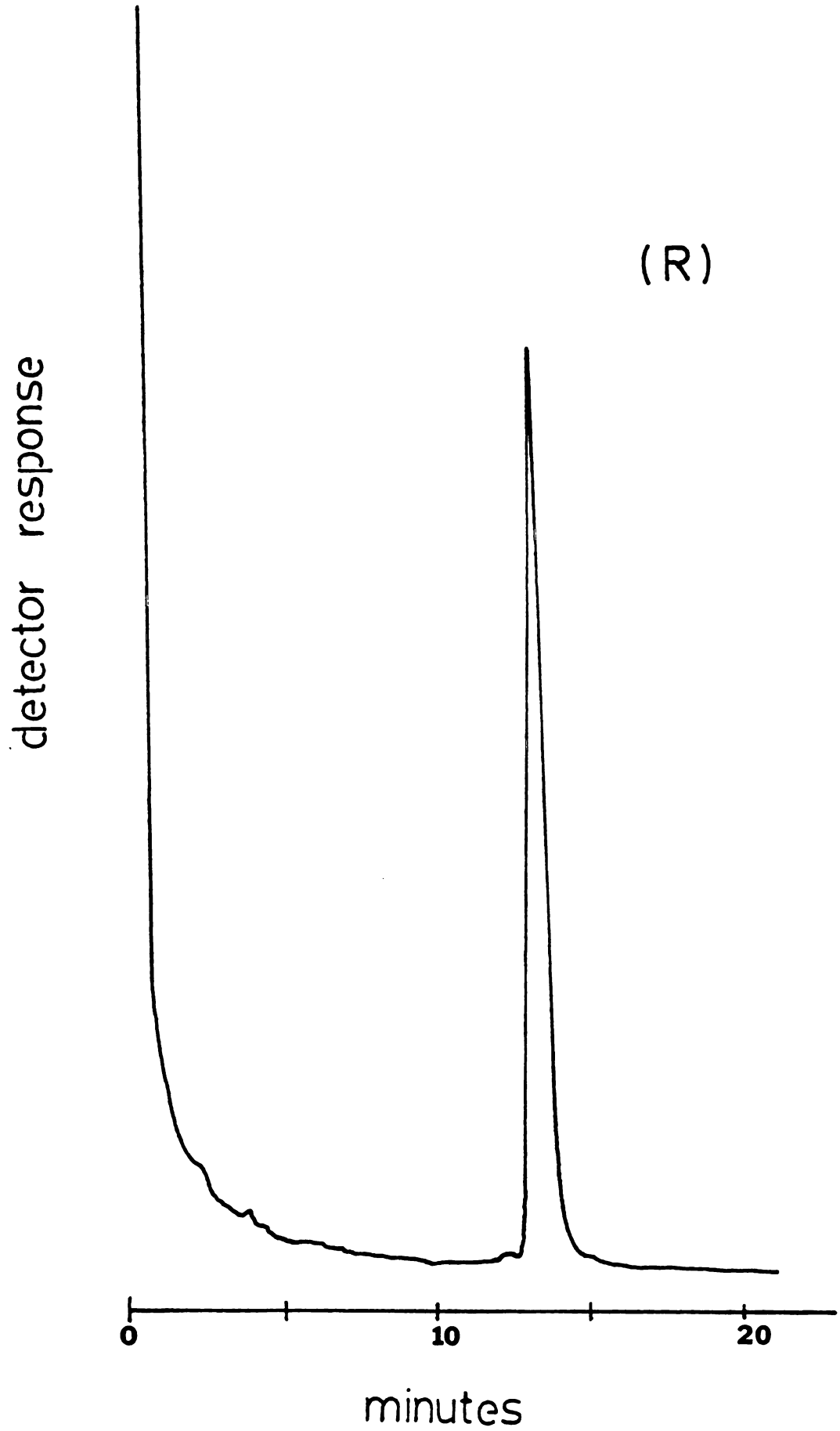
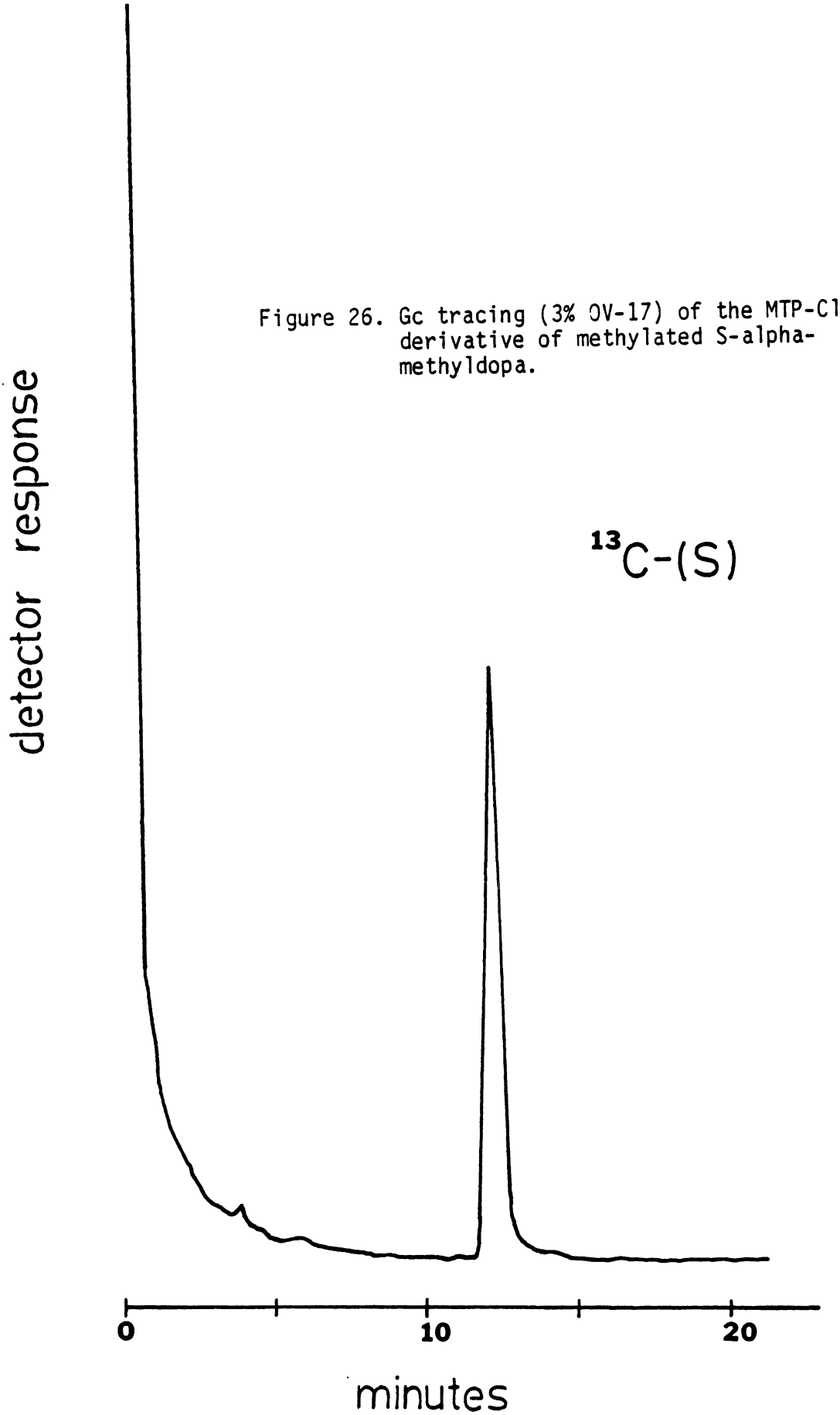


Figure 25. Gc tracing (3% OV-17) of the MTP-Cl derivative of methylated R-alpha-methyldopa.

Figure 26. Gc tracing (3% OV-17) of the MTP-Cl derivative of methylated S-alpha-methyldopa.



EXPERIMENTAL SECTION

Solvents were removed by means of a rotary evaporator under vacuum. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Literature melting points refer to the unlabeled ^{12}C compounds. Nmr spectra were recorded on a Varian A-60A instrument in the indicated solvent with TMS as the internal standard unless otherwise indicated. Chemical ionization mass spectra were taken on an AEI MS 902 double focusing mass spectrometer equipped with a direct inlet system and modified for chemical ionization mass spectrometry. The reagent gas was isobutane at an ion chamber pressure of 0.5 to 1.0 torr, at the source temperature indicated. Glpc were run on a Varian Model 2100 with a U-shaped 2m x 2mm Pyrex column packed with 3% OV-1 on acid-washed, DMCS-treated Chromasorb W. Gc/ms were run on a Hewlett Packard 5980A quadrupole mass spectrometer (70 eV) with a source temperature of 200°, transfer line temperature of 250°, and separator temperature of 200°. The instrument was interfaced with a Varian Model 2100 gas chromatograph with a U-shaped 2m x 2mm Pyrex column packed with 3% OV-17 on Chromasorb Q. The column temperature was 200°, injection port temperature 240°, and detector temperature 260°. Specific rotation measurements were taken on a Perkin-Elmer 141 electronic polarimeter. UV were taken on a Cary 14 spectrophotometer. IR were obtained on a Perkin-Elmer 337 spectrophotometer. Microanalyses were performed by the Microanalytical Labs, University of California, Berkeley, California. Gc/ms data was obtained at the University of California at Los Angeles, Department of Pharmacology, School of Medicine.

^{13}C-Labeled barium carbonate (89 atom per cent). In a large flask, 4 liters of distilled H_2O were boiled and then cooled under an atmosphere of N_2. When the temperature was approximately 50°, the H_2O (3.1 l) was transferred to a smaller flask and $Ba(OH)_2 \cdot 8H_2O$ (246.8 g, 0.78 moles) added to the flask. The milky solution was stirred for 30 min and then filtered under vacuum and an atmosphere of N_2 into a 3-neck 4 liter round bottom flask equipped with a mechanical stirrer, nitrogen bubbler, and thermometer. Meanwhile, a saturated solution of $Ba(OH)_2 \cdot 8H_2O$ (400 ml) was prepared under the conditions described above, and placed in a beaker equipped with a sidearm, and sealed with a stopper. This beaker was then attached to the large round bottom flask by means of a gas line which replaced the thermometer in the large flask. The gas cylinder containing the 95% $^{13}CO_2$ was then connected to the large round bottom flask, and all seals in the flask, gas cylinder, and safety trap checked. ith the temperature at 42°, the gas flow was slowly initiated with stirring and nitrogen flowing through the system. After several minutes, white precipitate began to appear in the large flask; the gas flow was thus continued for 2 hr. It had been assumed that the gas cylinder contained the stated amount of $^{13}CO_2$ (26 g, 0.59 moles), and that figure was used to determine the amount of barium hydroxide used, a 32% exess. It was later discovered that the cylinder contained a much larger amount of gas. Thus, the excess barium hydroxide was consumed in this reaction. The white solids were allowed to cool overnight, and then filtered under a cone of N_2. The solids were then suspended in distilled H_2O, well stirred, and filtered under a cone of N_2. The solids were dried on the filter for 24 hr, ground in a mortar and pestle, and dried 3 days in a

vacuum dessicator under vacuum to yield the white solids: 150.3 g, 132%). No analysis of the solids was performed, but the solids were employed in the carbonation of 3,4-dibenzyloxybromobenzene (58) described below.

3,4-Dibenzyloxybenzoic -7-^{13}C-Acid (60). Into a dry reaction vessel equipped with magnetic stirrer was placed the solid 3,4-dibenzyloxybromobenzene (58, 2.87 g, 7.8 mmoles) and dry (distilled from $LiAlH_4$) ether (50 ml). The stirring solution was purged with dry N_2 and then placed in a dry ice/MeOH bath and cooled (-80°). To the cooling and stirring solution was added n-BuLi (2.4 ml, 5.50 mmoles) as a 2.28 M hexane solution via a 5 cc syringe. The reaction vessel was then transferred to the vacuum manifold, the contents frozen in a liquid N_2 bath, and placed under vacuum with the reaction vessel and manifold stopcocks open.

During the 15 min metallation reaction, the ^{13}C-labeled barium carbonate (64 atom per cent or 95 atom percent, 0.988 g, 5.0 mmoles) was placed in the dry gas generator and covered with 3 glass wool plugs. Concd H_2SO_4 (40 ml) was added to the acid flask. The acid flask, generator, drying tower, and stopcock were all assembled, placed on the vacuum manifold, and placed under vacuum with the generator and manifold stopcoks open. When the metallation reaction was finished, and both reaction vessel and generator assemblies had reached a pressure of 5 microns Hg, the generator and manifold stopcocks were closed and the acid slowly poured into the generator containing the $Ba^{13}CO_3$. The generator apparatus was removed from the vacuum manifold under vacuum (the small 14/20 joint between the manifold and generator stopcocks

can be easily broken under vacuum) and the bubbling acid-carbonate mixture swirled until all solid had disappeared (10 min). The generator assembly was then reattached and placed under vacuum by opening only the manifold stopcock, thereby retaining the $^{13}CO_2$ in the generator assembly.

With the reaction vessel frozen in liquid N_2, the pressure in the system was reduced to less than 5 microns Hg, and the large main manifold stopcock closed (isolating the system from the vacuum pump). The generator stoptock was then slowly opened allowing the $^{13}CO_2$ to distill into the reaction vessel. After 10 min, a heat gun was employed to drive the $^{13}CO_2$ out of the generator assembly and manifold. After a total time of 15 min all 4 stopcocks were closed, and the reaction vessel transferred to a dry ice/MeOH bath (-80^{o}). The carbonation was allowed to proceed with stirring for 15 min.

The reaction vessel was removed from the bath and vented to the atmosphere, and 5N HCl (10 ml) added. After 10 min stirring at room temperature, the still cold contents of the reaction vessel (now containing white solids) were transferred to a separatory funnel (125 ml) with rinsing by distilled H_2O (25 ml) and ether (25 ml). The clear, colorless, aqueous layer was drawn off, and the white solids and yellow ethereal layer washed with additional H_2O (25 ml, drawn off and also discarded). The remaining solids and organic layer were extracted with 1N NaOH (3 x 25 ml), and the combined basic fractions acidified with concd HCl (10 ml) to yield a white solid. the yellow organic layer was discarded. The mixture was cooled and vacuum filtered with rinsing (cold H_2O). The slightly discolored solid was dried on the vacuum filter overnight, yielding a dry slightly off-white solid

(1.15 g, 69%, mp 175-180°). Combined crops of crude acid 60 were recrystallized from EtOAc in 5 g batches to give an average yield of 3.5 g, for an overall average yield of 50%, based on an average crude yield of 70%. The data for such combined purified acid 60: mp 183-185° (lit.[131] mp 186°); nmr (DMSO-d_6) δ 7.82-7.08 (m, 13, aromatic), 5.25 (s, 2, O-C$\underline{H}_2$-C_6H_5), and 5.22 ppm (s, 2, O-C$\underline{H}_2$-C_6H_5); cims (300°) m/e (rel intensity) 336(89), 335(50), 318(17), 317(11), 291(50), 245(33), 244(56), 243(34), 181(22), 91(100).

3,4-Dibenzyloxybenzyl Alcohol (^{12}C-61). (a). From acid ^{12}C-60.

To an ice-cold flask containing dry, distilled THF (50 ml) was added $LiAlH_4$ (0.46 g, 12 mmoles). The system was purged with N_2 and additional THF (25 ml) added to the heterogeneous solution. The acid (^{12}C-60, 1.0 g, 3 mmoles) was dissolved in THF (40 ml) and added dropwise to the cooled and stirring reaction mixture. The reaction was allowed to proceed for 15 hr, gradually warming to room temperature. Distilled H_2O (0.46 ml), 15% NaOH (0.46 ml), and additional H_2O (1.37 ml) were added slowly and sequentially to the ice-cold solution. After stirring (30 min), the grey solids were filtered, and the solids leeched in hot ether (25 ml) for 30 min. The combined filtrates were dried ($MgSO_4$), filtered, and the solvent removed in vacuo to yield the white crude product (0.86 g, 90%). The crude product ^{12}C-61 was recrystallized from benzene-hexane to yield the pure alcohol (0.60 g, 62%): mp 69-70° (lit.[225] mp 71-71°); nmr ($CDCl_3$) δ 7.64-6.77 (m, 13, aromatic), 5.10 (s, 4, O-C$\underline{H}_2$-C_6H_5), 4.49 (s, 2, C$\underline{H}_2$-OH), and 1.97 ppm (broad s, 1, CH_2-O$\underline{H}$); uv max (95% EtOH) 225 nm (ε 1,225) and 279 nm (ε 2,889); ir (KBr) 3580, 3005, 2860, 1605, and 1380 cm^{-1}.

Anal. Calcd for $C_{21}H_{20}O_3$; C, 78.75; H, 6.25. Found C, 78.79; H, 6.36.

(b). From aldehyde ^{12}C-62. The ^{12}C-aldehyde (^{12}C-62, 10.0 g, 31.4 mmoles) was dissolved with gentle heating in absolute EtOH (1000 ml). $NaBH_4$ (4.75 g, 125.8 mmoles) was dissolved in absolute EtOH (200 ml), and the solution cooled with an ice bath. The aldehyde solution was dripped into the reaction vessel via a pressure equalizing dropping funnel over a 60 min period. The solution was stirred for 3 hr, and allowed to warm to room temperature. Distilled H_2O (200 ml) was then added, and the solution left at room temperature for 15 hr. The solvent was concentrated in vacuo leaving a suspension of white solids in aqueous solution. The mixture was then extracted with $CHCl_3$ (75,50, and 50 ml), and the combined cloudy extracts dried ($MgSO_4$). The solvent was then removed in vacuo to yield the crude product (11.0 g, 110%) which was recrystallized from benzene to afford the pure alcohol ^{12}C-62 (9.2 g, 92%): mp 70-72° (lit. mp 71-72°); nmr ($CDCl_3$) δ 7.64-6.80 (m, 13, aromatic), 5.11 (s, 4, O-C$\underline{H}_2$-OH), 4.49 (s, 2, C$\underline{H}_2$-OH), and 2.11 ppm (broad s, 1, CH_2-O$\underline{H}$).

3,4-Dibenzyloxybenzyl-7-^{13}C-Alcohol (61). The acid (60, 3.0 g, 9.0 mmoles) was taken up in dry THF (90 ml) and added dropwise under N_2 to an ice-cold stirred solution of $LiAlH_4$ (1.8 g, 48 mmoles) in dry THF (175 ml). After stirring an additional 18 hr at room temperature, the reaction mixture was cooled again and treated with distilled H_2O (1.9 ml), 15% NaOH (1.9 ml), and additional H_2O (5.2 ml). After stirring 15 min, the solids were vacuum filtered and digested

in boiling ether (25 ml). The combined clear organic filtrates were dried ($MgSO_4$), filtered, and the solvents evaporated to yield a white solid (3.65 g, 95%): mp 66-68° (lit. mp 71-72°); nmr ($CDCl_3$) δ 7.78-6.82 (m, 13, aromatic), 5.13 (s, 4, O-$\underline{CH_2}$-C_6H_5), 4.53 (s, 2, $^{12}\underline{CH_2}$-OH), 4.53 (d, 2, $\underline{J}_{^{13}C-H}$ = 144 Hz, $^{13}\underline{CH_2}$-OH), and 1.86 ppm (s, 1, CH_2-$\underline{OH}$); cims (210°) m/e (rel intensity) 322(12), 321(23), 320(23), 319(9), 304(60), 303(41), 291(100), 214(47), 213(35), 181(29), 91(41).

3,4-Dibenzyloxybenzaldehyde (^{12}C-62). (a). From alcohol ^{12}C-61 with Jones Reagent. The benzyl alcohol (^{12}C-61, 1.0 g, 3.0 mmoles) was dissolved in acetone (15 ml) and cooled with an ice bath. The Jones Reagent[134] was added dropwise to the ice-cold, stirring solution containing the alcohol until the milky green reaction mixture turned yellow (0.5 ml). Additional stirring returned the mixture to its green color, and this process was continued until the green color did not return (0.78 ml). After 20 min additional stirring, isopropyl alcohol was added dropwise until the solution again turned milky green. Stirring (5 min) was followed by vacuum filtration. The solids were leeched by stirring in hot acetone (10 ml, 5 min) and the filtrates combined. The organic filtrates were vacuum filtered again, and the solvent concentrated to a slurry in vacuo. Due to the presence of green solids in the slurry, $CHCl_3$ (25 ml) was added and the solution again filtered. The clear yellow liquid was dried ($MgSO_4$) and the solvent removed in vacuo to yield a yellow solid (1.0 g, 100%). Recrystallization was attempted with absolute EtOH and benzene-hexane, with both systems yielding a brown product. Therefore, the crude product obtained from another such preparation was sublimed (130°, 50

microns Hg) to yield white crystals of the aldehyde (0.72 g, 73%); mp 88-89° (lit. [131] mp 86°); nmr ($CDCl_3$) δ 9.83 (s, 1, CHO, 7.63-6.91 (m, 13, aromatic), 5.23 (s, 2, O-$C\underline{H}_2$-C_6H_5), and 5.20 ppm (s, 2, O-$C\underline{H}_2$-C_6H_5).

Attempts to repeat the above reaction resulted in decomposition of the product upon sublimation in most instances. Several modifications were attempted, as described below.

After filtrations of the chromium salts as described above, the organic liquid was refiltered through beds of $MgSO_4$ several times. The temperature of the sublimation was reduced to 110-115°. The amounts of isopropyl alcohol and Jones Reagent were varied. None of these efforts eliminated the unpredictable decomposition of the product.

(b). From alcohol ^{12}C-61 with chromium trioxide-graphite. The benzyl alcohol (^{12}C-61, 0.5 g, 1.6 mmoles) and chromium trioxide-graphite (Seloxcette, purchased from Alpha Products, Ventron Corporation, 0.29 g, 1.42 mmoles, 49% by weight) were heated to reflux in dry distilled toluene (20 ml). The heterogeneous reaction mixture was stirred at reflux for 72 hr under an atmosphere of N_2. Tlc on silical gel (benzene-$CHCl_3$, 2:1) revealed starting material. Accordingly, additional Seloxcette (0.2 g, 0.98 mmoles) was added, and the reaction allowed to proceed for an additional 48 hr. The mixture was then filtered through a bed of $MgSO_4$, and the solvent removed in vacuo to yield the pale brown crude product (0.34 g, 69%). A portion of the crude product (0.2 g) was sublimed (90°, 25 microns Hg) for 72 hr to yield the pure aldehyde (0.18 g, 98% of applied crude product): mp 85-87° (lit.[131] mp 86°);

nmr ($CDCl_3$) δ 9.83 (s, 1, CHO), 7.75-6.92 (m, 13, aromatic), 5.24 (s, 2, O-$C\underline{H}_2$-C_6H_5), and 5.21 ppm (s, 2, O-$C\underline{H}_2$-C_6H_5).

3,4-Dibenzyloxybenz-7-^{13}C-aldehyde (62). Without further purification, the crude alcohol (61, 3.8 g, 12.0 mmoles) and chromium trioxide-graphite (5.0 g, 27.5 mmoles, 49% by weight) were heated to reflux in dry toluene (75 ml) with stirring under an atmosphere of N_2 for 72 hr. The mixture was twice filtered through beds of $MgSO_4$, and after removing the solvent in vacuo, the residue was sublimed (120°, 50 microns Hg) to yield the white pure aldehyde (3.1 g, 81%): mp 84-86° (lit.[131] mp 86°); nmr ($CDCl_3$) δ 9.83 (d, 1, $J_{^{13}C-H}$ = 174 Hz, $^{13}C\underline{H}O$), 9.83 (s, 1, $^{12}C\underline{H}O$), 7.70-6.88 (m, 13, aromatic), 5.23 (s, 2, O-$C\underline{H}_2$-C_6H_5), and 5.20 ppm (s, 2, O-$C\underline{H}_2$-C_6H_5); cims (200°) m/e (rel intensity) 320 (100), 319(60), 230(50), 229(35), 228(28), 227(16), 91(12).

1-(3,4-Dibenzyloxyphenyl)-2-nitroprop-1-ene (^{12}C-63). The aldehyde (^{12}C-62, 1.0 g, 3.1 mmoles), ammonium acetate (0.12 g, 1.5 mmoles), and nitroethane (15 ml) were brought to reflux and the reaction allowed to proceed with stirring for 15 hr. Upon cooling and addition of anhydrous MeOH (50 ml), crystallization occurred. After further cooling (2 hr) over an ice bath, the dark yellow solids were filtered and dried to give the crude product (0.85 g, 72%): mp 116-117° (lit.[149] mp 117°). The crude product was recrystallized from MeOH to give the yellow phenylnitropropene (0.75 g, 64%): mp 117° (lit.[149] mp 117°); nmr ($CDCl_3$) δ 7.95 (broad s, 1, $C\underline{H}$=C), 7.68-7.27 (m, 10, aromatic), 6.97 (s, 3, aromatic from main phenyl ring), 5.17 (s, 4, O-$C\underline{H}_2$-C_6H_5), and 2.28 ppm (broad s, 3, C=$C\underline{H}_3$).

1-(3,4-Dibenzyloxyphenyl)-2-nitro-1-^{13}C-prop-1-ene (63). A mixture of the aldehyde (62, 10.0 g, 31.0 mmoles), ammonium acetate (1.2 g, 16.0 mmoles), and nitroethane (85 ml) was held at reflux with stirring and under an atmosphere of N_2 for 18 hr. Upon cooling, a solid was obtained which was crystallized from absolute MeOH to yield the crude nitrostyrene (63, 9.64 g, 83%). A second crop (0.5 g, 5%) was obtained from the concd filtrate. The following data applies to the first crop: mp 107-109° (lit.[149] mp 117°); nmr ($CDCl_3$) δ 7.99 (broad s, 1, ^{12}C$\underline{H}$=C), 7.99 (d of broad s, 1, $\underline{J}_{^{13}C-H}$ = 159 Hz, ^{13}C$\underline{H}$=C), 7.68-7.27 (m, 10, aromatic from benzyl substituents), 7.03 (s, 3, aromatic from main phenyl ring in ^{12}C molecule), 7.03 (d, 2, $\underline{J}_{^{13}C-H}$ = 3 Hz, ortho protons of main ring split by benzylic ^{13}C), 7.03 (s, 1, meta proton of main phenyl ring not split by benzylic ^{13}C), 5.23 (s, 2, O-C$\underline{H}_2$-C_6H_5), 5.21 (s, 2, O-C$\underline{H}_2$-C_6H_5), 2.32 (d, 3, $\underline{J}$ = 1 Hz, ^{12}CH=C(NO_2)C$\underline{H}_3$) and 2.32 ppm (d of d, 3, $J_{^{13}C-C-CH}$ = 4 Hz, ^{13}CH=C(NO_2)C$\underline{H}_3$); cims (230°) m/e (rel intensity) 377(100), 376(58), 287(27), 286(17), 285(7), 284(5), 181(10), 91(25).

1-(3,4-Dibenzyloxyphenyl)-2-propanone (^{12}C-64). Iron filings (20 mesh) were suspended with heating to reflux in glacial HOAc (100 ml) under an atmosphere of N_2 until a grey milky color was observed (30 min). To this was added the crude nitrostyrene (^{12}C-63, 25.0 g, 6.7 mmoles), after which the reaction was allowed to proceed with vigorous stirring for 2 hr. The hot reaction mixture was filtered through a bed of Celite and washed with hot glacial HOAc (350 ml). Distilled H_2O (1000 ml) was added to the filtrate and the milky orange solution extracted with CH_2Cl_2 (3 x 250 ml). The organic layer was washed with

5% $NaHCO_3$ (2 x 200 ml) and distilled H_2O (300 ml). The orange organic layer was dried ($MgSO_4$) and the solvent removed in vacuo to give an oil (26.24 g, greater than 100%). A small portion of the crude product was purified by molecular distillation (150°, 50 microns Hg) to give a pale yellow oil (80% of applied product): nmr ($CDCl_3$) δ 7.80-6.52 (m, 13, aromatic), 5.12 (s, 4, O-CH_2-C_6H_5), 3.53 (s, 2, CH_2-CO), and 2.03 ppm (s, 3, CO-CH_3); ir (CCl_4) 3090, 3065, 2940, 2870, and 1715 (C=O) cm^{-1}.

1-(3,4-Dibenzyloxyphenyl)-2-propanone oxime (67). To a small reaction flask was added $NH_2OH \cdot HCl$ (0.1 g, 1.4 mmoles) and ammonium acetate (0.14 g, 1.9 mmoles). After addition of distilled H_2O (1 ml) and 95% EtOH (5 ml), the ketone ^{12}C-64 was added (0.1 g, 0.3 mmoles). The solution was gently heated for 20 min and then cooled in an ice bath. The white solid was filtered, and the filtrate evaporated in vacuo. The solid residue was dissolved in H_2O and the solution extracted with benzene, the benzene layer dried ($MgSO_4$) and the solvent removed in vacuo, to give a pale yellow oil which could not be obtained in solid form: ir (CCl_4) 3250, 1680 (C=NOH), 960 cm^{-1}.

1-(3,4-Dibenzyloxyphenyl)-1-^{13}C-2-propanone (64). A mixture of iron filings (20 mesh, 12.55 g, 22.41 g atoms) and glacial HOAc (40 ml) was heated under reflux and an atmosphere of N_2 until a grey milky color was observed (30 min) at which time the crude nitrostyrene (63, 9.5 g, 2.5 mmoles) was added. After an additional 2 hr of heating and stirring, the hot mixture was filtered through a bed of Celite and washed with glacial HOAc (300 ml). Distilled H_2O (300 ml)

was added to the filtrate and the orange milky solution was extracted with CH_2Cl_2 (3 x 100 ml). The combined extracts were washed with 5% $NaHCO_3$ (3 x 100 ml) and H_2O (100 ml). The reddish-yellow organic layer was dried ($MgSO_4$), the solvent removed in vacuo, and the crude product (8.58 g, 98%) purified by molecular distillation (160°, 50 microns Hg) to yield a pale yellow oil (7.5 g, 86%): nmr ($CDCl_3$) δ 7.81-6.54 (m, 13, aromatic), 5.13 (s, 4, O-CH_2-C_6H_5), 3.53 (s, 2, $^{12}CH_2$-CO), 3.53 (d, 2, $J_{^{13}C-H}$ = 126 Hz, $^{13}CH_2$-CO), and 2.03 ppm (s, 3, CO-CH_3); cims (240°) m/e (rel intensity) 348(100), 347(64), 330(9), 329(6), 258(33), 257(22), 256(10), 255(6), 181(61), 180(47), 179(33), 91(39).

Anal. Calcd for $^{12}C_{22.36}{}^{13}C_{64}H_{22}O_3$; C, 79.78; H, 6.39. Found: C, 79.54; H, 6.36.

5-Methyl-5-(3,4-dibenzyloxy-7-^{13}C-benzyl) hydantoin (65). A mixture of the ketone (64, 7.5 g, 21.7 mmoles), absolute EtOH (150 ml), distilled H_2O (40 ml), KCN (1.8 g, 28.0 mmoles), and ammonium carbonate (18.25 g, 190 mmoles) was heated to reflux with stirring, and the reaction allowed to proceed for 6.5 hr. During this period solids which formed in the condenser were washed down with additional H_2O (10 ml). After standing 15 hr at room temperature, additional H_2O (20 ml) was added and then most of the EtOH removed in vacuo. The white solids in the remaining aqueous solution were vacuum filtered, washed with H_2O, and dried for 12 hr on the vacuum filter to yield the crude product (65, 8.57 g, 95%): mp 182-184° (lit.[149] mp 188°); nmr (DMSO-d_6) δ 7.98 (s, 1, CO-NH-CO), 7.22-6.60 (m, 13, aromatic), 5.12 (s, 4, O-CH_2-C_6H_5), 2.91 and 2.72 (AB quartet, 2, J_{AB} = 15 Hz, Ar-$^{12}CH_2$-C),

2.92 and 2.72 (d of AB quartet, 2, $J_{^{13}C-H}$ = 128 Hz, Ar-$^{13}CH_2$-C), and 1.37 ppm (s, 3, C-CH_3). The crude hydantoin, in the case of the 65 atom percent enriched compound, was then diluted by the formula of 72.5% crude ^{13}C-labeled hydantoin and 27.5% recrystallized ^{12}C-hydantoin. This mixture was then recrystallized from absolute EtOH with a recovery of 97%, for as estimated yield of the pure ^{13}C-labeled hydantoin of 82%: cims (250°) m/e (rel intensity) 418(100), 417(100), 328 (43), 327(46), 326(17), 325(16), 250(30), 249(30), 238(26), 237(27), 181(46), 91(46).

3-(3,4-Dibenzyloxyphenyl)-2-methylalanine (^{12}C-66). (a). From hydantoin (^{12}C-65) with NaOH and diglyme. A mixture of hydantoin (^{12}C-65, 3.0 g, 7.2 mmoles) in distilled H_2O (6 ml) and diglyme (4 ml) containing NaOH (6.0 g, 150 mmoles) was heated at reflux with stirring for 24 hr. Tlc (silica gel, $CHCl_3$/ether, 2:1) indicated the presence of starting material. Thus the reaction was allowed to continue for a total of 36 hr. The hot heterogeneous reaction mixture was transferred to a separatory funnel and the hot organic layer drawn off. Crystals separated from the cooled organic layer and ether (50 ml) was added. The mixture was filtered, and the solids washed with additional ether (25 ml). The solids were then dissolved in warm (40°) distilled H_2O (60 ml), the pH adjusted to 5.5 with 5% HOAc, and the mixture allowed to cool. The thick white solids were filtered and washed with cold H_2O. The solids were then suspended in absolute EtOH (80 ml) and heated until finely dispersed. The mixture was cooled i n an ice bath and the solids filtered, washed with cold ether and absolute EtOH, and dried to yield the amino acid (^{12}C-66, 1.95 g, 70%):

mp 220-230° with dec (lit.[149] mp 220-230° with dec).

(b). From hydantoin (^{12}C-65) with $Ba(OH)_2$ and ethylene glycol. To a flask containing the hydantoin (^{12}C-65, 2.0 g, 4.8 mmoles) was added distilled H_2O (40 ml) and ethylene glycol (20 ml). $Ba(OH)_2$ (25.0 g, 74.2 mmoles) was added, and the solution heated to reflux. The solids would not dissolve, thus additional H_2O (30 ml) and ethylene glycol (20 ml) were added to dissolve the solids. The reaction was allowed to proceed until evolution of NH_3 and tlc (silica gel, $CHCl_3$/ether, 2:1) indicated the reaction had proceeded to completion (72 hr). The pH of the reaction mixture was then adjusted to 1 with concd HCl, and H_2O (1000 ml) added to the milky solution. The pH was then readjusted to 5.5 with 15% NaOH. The mixture was cooled, the solids filtered, and dried on the filter to give the crude product (1.75 g, 93%): mp 205-210° (lit.[149] mp 220-230° with dec). A small portion of these solids were recrystallized from absolute EtOH to give the pure acid ^{12}C-66: mp 219-229° with dec (lit.[149] mp 219-229° with dec).

Variations of the above procedure included the use of EtOH and triethylene glycol as cosolvent with H_2O. Yields varied greatly, and often the hydantoin and/or the amino acid product formed a lacquer-like solid in the reaction flask from which the product could not be isolated. Thus, these methods were not further pursued.

3-(3,4-Dibenzyloxyphenyl)-3-^{13}C-2-methylalanine (66). A mixture of hydantoin (65, 0.87 g, 2.1 mmoles) in distilled H_2O (4 ml) and diglyme (2 ml) containing NaOH (2.0 g, 50 mmoles) was heated to reflux with stirring for 36 hr. The hot heterogeneous reaction mixture was trans-

ferred to a separatory funnel, and allowed to cool (40^{o}). The upper yellow organic layer was drawn off. Crystals separated from the cooled organic layer, and to these was added ether (20 ml). The solids were filtered and washed with additional ether (10 ml). The solids were then dissolved in warm (45^{o}) H_2O, and after filtering, the pH adjusted to 5.5 with 5% HOAc. After cooling, the thick white solids were filtered and washed with cold H_2O (10 ml). The solids were then suspended in absolute EtOH (30 ml) and heated until finely dispersed. The mixture was cooled in an ice bath, the solids filtered, and washed with small amounts of cold ether and absolute EtOH. The solids were dried on the vacuum filter to yield the crude amino acid 66 (0.56 g, 68%): mp 219-231^{o} with dec (lit.[149] mp 220-230^{o} with dec); cims (285^{o}) m/e (rel intensity) 393(100), 392(93), 257(15), 256(19), 255(11), 254(8).

3-(3,4-Dihydroxyphenyl)-3-^{13}C-2-methylalanine Hydrochloride (^{13}C-1). To the reaction flask containing the benzyloxy amino acid (66, 0.56 g, 1.4 mmoles) was added benzene (10 ml) and concd HCl (10 ml). The system was purged with N_2 and the heterogeneous reaction mixture vigorously stirred at room temperature for 18 hr. The solvents were removed in vacuo to yield an extremely hygroscopic white solid (0.35 g, 100%): nmr (D_2O-DSS) δ 7.23-6.67 (m, 3, aromatic), 3.32 and 3.12 (AB quartet, 2, J_{AB} = 15 Hz, Ar-$^{12}CH_2$-C), 3.32 and 3.12 (d of AB quartet, 2, $J_{^{13}C\text{-}H}$ = 133 Hz, Ar-$^{13}CH_2$-C), and 1.77 ppm (s, 3, C-CH_3); cims (290^{o}) m/e (rel intensity 213(100), 212(100), 167(42), 166(44), 124(36), 123(38).

S-(-)-3-(3,4-Dihydroxyphenyl)-2-methylalanine (1a). The hydrochloride salt of the amino acid 1a (0.34 g, 1.4 mmoles) was dissolved in N_2 purged distilled H_2O (1 ml), and the pale yellow solution purged with N_2. To this solution was slowly added a 40-50% excess of N_2 purged triethylamine (0.3 ml, 0.7255 g/ml, 2.2 mmoles), the whole system being continuously purged with N_2 during the triethylamine addition, and also cooled on an ice bath. The addition was stopped when the pH reached 8, at which time solids gradually formed from the brown solution. After 5 min the solids were filtered under a cone of N_2, rinsed with a min volume of ice-cold N_2 purged H_2O, and dried in vacuo to yield the white amino acid (68 mg, 23%): mp 280-300° with dec (lit.[155] mp 290-300° with dec).

RS-3-(3,4-Dihydroxyphenyl)-2-methylalanine (1). The hydrochloride salt of the amino acid 1 (0.89 g, 3.6 mmoles) was dissolved in N_2 purged distilled H_2O (1.2 ml), and the pale yellow solution purged with N_2 and cooled in an ice bath. To this solution was slowly added the molar equivt of triethylamine (0.5 ml, 0.7255 g/ml, 3.6 mmoles). The pH of the resulting solution was found to be 4. Therefore, additional triethylamine was added (total 0.8 ml, 5.8 mmoles), until the pH was 8. However, no solids were observed in the ice-cold brown solution after 12 hr in the cold. After 24 hr, a small amount of grey solids were filtered, and the solids found to be very hygroscopic. This reaction was repeated numberous times, without success.

RS-N-Acetyl-3-(3,4-diacetoxyphenyl)-2-methylalanine (^{12}C-69). (a). From amino acid 1·HCl with heating for 3 hr. A solution of RS-alpha-methyldopa hydrochloride (1·HCl, 1.35 g, 5.5 mmoles), acetic anhydride

(13 ml), and anhydrous pyridine (6 ml) was heated with stirring to a bath temperature of 92°. After 3 hr, the solvents were removed in vacuo to yield a pale brown-yellow oil. To this oil was added aceonte (5 ml), 3N HCl (5 ml), and distilled H_2O (20 ml). The mixture was left in an ice bath for 5 hr, but not solids appeared. The insoluble oily material was reisolated and submitted to cims analysis: cims (240°) m/e (rel intensity) 338(12), 296(52), 254(100), 250(14).* The reaction was repeated at room temperature for 48 hr, and again, no solids product was isolated.

*The cims analysis suggests minimal formation of the tri-acetyl product (MH+ = 338), with substantial amounts of mono-acetyl (MH+ = 296) and di-acetyl (MH+ = 254) impurities, suggesting incomplete acetylation.

(b). From amino acid 1a·HCl with heating for 15 hr. A solution of S-alpha-methyldopa hydrochloride (1a·HCl, 1.35 g, 5.5 mmoles), acetic anhyride (6 ml), and pyridine (6 ml) was heated to reflux under an atmosphere of N_2, and the reaction allowed to proceed for 15 hr. The solvents were removed in vacuo . Addition of toluene (15 ml) followed by removal of solvent in vacuo was repeated twice to remove traces of pyridine and acetic anhydride. The brown viscuous oil was cooled and triturated with 2N HCl (2 ml) which gave an off-white solid. The crude product was cooled for 2 hr, and extracted with EtOAc (3 x 25 ml). The combined extracts were washed with distilled H_2O (15 ml), the organic layer dried (Na_2SO_4), and the solvent removed to yield the crude product (^{12}C-69a, 1.6 g, 85%): mp 173-175° (lit.[155] mp 181-183°); nmr (pyr-d_5) δ 7.37 (s, 1, aromatic), 7.27 (s, 2, aromatic), 3.99 and

3.58 (AB quartet, 2, J_{AB} = 18.8 Hz, Ar-CH_2-C), 2.23 (s, 3, O-CO-CH_3), 2.20 (s, 3, O-CO-CH_3), 2.04 (s, 3, N-CO-CH_3), and 1.69 ppm (s, 3, C-CH_3); cims (240°) m/e (rel intensity) 338(100), 320(29), 292(6), 278(7), 250(9).

(c). From amino acid 1 without heating for 48 hr. A solution of alpha-methyldopa (1, 1.0 g, 4.7 mmoles), freshly distilled pyridine (8 ml), freshly distilled acetic anhydride (8 ml) was allowed to sit at room temperature in a reaction flask equipped with a drying tube ($CaSO_4$) for 48 hr. The solvents were then removed in vacuo. Addition of toluene (10 ml) followed by removal of solvent in vacuo was repeated twice to remove traces of pyridine and acetic anhydride. The brown viscuous oil was cooled and triturated with 2N HCl (2 ml) to give an off-white solid (after scratching with a glass rod). The crude product was cooled for 1 hr, filtered, washed with distilled H_2O, and dried on the filter pad (12 hr) to yield the crude product ^{12}C-69 (1.2 g, 75%): mp 192-194° (lit.[155] mp 197-199°).

RS-N-Acetyl-3-(3,4-diacetoxyphenyl)-3-^{13}C-2-methylalanine (69). To the flask containing the ^{13}C-labeled amino acid ^{13}C-1 as the hydrochloride salt (2.53 g, 10.2 mmoles) was added freshly distilled acetic anhydride (11 ml) and freshly distilled pyridine (11 ml). The mixture was heated to reflux under an atmosphere of N_2, and the reaction allowed to proceed for 13 hr. The solvents were removed in vacuo. Addition of toluene (20 ml) and removal of solvent in vacuo was repeated three times to remove traces of pyridine and acetic anhydride. The brown, slightly viscuous oil was placed under vacuum for 20 min, then cooled and triturated with 2N HCl (4 ml) to yield a mixture of solids and a

brown oil. The crude product was cooled (0-5°) for 3 hr, extracted with EtOAc (4x 50 ml), and the combined extracts washed with distilled H_2O (2 x 25 ml). The organic layer was dried (Na_2SO_4) and the solvent removed *in vacuo* to yield the brown crude product (3.28 g, 95%); mp 187-190°. The solids were recrystallized in a min volume of acetone at room temperature to yield the white product (1.85 g, 54%): mp 191-193° (lit.[155] mp 197-199°); nmr (pyr-d_5) δ 7.43-7.02 (m, 3, aromatic), 3.99 and 3.58 (AB quartet, 2, J_{AB} = 18.8 Hz, Ar-$^{12}CH_2$-C), 3.99 and 3.58 (d of AB quartet, 2, $J_{^{13}C-H}$ = 132 Hz, Ar-$^{13}CH_2$-C), 2.23 (s, 3, O-CO-CH_3), 2.20 (s, 3, O-CO-CH_3), 2.04 (s, 3, N-CO-CH_3), 1.69 (s, 3, Ar-^{12}C-C-CH_3), and 1.69 ppm (d, 3, $J_{^{13}C-C-CH}$ = 3.5 Hz, Ar-^{13}C-C-CH_3); cims (280°) *m/e* (rel intensity) 339(100), 338(11), 321(89), 320(7), 297(33), 296(4), 279(17), 278(4), 251(31), 250(4), 182(81), 181(4).

Quinine Salts of RS-N-Acetyl-3-(3,4-diacetoxyphenyl)-3-^{13}C-2-methylalanine 70a and 70 b. Quinine (3.35 g, 10.3 mmoles) and the triacetyl ^{13}C-labeled amino acid (69, 2.96 g, 8.76 mmoles) were dissolved in acetone (30 ml) , with stirring and mild heating. Additional stirring (10 min) resulted in the appearance of white solids. The solution was cooled (0-5°) for 3hr, the solids collected and rinsed with ice-cold acetone, and dried *in vacuo* to yield the S-triacetyl quinine salt 70a (1.85 g, 64% yield of the S-triacetyl diastereomer). The soluble R-triacetyl diastereomer 70b was recovered by removal of solvent *in vacuo* from the mother liquor.

Combined reaction products from several reactions were slurried in ice-cold acetone (20 ml) to further purify the salt. The solution was filtered, the solids rinsed with ice-cold acetone, and dried *in vacuo* to yield the S-triacetyl quinine salt 70a (3.08 g, 82%); mp

155-170° (lit.[155] mp 164-166°).

S-(-)-N-Acetyl-3-(3,4-diacetoxyphenyl)-3-^{13}C-2-methylalanine (69a). The S-triacetyl ^{13}C-labeled quinine salt 70a (3.08g, 4.7 mmoles) was dissolved in distilled H_2O (6 ml) and 2N HCl (9 ml) with stirring of the sticky solution. The yellow liquid was immediately extracted with EtOAc (4 x 30 ml), washed with 2N HCl (11 ml), and dried ($MgSO_4$). The solvents were evaporated in vacuo to yield a white solid (1.60 g, 103%): mp 178-179° (lit.[155] mp 181-183°); $[\alpha]_{589}^{25}$ -64.86° (c 2, 96% EtOH), (lit.[155] $[\alpha]_{589}^{25}$ -74.5°).

Quinine Salt of S-(-)-N-Acetyl-3-(3,4-diacetoxyphenyl)-3-^{13}C-2-methylalanine 70a. The S-(-)-triacetyl compound (69a, 1.66g. 4.9 mmoles) was treated with quinine (1.86 g, 5.7 mmoles) as described above for the racemic triacetyl compound 69, to yield the S-triacetyl quinine salt diastereomer (2.85 g, 88%).

S-(-)-N-Acetyl-3-(3,4-diacetoxyphenyl)-3-^{13}C-2-methylalanine (69a). The retreated quinine salt 70a (2.85 g, 4.3 mmoles) was treated with distilled H_2O (6 ml) and 2N HCl (9 ml) as described above to yield the resolved ^{13}C-triacetyl compound (69a, 1.3 g, 96%): mp 177-179°, (lit.[155] mp 181-183°); $[\alpha]_{589}^{25}$ -68.77° (c 2, 96% EtOH), (lit.[155] $[\alpha]_{589}^{25}$ -74.5°).

S-(-)-3-(3,4-Dihydroxyphenyl)-3-^{13}C-2-methylalanine hydrochloride (1a·HCl). To the flask containing the S-triacetyl ^{13}C-labeled amino acid (69a, 1.39 g, 2.1 mmoles) was added 6N HCl (35 ml). The solution was brought to reflux under an atmosphere of N_2 and the reaction allowed

to proceed for 2.5 hr. The solvent was removed in vacuo to yield the extremely hygroscopic product (0.97 g, 95%). This compound was not characterized at all, but directly treated to liberate the free amino acid S-(-)-^{13}C-1a as described below.

S-(-)-3-(3,4-Dihyroxyphenyl)-3-^{13}C-2-methylalanine (^{13}C-1a). The hydrochloride salt of ^{13}C-1a (0.97 g, 3.9 mmoles) was taken up in N_2 purged distilled H_2O (2 ml). The pale brown syrupy liquid was transferred to a tapered test tube and cooled in an ice bath under an atmosphere of N_2. To the N_2 purged solution was slowly added a 10% molar excess of triethylamine (0.6 ml, 0.7225 g/ml, 4.3 mmoles). The pH of the well-mixed solution was found to be 6. The solution was left to cool for 3 hr in an ice bath, and the solids filtered under an atmosphere of N_2. The white solids were washed with ice-cold N_2 purged H_2O (3 ml) and dried in vacuo to yield the desired S-(-) enantiomer of the ^{13}C-labeled alpha-methyldopa ^{13}C-1a (0.72 g, 87%): mp 290-300^{o} with dec (lit.[155] mp 290-300^{o} with dec).

Preparation of 3-(3,4-Dimethoxyphenyl)-3-^{13}C-2-methylalanine Methyl Ester (^{13}C-75) for Glpc Enantiomeric Analysis. To a small reaction flask containing the ^{13}C-labeled alpha-methyldopa (^{13}C-1, 10.2 mg, 0.047 mmoles) was added anhydrous MeOH (2 ml). To this was added ethereal diazomethane (CH_2N_2, 5 ml, approximately 0.32M). The flask was loosely stoppered, protected from light, and left at room temperature. At 2, 4, and 6 days, additional CH_2N_2 (1 ml/addition) was added. After 7 days, the solvent was removed in vacuo, and the yellow oil frozen (-10^{o}) until further derivatization.

Studies with unlabeled 75 by cims analysis indicated incomplete

methylation (m/e 226 and 240) as well as some N-methylation (m/e 268 and 280). While the volatilities of these compounds varies, it was estimated that the optimum production of 75 was observed at 6-7 days in the room temperature reaction mixture. Gc/ms analysis of a 7 day methylation reaction products mixture was undertaken. With a 3% OV-17 column (column temp 200°). the tri-methyl derivative 75 had a retention time of 3.0 min, the tetra-methyl (mono-N-methyl) derivative 3.5 min, and the penta-methyl (N,N-dimethyl) derivative 4.7 min. Ms analysis (source temp 200°) indicated a ratio of approximately 5:1:0.2 for these derivatives, respectively (Table IV in text).

Preparation of the N-pentafluorobenzoyl-S-prolyl-1-imidazolide (72, PFBPI) Derivative of 3-(3,4-Dimethoxyphenyl)-2-methylalanine Methyl Ester (75). To 10 mg of the methylated alpha-methyldopa mixture dissolved in 0.1 ml $MeCl_2$ was added the PFBPI (39 mg, 0.11 mmoles) dissolved in 0.1 ml $MeCl_2$. The mixture was left for 2 days at room temperature. Gc analysis (3% OV-1 and 3% OV-17, temp 200-280°) revealed a complex mixture with no evidence of separation of the diasteromeric derivatives. The gc analysis was repeated after washing with acid (0.1N HCl) and base (0.1N NaOH) with similar results. Cims analysis did reveal some formation of a species with the proper MW for the derivative (MH^+_2 = 545).

Preparation of the Alpha-methoxy-alpha-trifluoromethylphenyl-aceticacid Chloride (MTP-Cl, 76) Derivative of 3-(3,4-Dimethoxyphenyl-2-methylalanine Methyl Ester (75) or the 3-^{13}C Analog (^{13}C-75). To 1 mg of the methylated alpha-methyldopa or ^{13}C-alpha-methyldopa mixture dissolved in 0.1 ml $EtCl_2$ was added 0.1 ml of the MTP-Cl reagent

solution (1 mg MTP-Cl in 10 microl $EtCl_2$, 10 mg of MTP-Cl_2) followed by 0.02 ml pyridine. The solution was heated (70^o) for 30 min. After cooling in an ice bath, HCl (1N, 1 ml) was added, and the mixture shaken for 5 min. The solution was then centrifuged (3000g, 10 min), the aqueous layer aspirated off, and saturated Na_2CO_3 (1 ml plus 0.3 ml $EtCl_2$) added. The mixture was again shaken 5 min, centrifuged 10 min, and the bottom layer submitted to gc/ms analysis.

Gc/ms analysis (column temp 235^o, 3% OV-17 and ms source temp 200^o) indicated a retention time of 12.5 min for the derivative of S-alpha-methyldopa and 13.75 min for the derivative of R-alpha-methyldopa (Figures 24,25, and 26). Ms analysis of the gc peaks confirmed the presence of the derivatives ($M^+ = 468$).

PART III

ANALYTICAL PROCEDURES

The metabolic studies with alpha-methyldopa we envisioned required both sensitive and specific assay procedures. The analyses of 1 and its metabolites discussed in Part I consisted primarily of tlc and paper chromatography, ion exchange chromatography, and detection by spectrophotofluorimetric assay. Careful review of these methods made it clear that for the anticipated small quantities of structurally related molecules in which we were interested, these methods were not satisfactory. We required not only sensitivity and specificity, but also an assay which minimized handling of the chemically sensitive catecholamines and catecholamino acids. We felt that methods based on mass spectrometry (ms) offered the best combinations of these features.

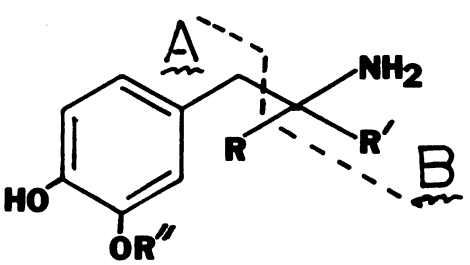
The recent literature contains many reports utilizing various ms applications for metabolism studies. A survey of the pertinent literature established that the majority of such studies employed electron impact mass spectrometry (eims). Almost all of the eims studies utilized gc separation of the samples prior to their introduction into the source of the mass spectrometer (gc/ms), with selected ion monitoring of the gc effluent. In such studies, the mass spectrometer was used as a sensitive detector for the gc effluent, monitoring either the total ion current or ions specifically associated with the molecules of interest in the sample.

Using selected ion monitoring gc/ms methods, Claeys et al[172] detected epinine in the adrenal medulla, Koslow and Green[173] identified indoles in the rat pineal gland, and Cattabeni et al[174] detected serotonin and its metabolites in the rat pineal gland. Selected ion monitoring gc/ms techniques have been used for the analysis of catecholamines, including dopamine and norepinephrine.[175,176,177] The

metabolism of such related molecules as phentermine[178] and carbidopa (8)[179] have been studied with these techniques. Stable isotopically labeled internal standards are also frequently employed in such studies, both for qualitative and quantitative purposes. The combined use of gc/ms and stable isotope labeling has been reviewed for metabolism studies,[180] including applications with psychoactive drugs[181] and catecholamines.[182]

While these assays are very sensitive and specific, they did pose several problems for the studies we envisioned. As a consequence of the high energy imparted to the sample molecules during eims, extensive fragmentation occurs and necessitates monitoring fragment ions rather than the molecular ions. The eims spectrum of almost all molecules display many fragment ion signals, especially at the lower masses. If a particular assay is concerned with the detection of several chemically related molecules in one sample, fragmentation may give rise to ions of the same mass. If these species happen to be major fragments (well suited for ion monitoring), the assay may be dependent upon complete separation of the molecules of interest prior to introduction into the mass spectrometer.

Catecholamines (the group with which this thesis is concerned) present an excellent example of fragmentation difficulties with gc/ms analysis. One of the primary modes of fragmentation in catecholamines is α-β cleavage to give fragments A and B.[183]



Many catecholamine assays[172,175,176] utilize these fragments for gc/ms analysis. It is apparant that detection of fragment A would not distinguish structural differences in the side chain of related catecholamines, and that the detection of fragment B would not distinguish structural differences in the ring portion of related catecholamines. Therefore, one must either monitor several peaks for each molecule, which along with internal standards will greatly increase the necessary monitoring capability, or completely separate the related molecules by gc. The latter approach represents a limitation similar to that noted in fluorimetric assay procedures--the analytical assay is actually dependent on prior separation techniques. Our interest in the simultaneous assay of both endogenous and alpha-methylamines (which with internal standards total eight molecules as discussed in the metabolism sections below), and our concern over the efficiency of separation of these closely related molecules led us to examine alternative ms analytical procedures.

Cims[184] has found growing application in drug metabolism studies.[185,186,187,188,189,190] As will be explained below, the primary advantage cims is the ability to observe the parent ions for most molecules. Thus, ion monitoring and prior gc separation (gc/ms) are often unnecessary. The ability to observe parent ions has also led to a widespread use of stable isotopically labeled internal standards, coupled with direct cims analysis of metabolites. Thus, warfarin,[187] amphetamine derivatives,[186] lidocaine,[190] quinine,[190] and steroids[189] have all been studied by the combined stable isotope-cims analytical procedures.

Cims differs fundamentally from eims in the method of ionization of the sample molecules. In eims, a high energy (70 eV) electron beam interacts directly with the sample molecules. The radical ion products of these interactions contain considerable excess energy which may be expended by further bond breaking.[184,191] Thus, the parent ion may often be observed as a very minor component of the spectrum. In cims, the electron beam interacts with a high pressure (0.5-1.0 torr) reactant gas rather than the sample molecules, since the latter are present at a much lower concentration (1 part per 1000). The ionized reactant gas undergoes further ion molecule reactions, forming a steady state plasma which then reacts with the sample molecules.[184,191] This process results in molecular ions of much lower energy and hence a marked reduction in the tendency of the parent ions to fragment. As a result, one obtains greater parent ion intensities, the parent ion often being the base peak of the spectrum.

To briefly illustrate the molecular events associated with cims ionization, the sequence of events occuring upon reaction of the electron beam with the reactant gas (in this case isobutane) is illustrated in Figure 27.[192,193] The reactive species $\underset{\sim}{C}$ may act either as a hydride ion extractor to yield M+ (where MH = sample molecule), or more commonly, as a proton donor to yield MH_2^+. In a study of abused drugs, Milne *et al*[194] reported that 75% of the drugs analyzed by cims gave a single protenated molecular ion (MH_2^+) in the cims spectrum.

With the ability of cims to produce parent ions, we proceeded to develop analytical procedures in which complex mixtures were directly introduced into the ion source, and the amine or amino acid constituents

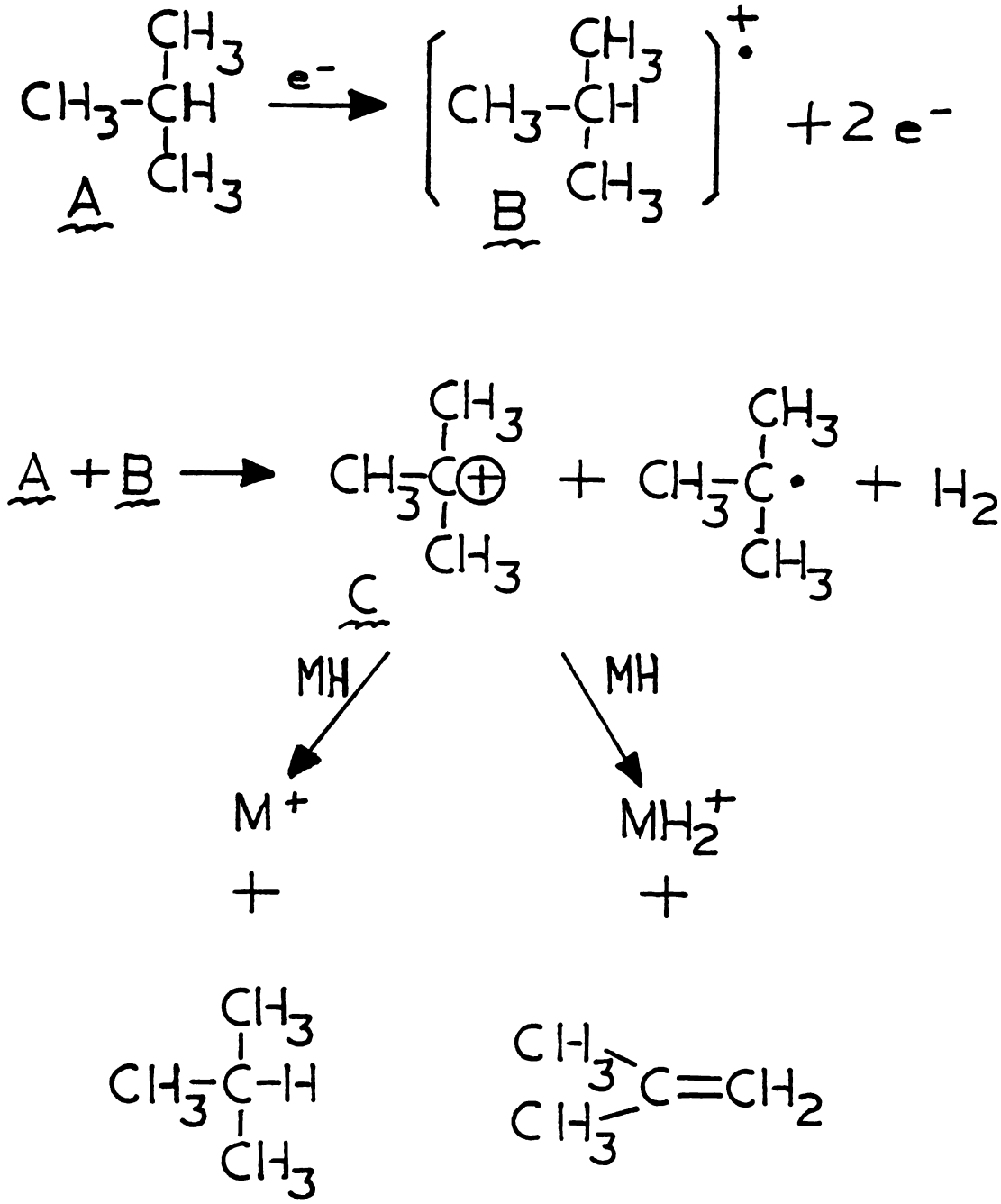
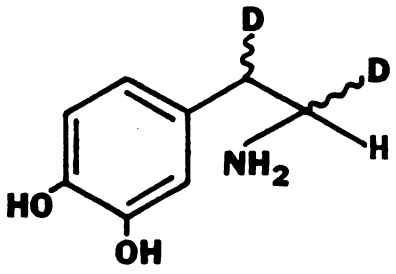
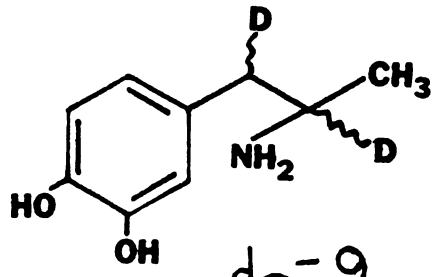


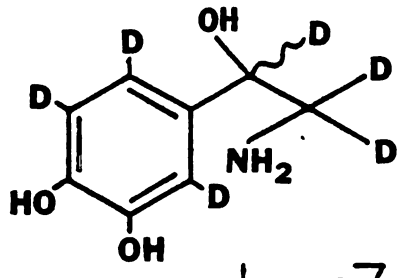
Figure 27. Molecular events associated with cims ionization with isobutane as the reactant gas (Ref. 192, 193).



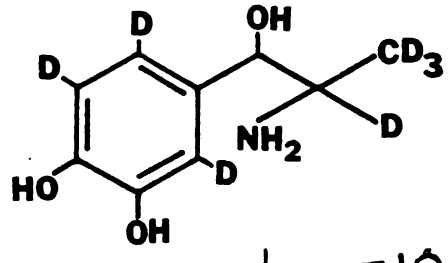
d_2-6



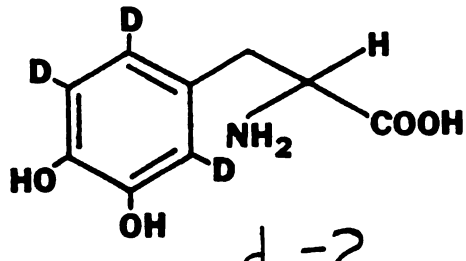
d_2-9



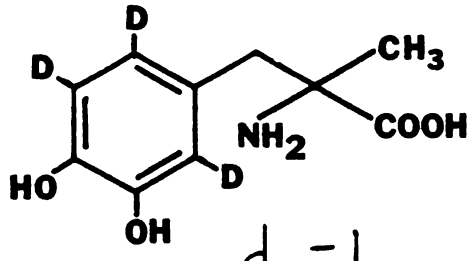
$d_{6,5}$-7



$d_{7,6,5}$-10



d_3-2



d_3-1

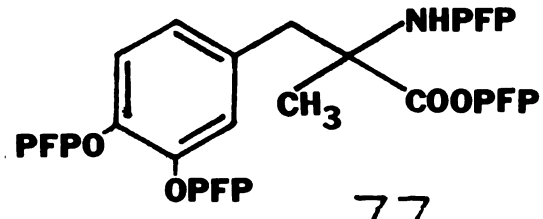
Figure 28. Structures of deuterium labeled internal standard amines and amino acids. Details of structure assignments for $d_{6,5}$-7 and $d_{7,6,5}$-10 will be published elsewhere (Ref. 226).

were analyzed as their protenated molecular ions (MH_2^+). In this way the chemical ionization mass spectrometer was used both to separate and detect the components of interest in the biological mixtures. The simultaneous use of labeled compounds (deuterium and ^{13}C) as internal standards and/or labeled substrates also allowed quantitative studies with the amines and amino acids in biological samples.

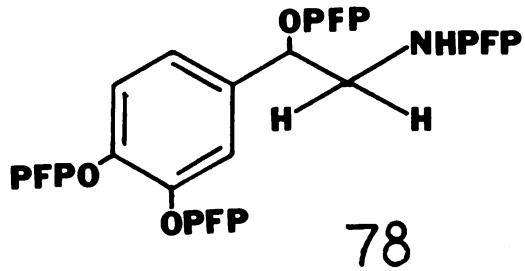
We wished to apply these methods to the determination of alpha-methyldopa, alpha-methyldopamine (9), alpha-methylnorepinephrine (10), and their endogenous counterparts DOPA, dopamine (6), and norepinephrine (7). The internal standards used for the in vivo determination of amine metabolites were d_2-dopamine, d_2-alpha-methyldopamine, $d_{6,5}$-norepinephrine, and $d_{7,6,5}$-alpha-methylnorepinephrine (Figure 28), all synthesized in our laboratory.[195,196] D_3-alpha-methyldopa was used for in vivo amino acid studies (with problems discussed in the metabolism section), and ^{13}C-alpha-methyldopa[130] employed for both in vivo and in vitro studies.

In gc and ms procedures, catecholamines and related compounds are routinely derivatized prior to analysis in order to provide increased volatility, chemical stability, and for ms, ions a higher mass regions where fewer interfering ions will be found. Numerous derivatives and derivatizing reagents have been studied for these purposes. Derivatives commonly used are trimethylsilyl (TMS),[172,197] trifluoroacetyl (TFA),[172,176,197] pentafluoropropionyl (PFP),[172,176,197,198] and heptafluorobutyrl (HFB).[176,197,198] These derivatives are formed through the oxygen and nitrogen atoms of the catechol and side chain amine functions, respectively. While many literature reports have appeared describing the use of these and other derivatives, pentafluoropropionic anhydride (PFPA)

has been found to be particularly suitable for gc and ms catecholamine analysis, in terms of the previously mentioned criteria.[172,176,197,198] We therefore selected the PFP derivatives for use in our cims studies. Two of these derivatives, formed from alpha-methyldopa (77) and norepinephrine (78) are shown below. The other amines and DOPA form derivatives in a similar manner.



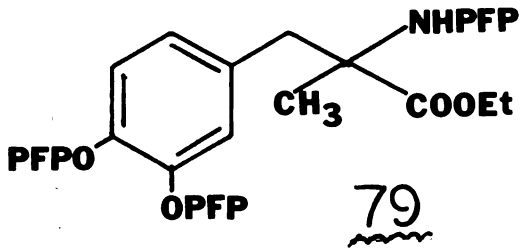
77



78

One further derivatization procedure was adopted in our studies. Two of the cited reports on derivatization[176,197] stated that samples containing amino acids were first treated with ethanolic HCl or diazomethane to yield the corresponding esters. Neither reference discussed the rationale for adopting such a procedure, but reported the gc and ms characteristics for the esters after derivatization with PFPA.

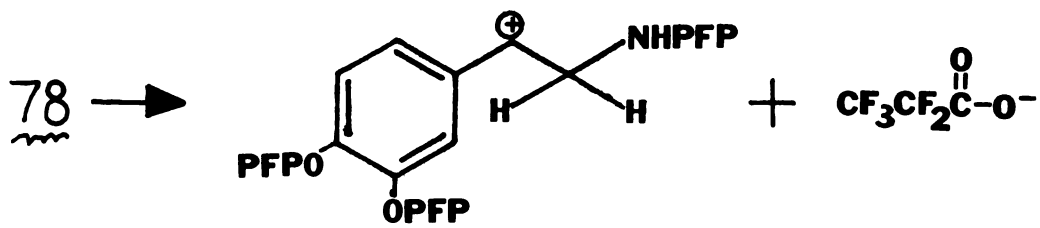
Preliminary studies on the derivatization procedures in our laboratory with DOPA and alpha-methyldopa by Curt Freed[196] demonstrated that esterification prior to treatment with PFPA gave a greatly enhanced intensity for the parent ion (MH_2^+) of the corresponding derivative 79.



79

It seems likely that the greater abundance of the parent ion is due to the greater stability of the ethyl ester 79 as compared to the mixed anhydride obtained upon direct PFPA derivatization of alpha-methyldopa (77) or DOPA. Further, the OPFP group is a particularly good leaving group.

In a similar manner, a study of the derivatization of norepinephrine[176] reported that treatment of 7 with methanolic HCl prior to PFPA derivatization gave a compound with longer gc retention time than the direct PFP product. The methanolic HCl treated derivative mass spectrum corresponded to the beta-methyl ether-PFP derivative. Again, the authors made no comments with regard to the importance of such treatment in the analytical assay of these compounds. Further studies by Curt Freed[196] with norepinephrine and alpha-methylnorepinephrine again showed that esterification with ethanolic HCl (we used ethanol in all cases rather than methanol, to avoid ambiguity between endogenous amines and amino acids and the alpha-methyl analogs) resulted in significantly higher proportions of parent ions in the ci mass spectrum of derivatized norepinephrine and alpha-methylnorepinephrine. It is likely that the PFP derivative 78 is much less stable due not only to the OPFP leaving group, but also due to the particularly stable carbonium ion formed in the thermal process:



Based on these studies, we treated all amine and amino acid samples with ethanolic HCl prior to PFPA derivatization and cims analysis.

Having selected the derivatization scheme, we then undertook a study of the derivatization and cims analysis of the amines and amino acids (and their internal standards) in the amounts anticipated for our *in vitro* and *in vivo* metabolism studies. These efforts allowed us to analyze the effectiveness of the methods for our particular studies, examine the spectra of the pure compounds and internal standard mixtures, and have the spectra for comparison with those obtained in the metabolism studies. Several examples of the cims spectra of the pure compounds and mixtures are presented here for the same reasons.

A spectrum typical of the amines is shown in Figure 29, a mixture of d_2- and d_0-alpha-methyldopamine, at m/e 620-450. The mixture was treated with ethanolic HCl (2N, 1 hr, 60°), the solvent removed with a stream of N_2, and the residue treated with PFPA (20 min, 60°). In the region of interest (the mixtures of amines gave parent ions over the range 657-592), there were no significant ion intensities. The only significant fragment (m/e 462,460) is due either to the loss of one PFP fragment (-146), or incomplete acylation in the derivatization procedure. To further emphasize this point, Figure 30 illustrates a mixture of all four labeled amine internal standards (d_2-dopamine MH_2^+ = 594, d_2-alpha-methyldopamine MH_2^+ = 608, $d_{6,5}$-norepinephrine MH_2^+ = 642,641, $d_{7,6,5}$-alpha-methylnorepinephrine MH_2^+ = 657,656,655). There are no interfering ion intensities, and only a few fragments in the spectrum from m/e 660-550.

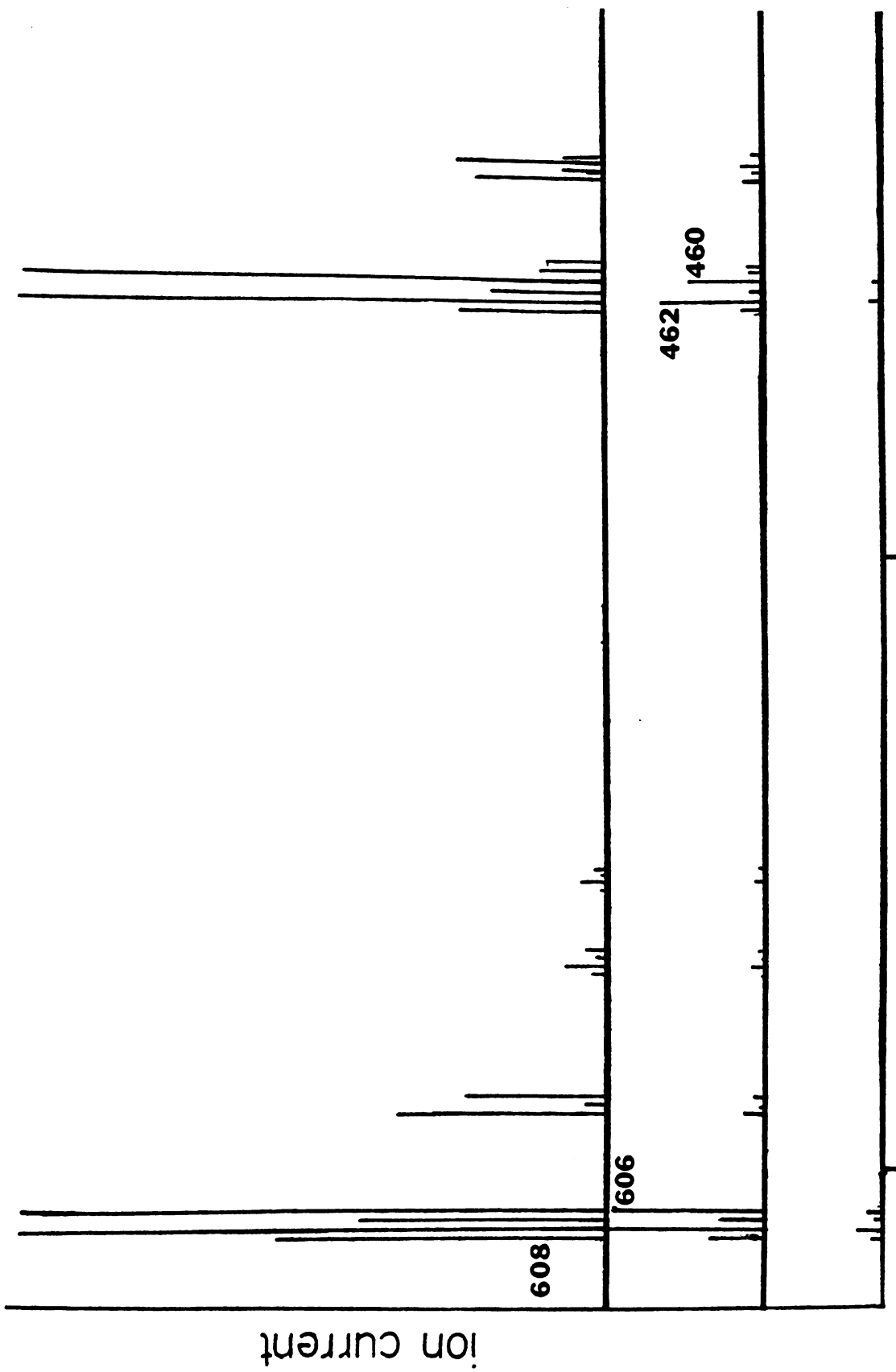


Figure 29. Cims spectrum (180°) of derivatized d_o- and d_2-alpha-methyldopamine.

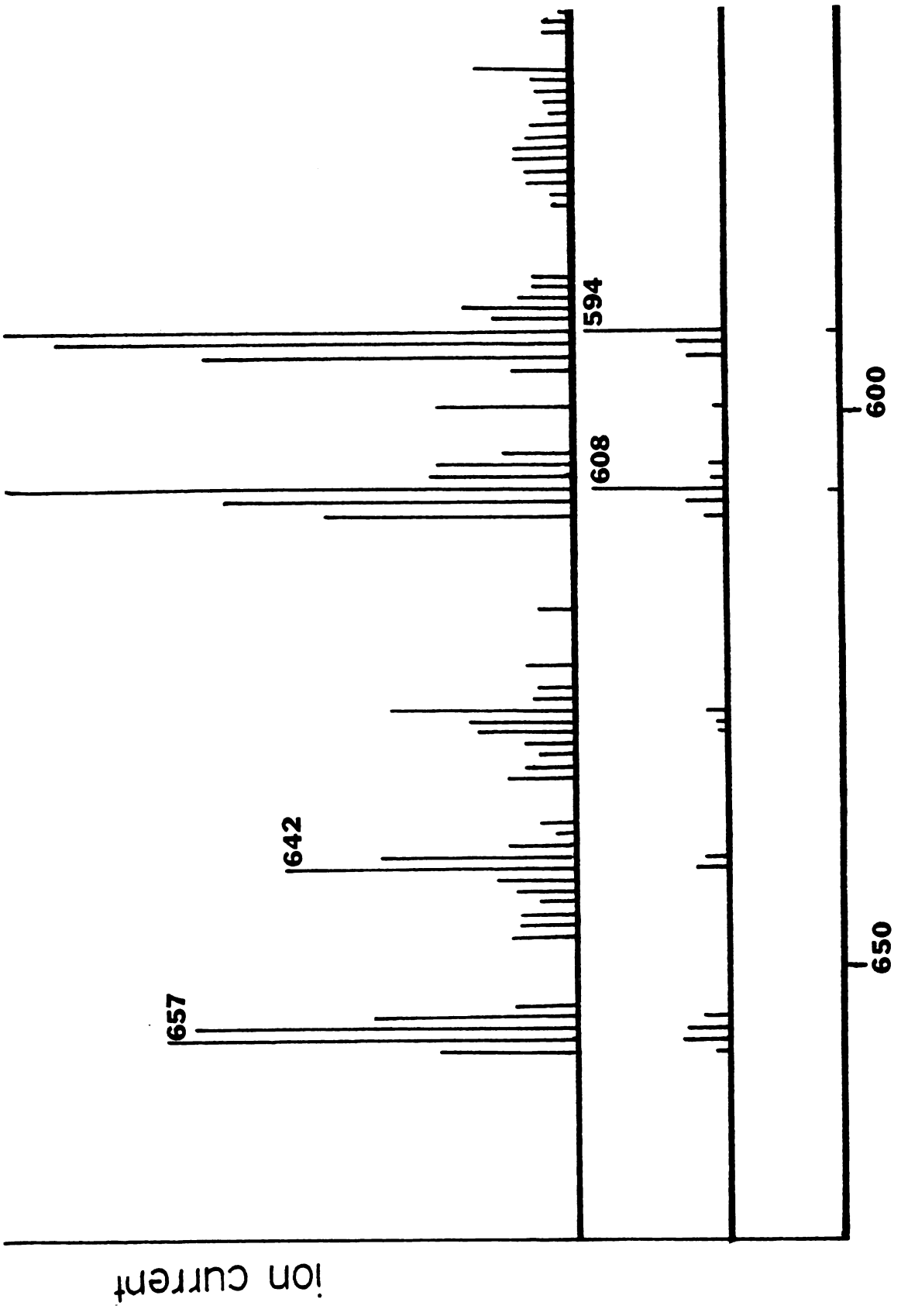
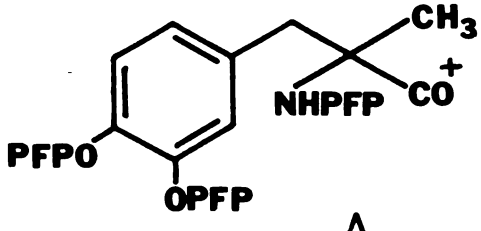


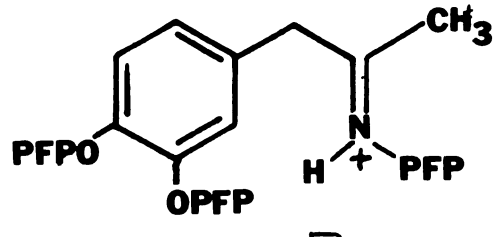
Figure 30. Cims spectrum (180°) of a mixture of the four derivatized and deuterium labeled amines.

To further characterize the appearance of a spectra which might be obtained from an in vivo metabolism experiment involving all eight amines, we obtained a spectrum of a mixture of the unlabeled and labeled amines (Figure 31). No significant ion intensities were observed beyond the eight molecular ions associated with the amines. When mixtures such as those in Figure 31 were scanned over a period of 5-10 minutes, it was found that the volatilities of the amines varied. However, there was no difference in volatility between the unlabeled and labeled amine pairs. This was true for mixtures down to the 1-5 nanomole per amine level. We thus felt that if there were no problems with interfering compounds in the tissues, we would be able to analyze the amines down to the 1 nanomole level.

In anticipation of the need to analyze alpha-methyldopa itself, we obtained cims spectra of this molecule after treatment with ethanolic HCl (2N, 2 hr, 70°), removal of the solvent with a stream of N_2, and treatment of the residue with PFPA (1 hr, 70°). One such spectrum is illustrated in Figure 32. Significant ion intensities were observed for the parent ion (MH_2^+ = 678), loss of ethanol (m/e 632, A) and loss of carboxyethyl (m/e 604, B):



A



B

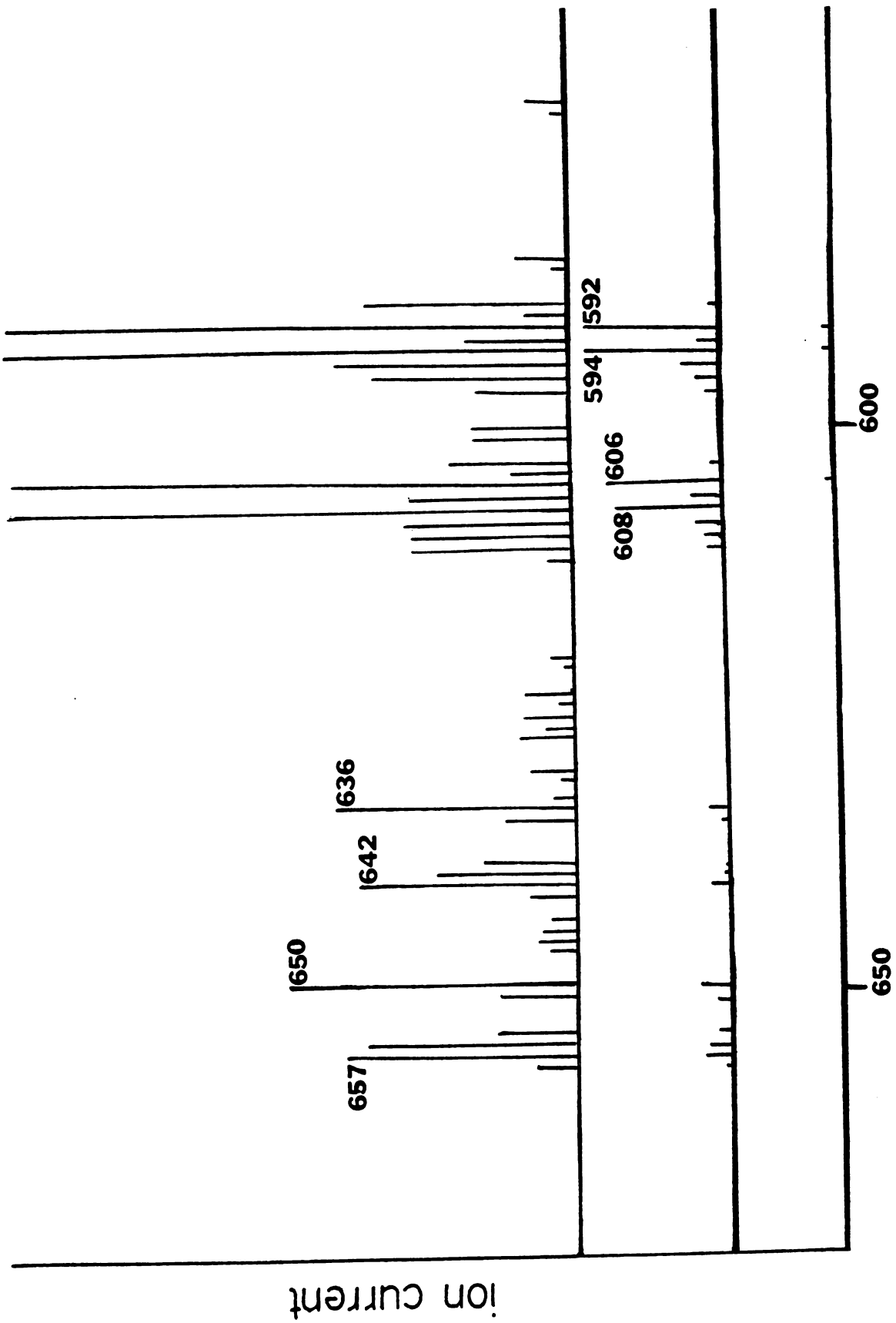


Figure 31. Cims spectrum (180^{0}) of a mixture of the eight labeled and unlabeled, derivatized amines.

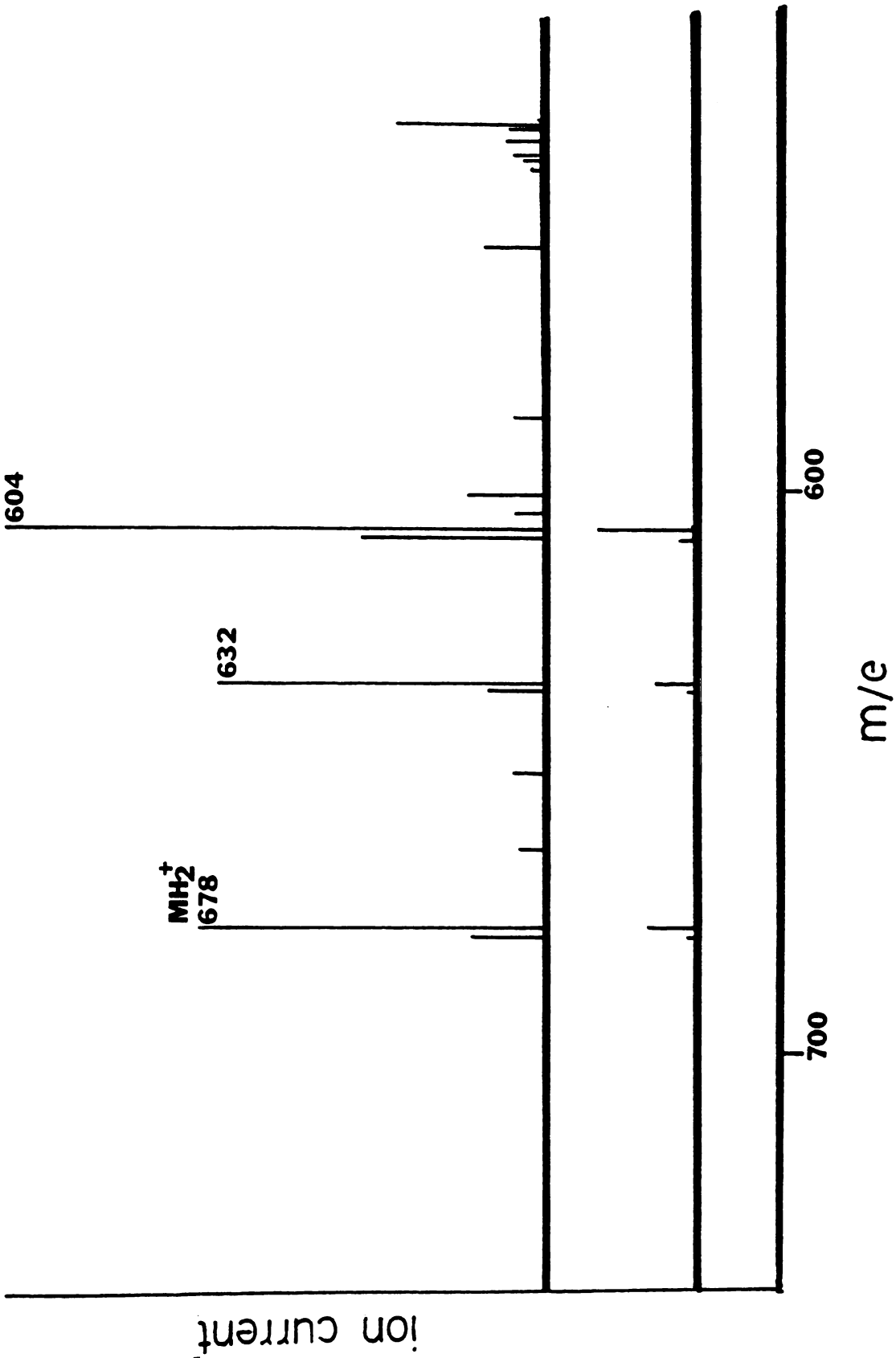


Figure 32. Cims spectrum (180^0) of derivatized alpha-methyldopa.

The spectrum illustrated in Figure 32 was obtained from a sample of 50 nanomoles of 1, an amount frequently used in the *in vitro* studies. However, when we later attempted to use the same procedures for the smaller amounts of drug utilized for *in vivo* studies (5 nanomoles), we were unable to detect either the internal standard or the substrate alpha-methyldopa.

An important consideration with regard to sensitivity in the cims analysis of alpha-methyldopa is the formation of the ethyl ester prior to PFPA derivatization. As part of his studies on the derivatization of alpha-methyldopa for cims analysis, Roger Cockerline demonstrated that the esterification was only approximately 10% complete after 2 hours at 70^{o}.[195] We therefore extended the esterification reaction time to 6 hours and obtained satisfactory spectra. This change in conditions, however, had important consequences with regard to our use of deuterium labeled alpha-methyldopa as an internal standard. We found that while the new conditions allowed us to readily detect the presence of 1 in the cims spectrum, the d_3-alpha-methyldopa gave a base peak at the unlabeled m/e value (678), with only small ion intensities at $MH_2^+ + 1$ and $MH_2^+ + 2$. Thus, back exchange appeared to be taking place under the more rigorous esterification conditions. As will be mentioned in the discussion of the metabolism experiments, we then turned to our ^{13}C-labeled alpha-methyldopa.

As was discussed earlier, cims analysis provided a means of direct mixture analysis without prior gc separation of biological samples. We therefore were faced with selecting an isolation procedure which would allow us to concentrate the amine and/or amino acid constituents of a

biological mixture. One of the most common isolation and separation procedures for catecholamines is cation exchange chromatography. It was especially well-suited for our studies in that it is a simple procedure, not overly time consuming, and is performed under acidic conditions which minimize the possibility of air-oxidation of the catecholamines.

Detailed procedures for the use of cation exchange resins have been published by several groups, including Bertler et al[30] and Haggendal,[199] both for amines and amino acids. Amines are retained on the sodium (Na+) form of the resin, while both amines and amino acids are retained on the hydrogen (H+) form of the resin. This method was therefore well-suited to our needs to isolate these particular fractions, and both forms of the resin were employed in our studies.

In our studies we employed both aqueous and anhydrous ethanolic HCl (2-3N) to elute the compounds of interest. We found that two micromoles of alpha-methyldopa and alpha-methylnorepinephrine were retained at pH 2 on 0.5-1.0 cc of resin (H+ form), and effectively eluted by 3N aqueous HCl (20 ml) as determined by uv analysis (Figure 33). These conditions were employed in the in vitro metabolic studies. Recoveries of 70-80% for the smaller amounts of amines (1-10 nanomoles) utilized for in vivo metabolic studies by elution from the sodium form of the resin with small volumes of ethanolic HCl (2N, 5-10 ml) have been demonstrated by Curt Freed.[196] The particular conditions utilized for the column isolation of these compounds in the metabolic experiments can be found in the metabolic discussion sections, and the experimental section. Details with regard to preparation of the biological samples (homogenization, pH, etc) may also be found there.

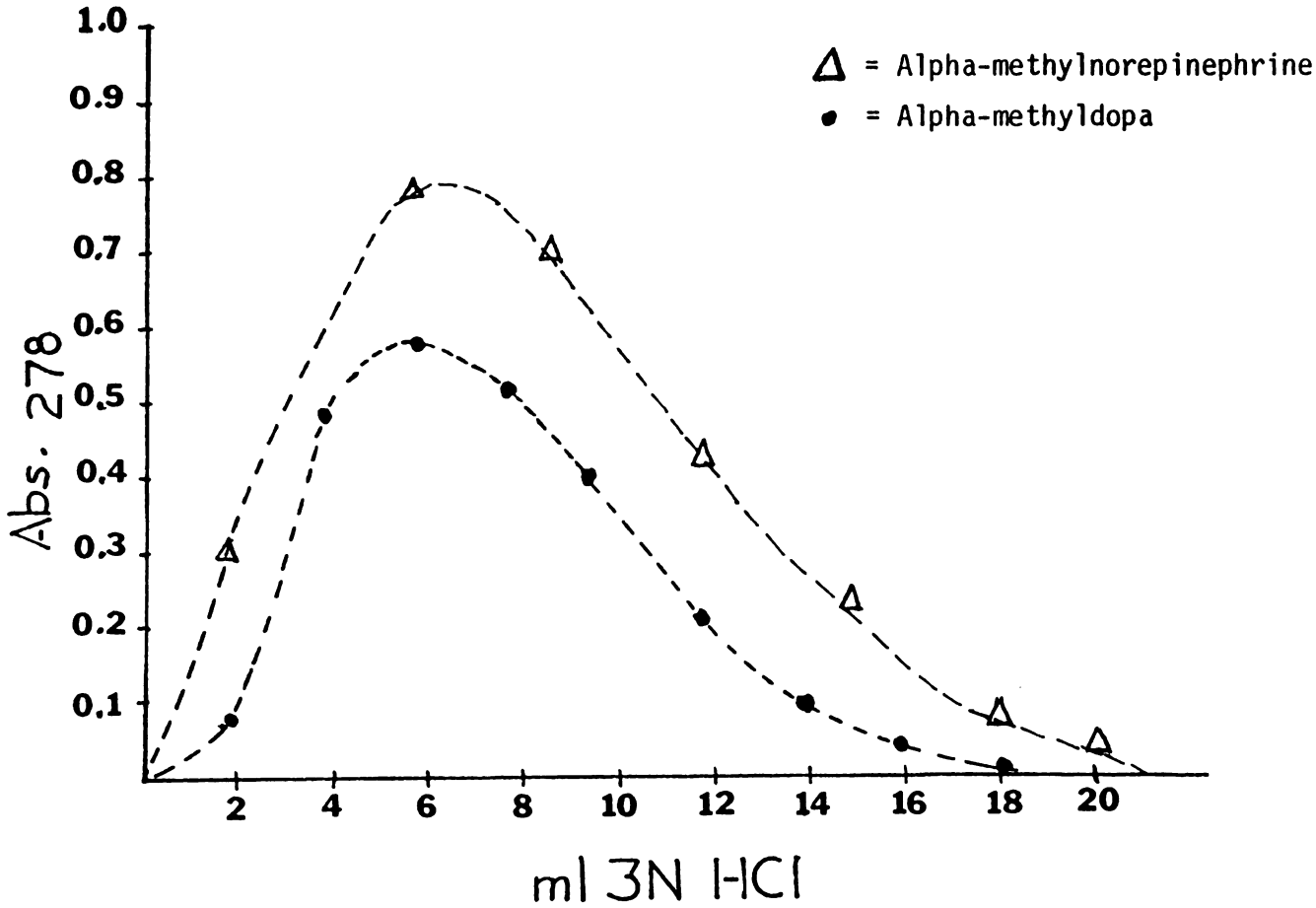
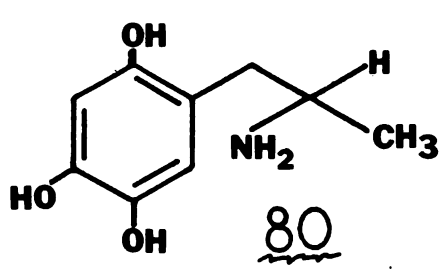


Figure 33. Elution of alpha-methyldopa and alpha-methylnorepinephrine from cation exchange column with 3N HCl.

IN VITRO METABOLISM OF ALPHA-METHYLDOPA

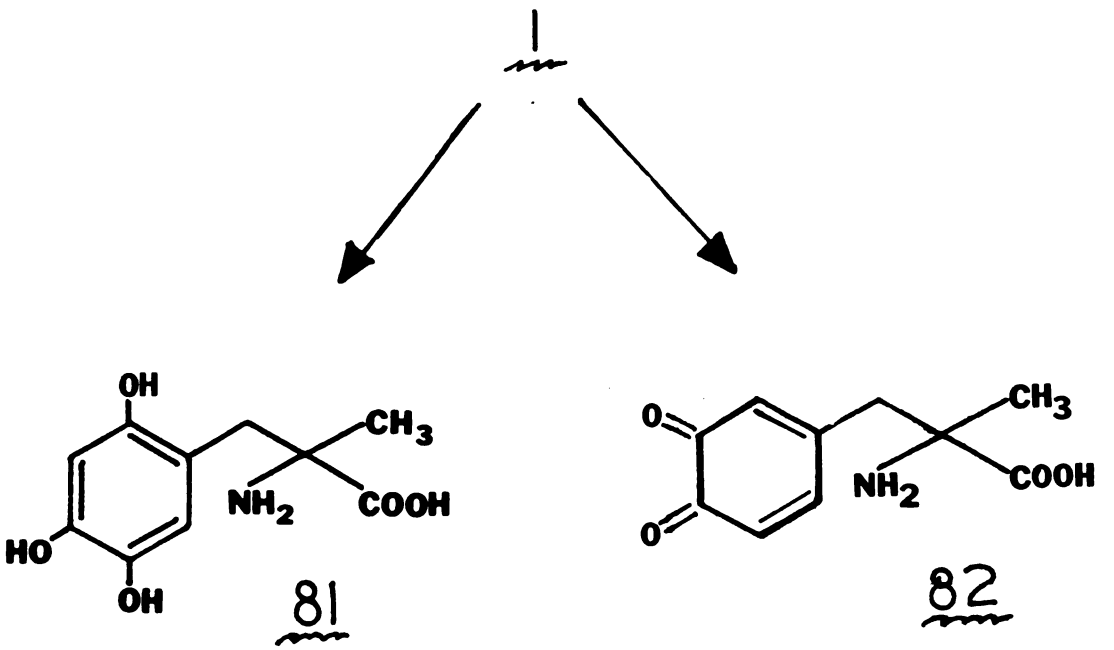
With the aid of our ^{13}C-enriched alpha-methyldopa and cims analytical procedures, we undertook the investigation of several aspects of the *in vitro* metabolism of alpha-methyldopa. Hypertensive therapy with 1 is sometimes accompanied by varying degrees of red blood cell and liver toxicity.[3,5,17,18,19] Positive Coomb's tests have been reported in up to 28% of the patients receiving alpha-methyldopa, with an average of 15%.[200] With this incidence of side effects, including liver toxicity, and an interest in the possible oxidative pathways of metabolism, we applied our cims procedures to the study of *in vitro* liver metabolism of alpha-methyldopa.

Several enzymes in the soluble fraction of liver have been reported to be involved in the metabolism of 1. For example, the enzymes responsible for 3-O-methylation (metabolites 36, 37)[201] and decarboxylation to give alpha-methyldopamine (9) and its metabolites[72] have activity in the soluble liver fraction. With regard to liver toxicity, and as a potentially significant metabolic pathway, we were more interested in the insoluble microsomal enzymes responsible for liver oxidations. The oxidative formation of 6-hydroxydopamine in an *in vitro* preparation[202] has been reported. The compound is a potent neurotoxic agent capable of selective destruction of sympathetic neurons. We were therefore interested in the possible metabolic or spontaneous formation of 6-hydroxy-alpha-methyldopamine (80), a potentially neurotoxic metabolite.



80

We also wished to investigate the possible formation of the hydroxylated amino acid 6-hydroxy-alpha-methyldopa (81). Finally, the alpha-methyldopa might be oxidized to the ortho-quinone 82, an electrophilic species. Addition of nucleophilic groups (-SH, -OH, etc) of liver proteins to the quinone was viewed by us as a possible mechanism for liver toxicity, as was the formation of 80 and 81.



While the liver toxicity of 1 and the preliminary report[202] of the in vitro formation of 6-hydroxydopamine stimulated our interest in the possible oxidation of alpha-methyldopa in the liver, there were indications that such an observation might not take place. A review of the literature revealed no example of microsomal liver oxidation of either catechols or catecholamino acids. Further, compounds with low solubility in organic phases and a high degree of

ionization at physiological pH are not well metabolized in microsomal systems.[205] Catechols have also been described as poor substrates and weak inhibitors of microsomal oxidation.[206]

While our primary interest was in microsomal oxidation, we selected a 10,000-12,000g supernatant liver fraction containing both microsomal and soluble enzyme systems. The preparation is quite simple and has been reported to have higher metabolic activity than isolated microsomes.[207] We also wished to look for oxidation of alpha-methyldopamine (as mentioned above), which would require both the soluble decarboxylase system and the insoluble microsomal enzyme system.

The conditions for the incubation system were selected after reviewing several liver incubation systems used for the metabolism studies of molecules similar to ours,[208,209,210] and the volume by La Du et al[207] on drug metabolism and disposition. Summaries of several such preparations (including our own) are presented in Table V. The procedure we adopted is shown schematically in Figure 34. The selection of Dutch male rabbits and the use of NADPH (rather than an NADPH generating system) were made on the basis of previous work on the metabolism of 1-phenyl-2-aminopropanes conducted in our laboratory.[211,212] La Du et al[207] found no fault with this method of NADPH addition, other than the need to add the NADPH in increments rather than in a total addition at the beginning of the incubation.

Based on the fact that alpha-methyldopa is a poor substrate for DOPA decarboxylase[40] and the probability that only small amounts of oxidized metabolites would be formed, we hoped to employ incubation times of 60-90 minutes. The characteristic instability of catechols, especially at higher pH values,[114] mentioned earlier prompted us to

Table V

A summary of various in vitro incubation systems for use in liver metabolism studies.*

Reference	208	209	210	Our Studies
Substrate	Oxindoles	Terbutaline	Oxadiazole	Methyl-dopa
Substrate (μmole)	6.5	4.0	4.0	2.37
Buffer (pH)	Phosphate (7.6)	Phosphate (7.4)	Phosphate (7.4)	Phosphate (7.4)
NADPH (μmole)	---	---	---	12
NADP (μmole)	0.4	---	4	---
Glucose-6-Phosphate (μmole)	20	---	20	---
Mg^{++} (μmole)	60	10	---	50-75
Animal	rat	rat	mouse	rabbit
Liver (gram)	0.6	0.3	0.2	1.0-2.0
Homogenate (ml)	2.0	0.8	1.0	10.0

*All figure apply to one incubation mixture.

1. Rabbit sacrificed by blow to head.
2. Liver removed and rinsed in cold 1.15% KCl.
3. Liver blotted, weighed, and added to buffer (phosphate, 0.1M, pH 7.4, 1 g/2 ml).
4. Tissue homogenized with Potter-Elvehjem tissue homogenizer.
5. Homogenate centrifuged (12,000g x 25 min) in the cold.
6. Supernatant fraction used for incubation, 1-2 g/ incubation.
7. Homogenate supernatant added to incubation flasks containing:
   4 ml above buffer
   1.5 ml $MgCl_2$ (0.05M)
8. ^{13}C-alpha-methyldopa added (2.37 or 0.47 micromoles, 1.0-3.0 ml).
9. Incubation started with addition of NADPH (3 mg of 12 mg total), in Dubnoff metabolic shaker at 38°.
10. Incubation allowed to continue at 38° for 60-90 minutes.
11. Incubation terminated by placing flask in ice bath.
12. Internal standard ^{12}C-alpha-methyldopa added (2.37 or 0.47 micromole).
13. TCA (25%, 0.5 ml) added to incubation flasks.
14. Samples centrifuged (10,000g x 15 min).
15. Supernatant applied to cation exchange column.
16. Cims analysis performed as described in text and experimental.

Figure 34. Procedure for in vitro rabbit liver incubation of alpha-methyldopa.

investigate the stability of alpha-methyldopa in solution under several conditions. Alpha-methyldopa has a reported absorbance of 281 nm, with a molar extinction coefficient of 2780.[213] Sassetti and Fudenberg[214] reported that air-oxidation at pH 10 of alpha-methyldopa caused this absorbance to shift to approximately 300 nm with a decreasing extinction coefficient. Concommitantly, an absorbance at longer wavelength (400-500 nm) appeared in the spectrum. With this information, we studied the stability of 1 by monitoring the uv absorbance at 278 nm (our experimental absorbance maximum) and 435 nm.

The uv spectrum of 1 in distilled H_2O (pH 5) displayed only one chromophore centered at 278 nm with a calculated molar extinction coefficient of 2495. Under these conditions, the spectrum of alpha-methyldopa did not change over the period of several hours. This was monitored by absorbance at 278 and 435 nm, and is shown in Table VI. Under basic conditions (pH 11) this absorbance shifted to approximately 300 nm, with a decreasing extinction coefficient, and was accompanied by the appearance of a broad absorbance centered at 420-435 nm. We then studied the stability of the drug under incubation conditions (phosphate buffer, pH 7.4), and found it to be stable for at least two hours (Table VI). The results of these studies suggested that incubation periods of up to 90 minutes could be undertaken without appreciable spontaneous oxidation of the substrate.

With incubation conditions and procedures selected (Figure 34, Table V), and the stability of the drug determined, we undertook two *in vitro* liver experiments utilizing the 12,000g supernatant fraction. The first incubation contained approximately 500 micro-

Table VI

I. Stability of alpha-methyldopa in distilled H_2O (pH 5) as determined by uv absorbance.*

Time (hr)	Absorbance (278 nm)	Absorbance (435 nm)
0	0.68	0.00
3	0.67	0.00

II. Stability of alpha-methyldopa in 0.2M potassium phosphate buffer (pH 7.4) as determined by uv absorbance.

Time (min)	Absorbance (278 nm)	Absorbance (435 nm)
0	0.62	0.00
3	0.61	0.00
9	0.62	0.00
20	0.62	0.00
45	0.63	0.00
60	0.63	0.00
120	0.66	0.00
210	0.68	0.05

*Addition of NaOH to the cuvettes (pH 11) resulted in decreased absorbance at 278 nm, with a shift in the absorbance maximum to 300nm. In the same time period, there was a new absorbance, very broad, at 420-435 nm.

grams of racemic 50% ^{13}C-enriched alpha-methyldopa, the second approximately 100 micrograms of the same substrate. The 50:50 mixture insured that metabolites derived from the substrate would be characterized by the appearance of a doublet at the appropriate MH_2^+ values in the cims spectrum.

The incubation was allowed to proceed for one hour, the reaction stopped, and equal amounts of ^{12}C internal standard 1 added to the mixture. Thus, if no metabolism took place, a 1:2 ratio of the $^{13}C/^{12}C$ mixture (MH_2+ = 679,678) would be observed in the cims spectrum. To check the interpretation of the $^{13}C/^{12}C$ ratio, controls (either no liver or boiled liver) were run with each experiment. After appropriate derivatization procedures the cims spectra of both experiments (illustrated by Figure 35, the spectrum of the 100 microgram experiment) showed (1) no 1:1 doublets in the entire spectrum indicative of metabolites, (2) ratios of approximately 1:2 (1.00:1.85 and 1.00:2.03 for the 500 and 100 microgram experiments, respectively) for the parent amino acids, and (3) identical ratios for control and viable liver incubation mixtures for the parent amino acids in both experiments.

The apparant absence of any significant metabolism in this system led us to repeat the experiment with 10 micrograms substrate in the hopes that even low levels of metabolism would be observed. In this instance, we were unable to obtain useful spectra; as reported in the analytical discussion it was only after we investigated the esterification conditions that these problems of sensitivity in the analysis of 1 were resolved. At the time of this study, we had not yet studied the reaction, and further liver studies were not attempted.

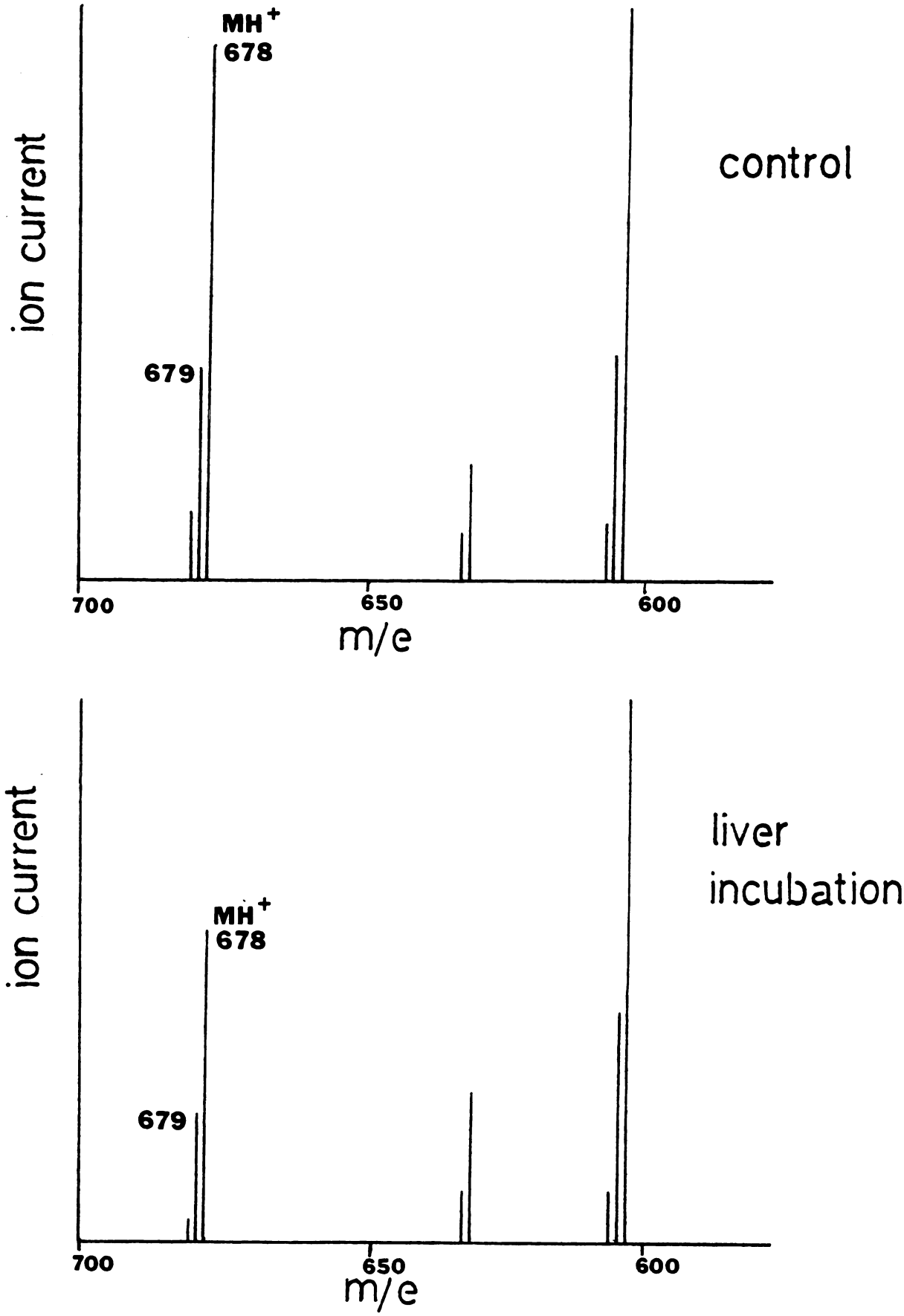


Figure 35. Cims spectrum (185°) of *in vitro* liver incubation with 50% ^{13}C-labeled alpha-methyldopa.

We were not greatly surprised at the lack of evidence of decarboxylation or other metabolism in the liver studies. As noted above, studies have shown that alpha-methyldopa is a poor substrate for the decarboxylase (1/200 the activity of DOPA)[40]. It has also been shown that the rabbit is a poor animal for decarboxylase activity (the rat has much higher activity in major organs such as heart, brain, kidney), and that the kidney has the greatest decarboxylase activity of the major organs.[40,215] Finally, essentially all decarboxylase studies reviewed added the cofactor pyridoxal-5-phosphate (47) to the incubation systems, with a 2-5 fold increase in activity. Thus, our choice of animal, organ, and lack of cofactor addition may have obviated potential decarboxylation. We were, of course, more disappointed at observing no oxidation of the drug.

In vitro studies were then undertaken with the potentially more active rat kidney preparation, fortified with pyridoxal-5-phosphate. a 75 minute incubation with 100 micrograms of the doublet mixture again provided no evidence of metabolite formation, and no change in the ratios of $^{13}C/^{12}C$ drug between control and viable liver incubations. However, with DOPA as substrate (0.4 mg and 2.1 mg), the cims spectrum indicated that significant amounts of dopamine (MH_2^+ = 592) had been formed (Figure 36). The ion multiplets observed for derivatized DOPA (MH_2^+ = 667-774) were due to the substrate and addition of internal standard d_3-DOPA. The esterification procedure led to partial back exchange of the deuterium, as mentioned in the analytical discussion. Fortunately, the significant amount of dopamine and the use of controls made the identification of dopamine possible.

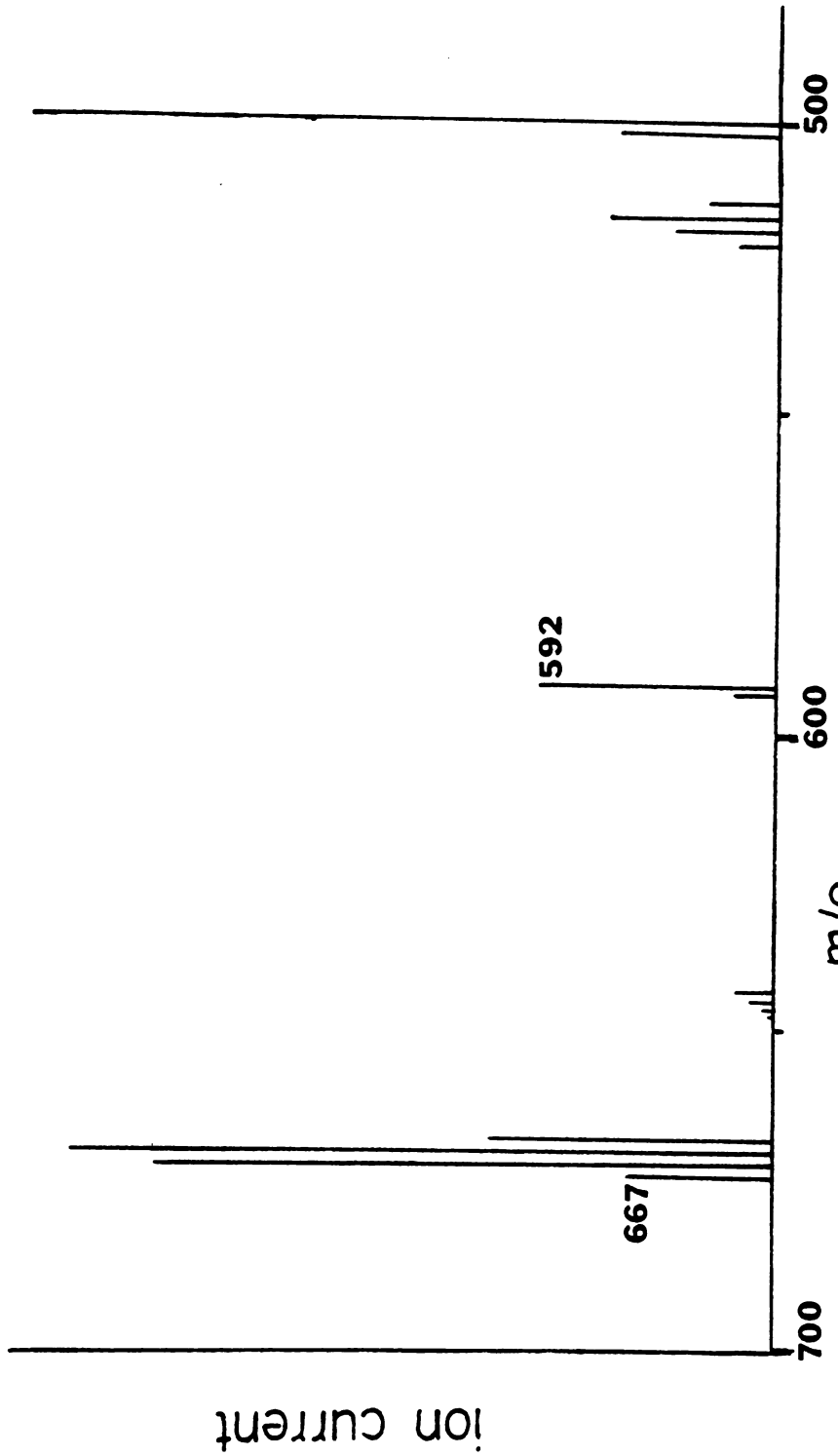


Figure 36. Cims spectrum (185°) of in vitro kidney incubation with DOPA as substrate, indicating formation of dopamine (MH_2^+ = 592).

We pursued no further *in vitro* metabolism studies and turned our attention to the *in vivo* experiments described below. However, recent studies have provided evidence for the type of liver metabolism we had envisioned in our *in vitro* studies. Dybing *et al*[216] have been able to bind tritium-labeled alpha-methyldopa to rat and mouse hepatic microsomes. Deletion of the NADPH generating system or the addition of an anti-oxidant (ascorbic acid) abolished the binding, suggesting that oxidation was a prerequisite. Addition of known *ortho*-quinone trapping agents or glutathione reduced the binding, implicating the *ortho*-quinone 82 as an intermediate in this process. Catechols such as dopamind or norepinephrine inhibited the binding, while 3-O-alpha-methyldopa had no effect and itself was not bound. The addition of catalase had no effect on the binding, while the addition of superoxide dismutase[217] abolished the binding, leading to the preliminary suggestion that the superoxide anion may be involved in the oxidation of alpha-methyldopa.

IN VIVO METABOLISM OF ALPHA-METHYLDOPA

Several pertinent aspects of the mechanism of action of alpha-methyldopa were discussed in the pharamcology and metabolism sections (Part I). It was shown quite conclusively that the mode of action is central in nature.[63,67,68,69] The central metabolism (decarboxylation) appears to be a prerequisite for pharmacological activity of the drug,[57,62,63] although the exact role of the metabolite(s) is not known.

The stereochemistry of the central decarboxylation is a crucial question in the consideration of the mechanism of action of alpha-methyldopa. No decarboxylated metabolites have been detected after administration of the R isomer,[81,85,92] with the exception of a trace amount of the phenylacetone 38.[85] DOPA decarboxylase preparations have been reported to decarboxylate only S amino acids.[72] However, as was noted in the literature review, there have been reports of decarboxylase activity separable from the decarboxylation of DOPA in several organs,[103,104] including the rat brain.[105]

We therefore undertook a stereochemical study of the decarboxylation of alpha-methyldopa in the rat brain. There are no reported studies of the stereochemical aspects of *in vivo* brain decarboxylation of alpha-methyldopa. When we started these studies, several questions were considered: (1) is the R isomer 1b decarboxylated in the rat brain to yield amine metabolites? (2) does the presence of the R isomer or its metabolites effect levels of the endogenous amines, dopamine and norepinephrine, in the brain? and (3) can the results of (1) and (2) be utilized as the basis for further studies into the stereochemical aspects of alpha-methyldopa metabolism.

With the aid of our cims analytical procedures, resolved ^{13}C-labeled (89%) alpha-methyldopa, and internal standards (deuterium and ^{13}C) we first studied the *in vivo* brain decarboxylation of R, S, and racemic alpha-methyldopa.The procedures for administration (ip) of the drugs and analysis of the amine metabolites were developed by Curt Freed in his studies on the regional metabolism of alpha-methyldopa, and will be published elsewhere.[196] Male Wistar rats were injected with approximately 60 mg of R, S, or racemic **1**, and sacrificed 6 hours later. The hypothalamus and caudate nucleus were removed immediately and frozen (-70^{o}) until analysis. The tissues were thawed and the internal standard amines (d_2-dopamine, d_2-alpha-methyldopamine, $d_{6,5}$-norepinephrine, $d_{7,6,5}$-alpha-methylnorepinephrine, Figure 28) added to the tissue. After homogenization with TCA, centrifugation, isolation via cation exchange chromatography, and derivatization, the cims spectra were obtained. Each sample was scanned 2-5 times, and the appropriate peak heights measured with any heights less than 10 mm discarded for quantitative purposes. The average value for each sample was obtained by the arithmetic average, thereby favoring the larger (and more reliably calculated) peak heights. Samples in which the variation of peak heights between internal standard and unknown was greater than 10% also were not used for quantitative purposes. Peak height measurements were taken above the average background signal for the particular sample.

The results of all the *in vivo* experiments in which quantitative data was used are summarized in Table VII. When S-alpha-methyldopa was administered, the caudate nucleus was found to contain significant

Table VII

Levels of dopamine and alpha-methyldopamine (nanomoles/gram) in the caudate nucleus after administration (ip except where noted) of R, S, or racemic alpha-methyldopa.#

Drug Administered	Dopamine (nanomoles/gram)	Alpha-methyldopamine (nanomoles/gram)
S-alpha-methyldopa	33.5	46.0
S-alpha-methyldopa	15.4	33.5
R-alpha-methyldopa	66.4	<1.0
R-alpha-methyldopa	62.3	<2.0
No drug (control)	42.3	<1.0
Literature value[218] for no drug (control)	62	<2.0
RS-alpha-methyldopa	44.3	54.4
RS-alpha-methyldopa	51.1	56.8
^{13}C-S-alpha-methyl dopa	21.1	81.7
S-alpha-methyldopa*	33.2	23.2
R-alpha-methyldopa*	52.3	<5.0

*Drug administered directly into the third ventricle of the brain.
#Administration of S-alpha-methyldopa, followed by analysis for the parent amino acid gave a value of 105 nanomoles/gram. The same procedure for the R amino acid gave 12 nanomoles/gram.

amounts of alpha-methyldopamine (30-60 nanomoles/gram, MH_2^+ = 606), and dopamine levels were depressed from normal values (20-40 nanomoles/gram, normal values approximately 60 nanomoles/gram, MH_2^+ = 592), as seen in Figure 37a. The caudate nucleus contains only small amounts of beta-hydroxylated compounds, and we were unable to detect the presence of norepinephrine (MH_2^+ = 636) or alpha-methylnorepinephrine (MH_2^+ = 650). These results may be compared to those obtained after administration of the R isomer (Figure 37b). Negligible amounts of alpha-methyldopamine (0-3 nanomoles/gram) were detected, while dopamine levels were similar to control values (60-65 nanomoles/gram). Again, no beta-hydroxylated compounds were detected. Note that there was no ambiguity in determining the absence of alpha-methyldopamine, and that the spectra are quite clean in appearance.

As a check on these results, we performed two additional experments. First, we administered racemic alpha-methyldopa in order to compare the levels of dopamine and alpha-methyldopamine with those obtained from the S isomer alone. Values were slightly higher than those obtained from the S isomer, but not unreasonable for different animals and different experiments (Table VII). To insure that we were attributing ion intensities to ions arising from our compounds (control animals also were used for this purpose), we administered ^{13}C-enriched (89%)-S-alpha-methyldopa. In this experiment, alpha-methyldopamine was indeed observed at MH_2^+ = 607, with little ion intensity at m/e 606 (corresponding to the 11% unlabled metabolite, Figure 38. The scan shown in Figure 38 was taken from a sample of the hypothalamus, and accordingly, alpha-methylnorepinephrine can be observed at MH_2^+ = 651 (rather than 650 due to the ^{13}C).

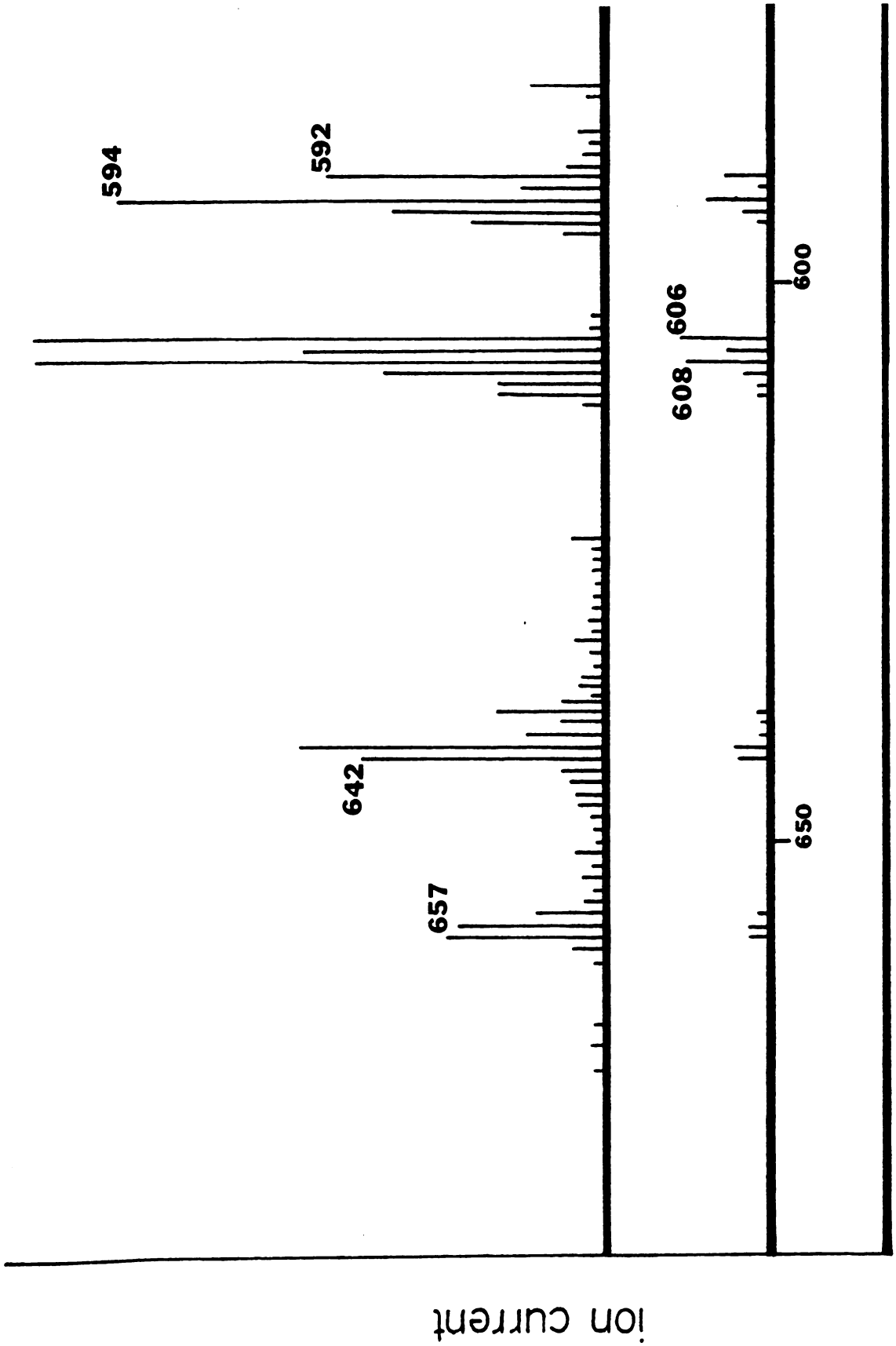


Figure 37a. Cims spectrum (180°) of amines in caudate nucleus after administration of S-alpha-methyldopa.

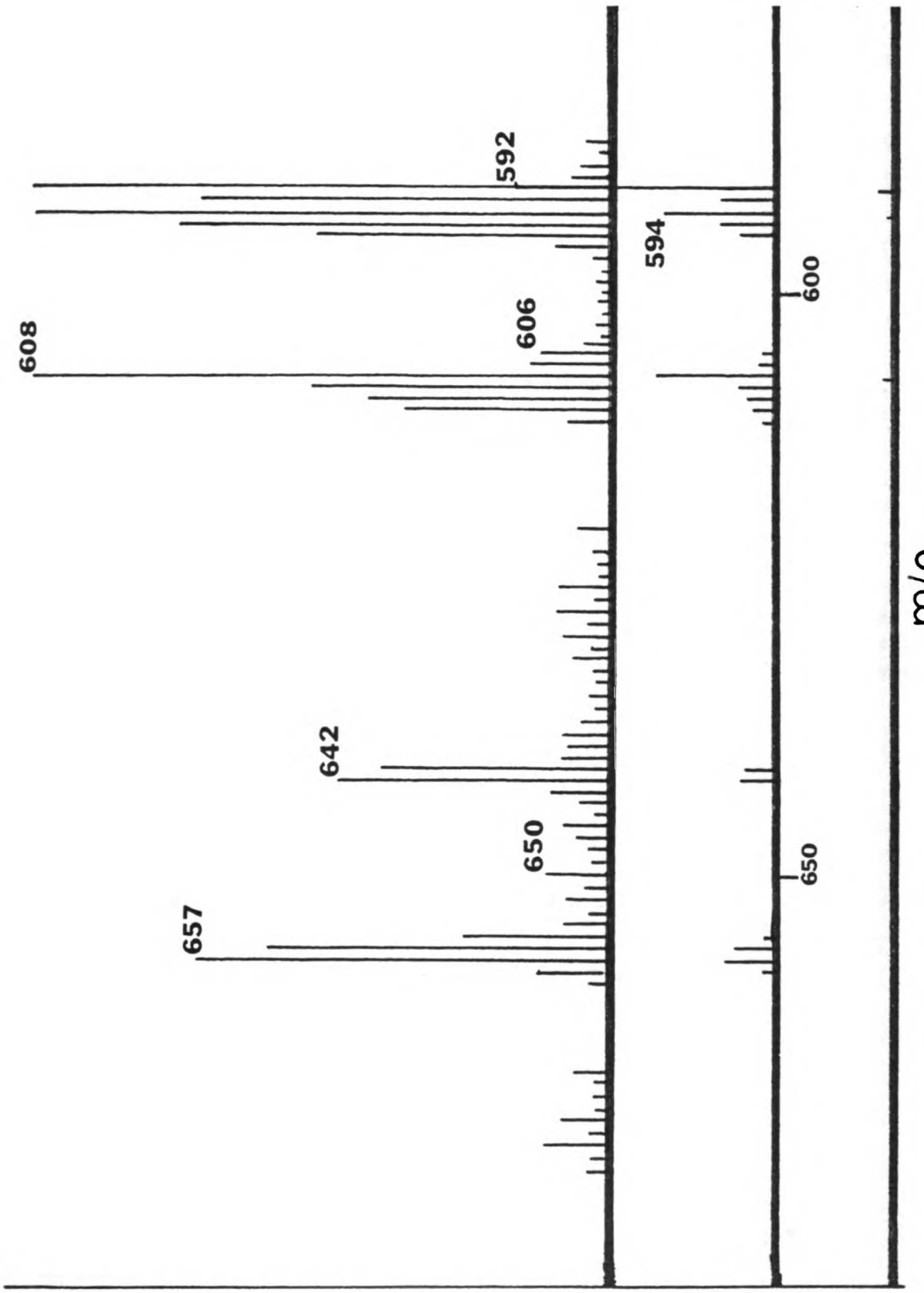


Figure 37b. Cims spectrum (1800) of amines in caudate nucleus after administration of R-alpha-methyldopa.

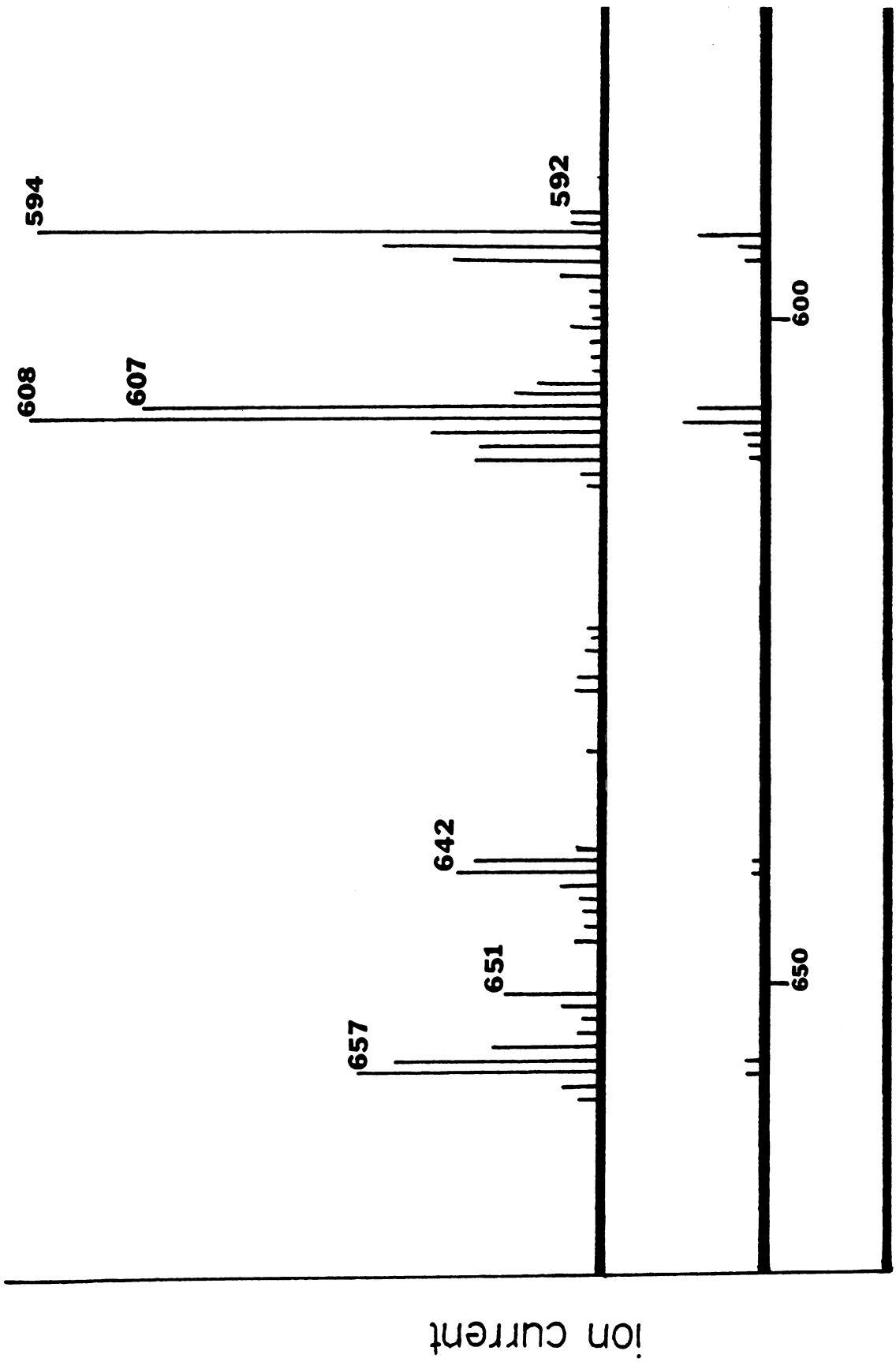


Figure 38. Cims spectrum (180°) of amines in hypothalamus after administration of ^{13}C-S-alpha-methyldopa.

Note the total displacement of norepinephrine (MH_2^+ = 636) and the small amount of dopamine (MH_2^+ = 592) in this region of the brain. This was also observed by Curt Freed in his studies.[218] This particular scan showed the presence of both the beta-hydroxylated and non-beta-hydroxylated compounds. By taking advantage of the differing volatilities of these compounds, the beta-hydroxylated compounds (MH_2^+ = 657, 651, 642) could be observed at a much greater intensity, at the expense of the other amines (MH_2^+ = 608, 607, 594, 592). No quantitative data was obtained from this experiment due to problems with the mass spectrometer at this time.

As a final check, we also administered a pseudo-racemic mixture of ^{13}C-S-alpha-methyldopa and ^{12}C-R-alpha-methyldopa. Alpha-methyldopamine was again observed at MH_2^+ = 607, while m/e 606 was not observed above background. Finally, we should note that control animals (those which received no drugs, but with added internal standards) resulted in spectra in which no ion intensities could be observed for any m/e values corresponding to the amine metabolites discussed here. The internal standards were calibrated against standard solutions of unlabeled amines (giving spectra similar to that in Figure 31) for quantitation. It should be stated that more rigorous quantitative procedures (such as standard curves) were not required for our studies. There is no ambiguity in the identification of R and/or S-alpha-methyldopa decarboxylation products in these studies.

With these results, we felt it would be of even more interest to determine the fate of the parent amino acids themselves in the rat brain. As was discussed in the analytical and <u>in vitro</u> sections,

we had previously been unable to identify small amounts (1-10 nanomoles) of the amino acid with our cims assay. Upon learning of the need to esterify for longer periods of time (6 hours), we decided to pursue studies of the fate of the amino acid in the brain.

Our first attempt was to simply add d_3-alpha-methyldopa to several of the samples used in the above amine studies, and pass the ion exchange eluent through a second cation exchange column in the hydrogen form to isolate the amino acids. After derivatization, however, the cims spectra of such samples contained only small ion intensities at the m/e value associated with unlabeled alpha-methyldopa (MH_2^+ = 678). We attributed this to back exchange of the deuterium labeled drug under the harsher esterification conditions (60^o, 6 hr).

In a further attempt to study the fate of 1, we turned to the non-exchangeable ^{13}C-labeled 1 for use as an internal standard rather than as a substrate. We administered ^{12}C-R or ^{12}C-S substrate, and added the ^{13}C-labeled compound as the internal standard. Derivatization was accomplished after the amino acids were isolated via the hydrogen form of the cation exchange columns. In these experiments, the animals were sacrificed after approximately one hour rather than six hours, in hopes of observing more of the unmetabolized drug.

The results of administration of the two isomers separately may be observed in Figure 39. While a significant amount of the S amino acid was observed, very little (allowing for the 11% unlabled 1) of the S isomer was seen. This suggested that the R amino acid was not crossing the blood-brain barrier, or at least was not being taken up by the caudate nucleus. Administration of racemic 1 gave spectra si-

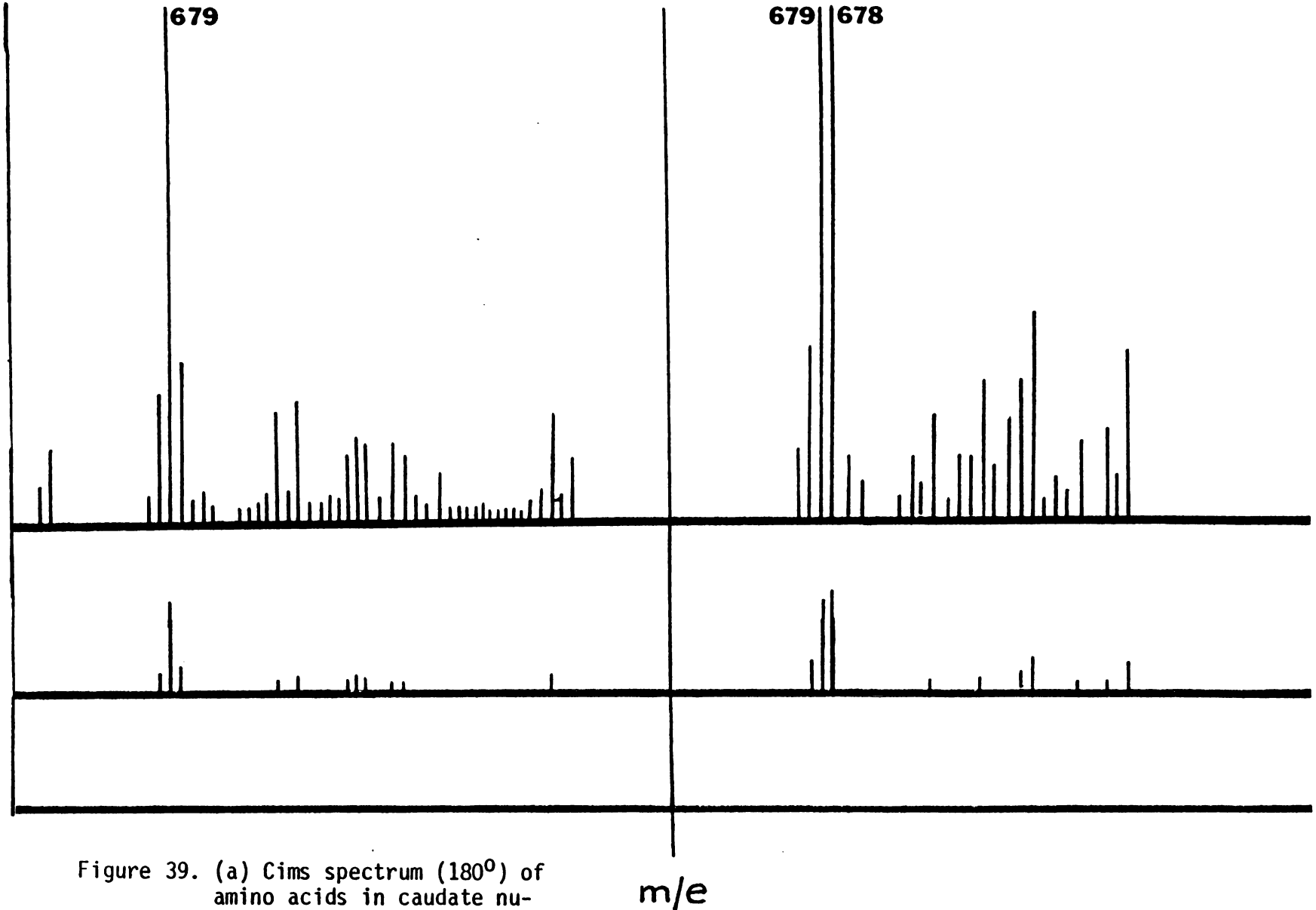


Figure 39. (a) Cims spectrum (180°) of amino acids in caudate nucleus after administration of S-alpha-methyldopa. (b) Spectrum after R-alpha-methyldopa.

milar to that observed for the S isomer.

These findings on the stereoselective nature of the uptake of 1 by the brain (caudate nucleus) prompted a literature review on the transport of amino acids (especially aromatic amino acids) into the central nervous system. While many studies were found on the subject, few investigators have studied the stereoselective aspects of amino acid transport across the blood-brain barrier, and fewer yet the transport of DOPA. No such studies were found with regard to the foreign amino acid alpha-methyldopa.

In 1962, Guroff and Udenfriend[219] studied the uptake of aromatic amino acids into the brain as a function of stereochemistry. Stereoselectivity was observed (always favoring the S isomers) for phenylalanine, tyrosine, and tryptophan by radioactive techniques. Inhibition by the R isomer was not observed for these acids. DOPA was not studied in these experiments. Several similar studies on the aromatic amino acid uptake found that the process was carrier mediated[220] and that there were separate mechanisms for the neutral and basic amino acids.[221]

Two studies were reviewed which did examine the uptake of DOPA into the brain. In a large study of 28 amino acids and 13 amines,[222] it was again suggested that there were separate mechanisms for neutral and basic amino acids. Amino acids from either group would inhibit others of the same group, but there was no cross inhibition. DOPA was included in this study, but no stereoselective data was reported. The second study by Oldendorf[223] did report findings on the stereoselective aspects of the uptake. Among the 12 amino acids

studied were DOPA, tyrosine, and tryptophan. For those amino acids which were available with radioactive labels for both isomers, it was found that the S isomer was preferentially transported into the brain. For example, S-tyrosine was taken up 53% compared to 8% for the R isomer, based on water as 100% uptake. However, for the majority of the acids, including DOPA, the labeled R isomer was not available. The author reported DOPA transport to be the most selective of all tested amino acids based on the fact that labeled S-DOPA was least effected by prior administration of R-DOPA; the assumption being that if the R isomer were taken up to a significant degree, its presence would lessen the degree to which the S isomer was taken up.

Finally, a histochemical study by Bertler et al[224] examined the cerebral capillaries for fluorescence after administration of R-DOPA and S-DOPA. The authors reported that whereas significant fluorescence was found after administration of the S isomer, no such fluorescence was observed after administration of the R isomer. It was concluded from this and other experiments that the S isomer, but not the R isomer, penetrated the capillary walls of cerebral blood vessels.

While these studies were not definitive with regard to alpha-methyldopa, they did suggest that our observations were probably correct, and that the R isomer of alpha-methyldopa had very little access to enzymes in the central nervous system. We felt it would therefore be important to see how the R isomer would be handled by decarboxylase if it were administered directly into the brain. With the help of Dr. Martha Hamlet, we undertook a study designed to introduce both isomers into the rat brain, and observe the metabolism of

each isomer.

The necessary surgical procedures required implantation of a cannula into the third ventricle. This was performed on two male Wistar rats after administration of a general anesthetic. After allowing 24 hours for their recovery, two injections of either R or S drug (300 micrograms in 10 microliters) were administered, one hour apart. Approximately 90 minutes later, the animals were sacrificed, each caudate nucleus removed, and the samples treated as before for analysis of amine metabolites.

After intraventricular injection of S-alpha-methyldopa, a significant ion intensity was observed for alpha-methyldopamine (Figure 40a, MH_2^+ = 606, 23 nanomoles/gram). The value of alpha-methyldopamine is somewhat lower, perhaps due to the shorter time between administration of the drug and sacrifice of the animal. It should also be pointed out that these studies were performed on one animal for each isomer; the numerical values are given only as rough estimates. In agreement with the value for alpha-methyldopamine, the dopamine level (MH_2^+ = 592, 33 nanomoles/gram) is lower than the earlier mentioned control values (approximately 60 nanomoles/gram).

In contrast to these results, administration of the R isomer resulted in negligible peak heights at MH_2^+ = 606, for alpha-methyldopamine. If this value is calculated, it is less than 5 nanomoles/gram of tissue. And, as expected, the dopamine levels are much higher (MH_2^+ = 592, 55 nanomoles/gram), as seen in Figure 40b. The values may be compared in Table VII.

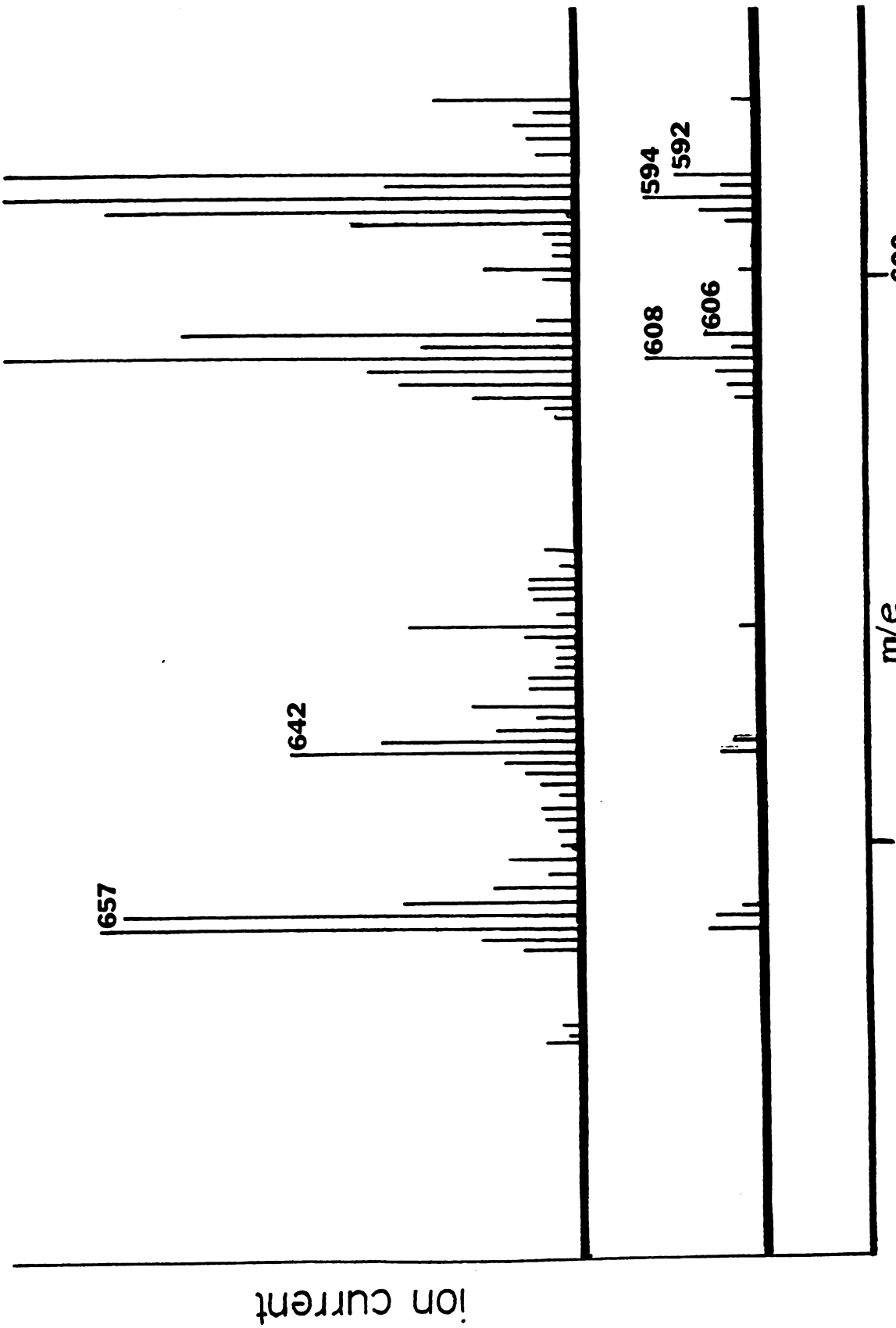


Figure 40a. Cims spectrum (180°) of amines in caudate nucleus after intraventricular administration of S-alpha-methyldopa.

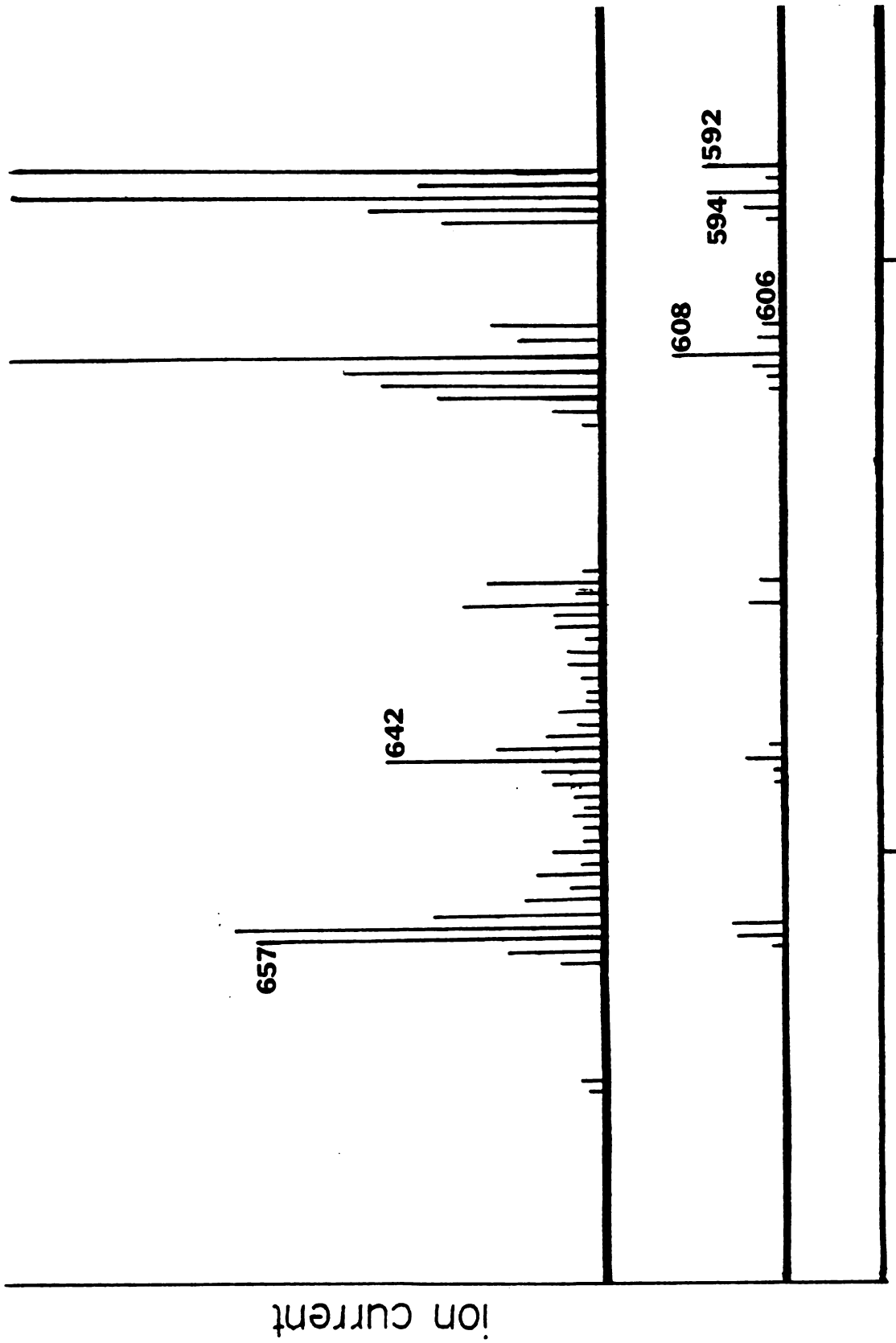


Figure 40b. Cims spectrum (180°) of amines in caudate nucleus after intraventricular administration of R-alpha-methyldopa.

The results of our *in vivo* experiments may be simply summarized. Peripheral administration of R-alpha-methyldopa does not lead to decarboxylated amine metabolites, nor does it effect the endogenous levels of dopamine in the caudate nucleus. The R isomer does not appear to cross the blood brain barrier; at least it does not penetrate the caudate nucleus under the conditions of our experiments. Furthermore, when administered centrally into the third ventricle, only the S-enantiomer of alpha-methyldopa undergoes decarboxylation.

These experiments were undertaken to better define the stereochemical control operating in the *in vivo* brain metabolism of a chiral amino acid which is not naturally occurring. Had R-alpha-methyldopa proved a substrate for brain decarboxylase then an additional parameter would have been available to unravel the detailed molecular events associated with the antihypertensive action of a clinically useful drug. Our studies show however, that not only are the enzymatic conversions of alpha-methyldopa under strict chemical control but also that the transport of this amino acid is under that control. Thus, biological specificity with respect to enantiomeric differences are as definitive for this foreign substance as for the closely related endogenous amino acid.

EXPERIMENTAL SECTION

Solvents were removed by means of a rotary evaporator under vacuum, or with a stream of N_2 by means of a Pasteur pipet, as indicated. Melting points were taken on a Thomas-Hoover apparatus and are uncorrected. Literature melting points refer to the unlabeled ^{12}C compounds. Nmr spectra were recorded on a Varian A-60A instrument in the indicated solvent with TMS as the internal standard unless otherwise indicated. Chemical ionization mass spectra were taken on an AEI MS-902 double focusing mass spectrometer equipped with a direct inlet system and modified for chemical ionization mass spectrometry. The reagent gas was isobutane at an ion chamber pressure of 0.5-1.0 torr, at the source temperature indicated. Glpc were run on a Varian Model 2100 with a U-shaped 2m x 2mm Pyrex column packed with the indicated phases. Gc/ms were run on a Hewlett-Packard 5980A quadrupole mass spectrometer (70 eV) with a source temperature of 200^{o}, transfer line temperature of 250^{o}, and separator temperature of 200^{o}. The instrument was interfaced with a Varian Model 2100 gas chromatograph with a U-shaped 2m x 2mm Pyrex column packed with 3% OV-17 on Chromasorb Q. The column temperature was 200^{o}, injection port temperature 240^{o}, and the detector temperature 260^{o}. Specific rotation measurements were taken on a Perkin-Elmer 141 electronic polarimeter. UV were taken on a Cary 14 spectrophotometer. IR were obtained on a Perkin-Elmer 337 specyrophotometer. Microanalyses were performed by the Microanalytical Labs, University of California, Berkeley, California. Gc/ms data were obtained at the University of California at Los Angeles, Department of Pharmacology, School of Medicine.

Derivatization Studies with Alpha-methyldopamine and d_2-Alpha-methyldopamine. Into individual round-bottom flasks (100 ml) were placed alpha-methyldopamine (100 microg, 0.60 micromoles), d_2-alpha-methyldopamine (110 microg, 0.66 micromoles), and a mixture of d_0- and d_2- alpha-methyldopamine (106 microg, 0.64 micromoles of each). To each of these flasks was added freshly prepared ethanolic HCl (approximately 2N, 2 ml). The flasks were heated (55^o) for 2 hr under an atmosphere of N_2. The solvent was removed by means of a stream of N_2, and to each flask was added PFPA (1.0 ml). The derivatization was allowed to proceed at room temperature for 25 min. Cims analysis was performed at 180-200^o on the reaction mixture (Figure 29 in text).

Derivatization Studies with Alpha-methylnorepinephrine. The derivatization was accomplished as described above for alpha-methyldopamine.

Derivatization Studies of a Mixture of Dopamine, Alpha-methyldopamine, Norepinephrine, and Alpha-methylnorepinephrine. To small nipple tubes (glass, 10 ml, nipple volume approximately 10 microl) were added either a solution of unlabeled dopamine, alpha-methyldopamine, norepinephrine, and alpha-methylnorepinephrine (100 microl of a standard 10 nanomoles/ml solution), a solution of d_2-alpha-methyldopamine, d_2-dopamine, $d_{6,5}$-norepinephrine, and $d_{7,6,5}$-alpha-methylnorepinephrine (100 microl of a standard 10 nanomoles/ml solution), or a mixture of both solutions in varying ratios (100-200 microl of each solution). The samples were lyophilized for 15 hr.

To each tube was then added ethanolic HCl (300 microl, 2N), the tubes capped, and heated (60^{o}) for 1 hr. The solvent was then removed by a stream of N_2 at 60^{o}, and PFPA (300 microl) added. The tubes were again capped, heated (60^{o}), and the derivatization reaction allowed to proceed for 20 min. The volume was then reduced to about half the volume of the nipple (5 microl), and the cims spectra obtained. (Figure 30,31 in text).

Derivatization Studies with Alpha-methyldopa. Into a round-bottom flask (100 ml) was placed the alpha-methyldopa (100 microg, 0.5 micromoles) and to the flask was added ethanolic HCl (2.0 ml, 2N). The flask was heated (60^{o}) for 2 hr, and the solvent removed by means of a stream of N_2. PFPA (1.0 ml) was added, and the derivatization reaction allowed to proceed at room temperature for 30 min. Cims spectra were then obtained at 180-200^{o} (Figure 32 in text).

These reaction conditions, when applied to much smaller amounts of alpha-methyldopa (0.4-1.0 microg) did not yield usable spectra. When, however, the reaction time of the esterification reaction with ethanolic HCl was lengthened to 6 hr, and the temperature increased to 70^{o}, the derivatization was successful, as described in the metabolic experimental details (see below), and discussed in the text.

Isolation of Amines and Amino Acids on Strong Cation Exchange Resin. Prior to column preparation, the resin (AG 50W-X8, Bio-Rad Coporation, Richmond, Ca.) was placed in a beaker (1 l) and rinsed several times with volumes of distilled H_2O (200-500 ml). The fine particles were poured off after each rinsing, allowing about 5 min

settling time. The hydrogen (H+) form of the resin was then prepared by addition and decantation of NaOH (2N), distilled H^2O, HCl (2N), and distilled H_2O. The resin was stored in the cold under distilled H_2O until use.

For *in vitro* studies, the resin (approximately 1.3 cc) was poured into small columns (9 x 100 mm) over a glass wool bed support. For *in vivo* studies, the resin (approximately 0.5 cc) was poured into small vitamin columns (4 x 200 mm) over a glass wool bed support. For use in the column as the hydrogen (H+) form, the resin was then rinsed with NaOH (2N, 10 ml), distilled H_2O (10 ml), HCL (2N, 10 ml), and distilled H_2O (until pH equaled that of distilled H_2O). For conversion to, and use as, the sodium (Na+), the order of the HCl and NaOH rinsings in the above procedure were reversed.

To examine the elution of alpha-methyldopa and alpha-methylnorepinephrine (being representative of the compounds isolated in the metabolism studies) under conditions utilized in the *in vitro* studies, alpha-methyldopa or alpha-methylnorepinephrine (500 microg, approximately 2 micromoles) was applied to the column in a solution (10 ml, pH 1-2) containing TCA (0.5 ml, 25%) and $MgCl_2$ (10 mg). The column was then washed with distilled water (until pH equaled that of distilled H_2O), and the compound eluted with HCl (3N, 20 ml). The applied solution, distilled H_2O wash, and acid elution (2 ml fractions) were analyzed by uv absorption at 278 nm (See Figure 33 in text). Elution studies with the amines under conditions employed in the *in vivo* studies were undertaken in this laboratory, and those results have been reported elsewhere.

Determination of Alpha-methyldopa Experimental Extinction Coefficient (Molar Absorptivity) for Use in Metabolism Studies. Due to the hygroscopicity of the alpha-methyldopa hydrochloride salt (^{13}C-1·HCl) used in the in vitro metabolic studies, solutions were made up to known concentrations by means of uv absorbance at 278 nm. Therefore, the experimental extinction coefficient was determined.

Using an electrobalance, alpha-methyldopa (5.46 mg, 25.85 micromoles) was placed in a volumetric (10 ml) flask, and diluted to volume with distilled H_2O containing 2 drops HCl (2N). A tenfold dilution of this solution was prepared with distilled H_2O to yield a 2.585 x 10^{-4}M solution. The uv absorbance was determined at 278 nm:

$$= \frac{A_{ave}}{c} \cdot 1 = \frac{0.645}{2.585 \times 10^{-4}} = 2495$$

This value (2495, lit. value 2780) was used to prepare solutions of alpha-methyldopa of known concentrations in metabolic studies, when the drug was available as the hydrochloride salt.

Alpha-methyldopa Stability Studies in Incubation Mixtures Without Tissue Homogenates. A solution of alpha-methyldopa (52.8 mg in 10 ml, 0.025M) was prepared and diluted 1:100 to give a 2.5 x 10^{-4}M solution. The uv absorbance of this solution was taken at 0 and 3 hr, both determinations showing one absorbance with a max at 278 nm, = 2495. Two drops of NaOH (1N) were then added to the cuvettes, and the uv absorbance again determined at 0, 3, and 10 min. The absorbance at 278 nm gradually shifted to approximately 300 nm, and a new very broad absorbance was detected at 420-435 nm with a gradual appearance of

color (red-purple) in the solution. Two additional solutions of alpha-methyldopa (2.5×10^{-4}M) were prepared in potassium phosphate buffer (0.1M, pH 7.4) and the uv absorbance spectra determined at varying times from 0-3.5 hr (see Table VI in text).

Rabbit Liver 12,000g Supernatant Incubation Studies With ^{13}C-Enriched (50%) Alpha-methyldopa. Six month old Dutch male rabbits were sacrificed by a blow to the head, and the livers removed and rinsed in 1.15% KCl. The livers were minced in phosphate buffer (0.1M, pH 7.4) using 2 ml buffer/gram liver. The minced tissue was homogenized with 6-8 vertical strokes in a Potter-Elvehjem teflon pestle-glass tube homogenizer. All above operations were performed in the cold room, and all further operations performed at 0-4^{o}, until the incubation was started. The homogenate was centrifuged (12,000g, 25 min), and the resulting supernatant used in the incubations, care being taken to avoid the fatty surface layer.

Study #1. The above supernatant (2 ml, 1 g liver) was added to one of two incubation flasks (50 ml), both flasks containing phosphate buffer (4 ml, 0.1M, pH 7.4) and $MgCl_2$ (1 ml, 0.05M, 50 micromoles). To both flasks was added a distilled H_2O solution (1.2 ml) containing the 50% ^{13}C-enriched alpha-methyldopa (492 microg, 2.33 micromoles) as the hydrochloride salt. This solution was prepared earlier by dissolving approximately 5 mg of the drug in distilled H_2O (10 ml) and determining the concentration by uv absorbance at 278 nm as described above. After adding NADPH (4 mg), the incubation (38^{o}) was begun in a Dubnoff metabolic shaker. The incubation was allowed to proceed 60 min, with addition of NADPH (3 mg/addition) at 20 and 40 min for a total amount of

NADPH of 10 mg (12 micromoles). The incubation was stopped by placing the flasks in an ice bath. Internal standard ^{12}C-alpha-methyldopa was then added to each flask (515 microg, 2.35 micromoles to flask containing tissue, 487 microg, 2.31 micromoles to control). Analysis was then performed as described below.

Study #2. The earlier described supernatant (3.4 ml, 2 g liver) was added to each of two incubation flasks (50 ml) containing phosphate buffer (4 ml, 0.1M, pH 7.4) and $MgCl_2$ (1.5 ml, 0.05M, 75 micromoles). The control flask was then heated until the contents boiled for 30 sec over a flame. To both flasks was added a distilled H_2O solution (3.0 ml) containing the 50% ^{13}C-enriched alpha-methyldopa (104 microg, 0.47 micromoles) as the hydrochloride salt. The solution was prepared as described above for Study #1. The incubation was begun (38^{o}) in a Dubnoff metabolic shaker after the addition of NADPH (4 mg). The incubation was allowed to proceed for 60 min, with additions of NADPH at 20 and 40 min (3 mg/addition) for a total amount of NADPH of 10 mg (12 micromoles total). The incubations were again stopped by placing the flasks in an ice bath. Internal standard ^{12}C-alpha-methyldopa (115 microg, 0.55 micromoles to the live incubation, 119 microg, 0.56 micromoles to the control incubation) was then added to each flask.

In each study (1 and 2), addition of the internal standards was followed by TCA (0.5 ml 25%), after which the flasks were allowed to stand for 10 min. All samples were then centrifuged (10,000g, 5 min), and the supernatants pipetted directly onto the cation exchange columns (H+ form) prepared as described above. After applying the solutions, distilled H_2O was washed through the columns until the pH was 4-5.

The columns were then eluted directly into round-bottom flasks (100 or 250 ml) with HCl (3N, 25 ml). The clear, colorless eluants were then lyophilized. The dried solids were then treated with ethanolic HCl (2N, 3 ml) at 55° under an atmosphere of N_2 for 2 hr. After removal of the solvent with a stream of N_2 at 55°, PFPA (2 ml) was added, and the derivatization reaction allowed to proceed at room temperature for 30 min. Cims analysis was immediately performed on the samples. Results of these studies are reported in the text (Figure 35).

Rat Kidney 12,000g Homogenate Incubation Studies with ^{13}C-Enriched Alpha-methyldopa. Adult male Wistar rats were sacrificed by stunning and decapitation after which the kidneys were removed. The fatty tissue around the kidneys was removed, and the organs rinsed in distilled H_2O. The tissue was immediately minced in distilled H_2O (4 ml/gram kidney), and then homogenized in a Potter-Elvehjem tissue homogenizer. All above operations were performed in a cold room. To one of two incubation flasks (50 ml), both containing phosphate buffer (1.8 ml, 0.1M, pH 7.4) and a distilled H_2O solution (1 ml) containing pyridoxal-5-phosphate (29.5 microg, 45 nanomoles), was added the crude homogenate (1 ml, 0.25 g kidney). To the other flask (control) was added distilled H_2O (1 ml). The flasks were preincubated in a Dubnoff metabolic shaker (38°, 15 min), after which a distilled H_2O solution (0.15 ml) of 50% ^{13}C-enriched alpha-methyldopa (100 microg, 0.47 micromoles) was added to both flasks. The alpha-methyldopa solution was prepared as described above for the liver incubation studies. The incubation (38°) was allowed to proceed for 75 min, after which the incubation was stopped by placing the flasks in an ice bath. To

both flasks was immediately added ^{12}C-alpha-methyldopa internal standard (103 microg, 0.49 micromoles). TCA (0.55 ml, 25%) and phosphate buffer (5 ml, 0.1M, pH 7.4) was then added to both flasks, and they were allowed to sit for 10 min. The mixtures were then centrifuged (10,000g, 10 min), and the supernatants applied to the cation exchange columns (H + form) prepared as described earlier. The columns were rinsed with distilled H_2O (15 ml), after which the pH of the column rinse was determined to be the same as distilled H_2O (4-5). The columns were then eluted with HCl (3N, 25 ml), and the eluants lyophilized. The solids were then treated with ethanolic HCl (2N, 2 ml) for 2 hr at 55^o. After removal of solvent with a stream of N_2 at 55^o, PFPA (1 ml) was added to each flask, and the derivatization reaction allowed to proceed for 30 min at room temperature. The samples were immediately analyzed by cims (180-200^o). The results of these experiments are described in the text.

Rat Kidney 12,000g Homogenate Incubation with DOPA. Adult male Wistar rats were sacrificed by stunning and decapitation after which the kidneys were removed and rinsed in distilled H_2O. The fatty tissue was removed, and the kidneys minced in phosphate buffer (0.02M, pH 7.4, 4ml/gram kidney). The minced tissue was then homogenized with 6-8 strokes of a Potter-Elvehjem tissue homogenizer. All the above operations were performed in the cold. To three incubation flasks (25 ml) was added phosphate buffer (0.5 ml, 0.02M, pH 7.4) containing pyridoxal-5-phosphate (11 microg, 45 nanomoles). To two of the three flasks was then added the crude homogenate (1 ml, 0.21 g tissue), and to the third flask was added distilled H_2O (1 ml). While the flasks

were preincubating for 10 min (38°) in a Dubnoff metabolic shaker, the substrate DOPA (6.33 mg, 31.1 micromoles) was dissolved in phosphate buffer (1.5 ml, 0.02M, pH 7.4). After 10 min preincubation, the incubations were begun by the addition of the DOPA solution to flask A (0.5 ml, 2.11 mg, 10.7 micromoles), addition of the DOPA solution to flask B (0.1 ml, 0.422 mg, 2.14 micromoles), and addition of the DOPA solution (0.5 ml, 2.11 mg, 10.7 micromoles) to flask C (control). The incubation was allowed to proceed for 50 min, after which the flasks were placed in an ice bath to stop the reaction. To flasks A and C was immediately added the d_3-DOPA internal standard (0.5 ml of a distilled H_2O solution, 2.10 mg, 10.66 micromoles). To flask B was added the same solution (0.1 ml, 0.421 mg, 2.10 micromoles). To all three flasks was then added TCA (0.2 ml, 25%), and the solutins allowed to sit for 10 min. The incubation mixtures were centrifuged (10,000g, 10 min), and the supernatants directly applied to the cation exhange column prepared as described previously. After rinsing with distilled H_2O (15 ml, pH of eluant then 4-5), the columns were eluted directly into round-bottom flasks (100 ml) with HCl (3N, 25 ml). The samples were lyophilized, and the dried solids treated with ethanolic HCl (2N, 1 ml) for 2 hr at 55°. The solvent was then removed with a stream of N_2 at 55°, and PFPA (1 ml) added. The flasks were allowed to sit at room temperature for 30 min, and the cims spectra immediately obtained (180-200°, see text and Figure 36).

In Vivo Studies, General Procedures. Adult male Wistar rats (300-350 g) were employed in all in vivo studies. Drugs were administered in a solution of normal saline (0.9%) which was also 0.1N in

HCl, by intraperitoneal (ip) injection, except where indicated otherwise. After appropriate times, the animals were sacrificed by stunning and decapitation. The brain was immediately removed, the caudate nucleus and hypothalamus removed, and the tissue frozen (-70^{o}) until analyzed.

For analysis of amines, the tissue was thawed and immediately homogenized after addition of TCA (3 ml, 5%) containing sodium metabisulfite (0.5% final concentration) and appropriate amounts of internal standards. The pH was then adjusted to 4.0-4.2 by addition of NaOAc buffer (0.5M, pH 5.95). Solutions were then centrifuged (15,000g, 15 min) and the supernatant fraction pipetted onto strong cation exchange resin columns (0.5 cc, prepared as described earlier) prepared as the sodium (Na+) form. To each vitamin column was then added sequentially:

5 ml 0.5M NaOAc/HOAc buffer (pH 5)
10 ml 0.1N HCl
10 ml anhydrous EtOH
8 ml 2N ethanolic HCl

The ethanolic HCl (8 ml, 2N) eluant was collected in nipple tubes and after cooling, lyophilized.

For analysis of amino acids, the thawed tissue was homogenized after addition of TCA (2.5 ml, 5%) containing sodium metabisulfite (0.5% final concentration) and appropriate amounts of internal standards. The pH was not adjusted prior to centrifugation (15,000g, 15 min). The supernatant fraction was pipetted onto strong cation exchange columns (0.5cc, prepared as described earlier) in the hydrogen (H+) form. To each column was then added sequentially:

10 ml 0.1N HCl
5 ml anhydrous EtOH
10 ml 2N ethanolic HCl

The ethanolic HCl (10 ml, 2N) eluant was collected in nipple tubes and after cooling, lyophilized-

To all lyophilized samples (amines and amino acids) were added ethanolic HCl (0.25 ml, 2N). The nipple tubes were capped and heated (60^{o}), the amine samples for 1 hr and the amino acid samples for 2-6 hr (see below), and the solvent removed with heating (60^{o}) with a stream of N_2. PFPA (0.25 ml) was then added, the tubes capped, and the derivatization reaction allowed to proceed with heating (60^{o}) for 20 min. The volume was then reduced to 10 microliters with a stream of N_2 and heating (60^{o}).

Cims spectra were then obtained by the direct probe insertion technique at 170-185^{o}. The appropriate portion of the spectra were scanned 2-5 times, and peak heights measured with a mm ruler. No ion intensities of less than 10 mm above the average background signal (noise) were used for quantitative data. Values described in the text for the amines and amino acids in tissues were determined as described there, by averaging the values of the 2-5 scans from each sample.

Determination of Amine Metabolites in Caudate Nucleus and Hypothalamus after Administration of ^{12}C-R, ^{12}C-S, ^{12}C-RS, ^{13}C-S, and ^{13}C-s/^{12}C-R Alpha-methyldopa. ^{12}C-R or ^{12}C-S alpha-methyldopa (approximately 60 mg) was administered ip in a total volume of 2.0 ml (0.9% saline-0.1N HCl) while ^{12}C-RS drug (30-40 mg of each isomer) was administered in 2.5-3.0 ml of the same solution. ^{13}C-S and ^{13}C-S/^{12}C-R compounds were administered in the same manner. The rats were sacrificed 4-6 hr after administration of the drug. Before homogen-

ization, internal standards (d_2-dopamine, d_2-alpha-methyldopamine, $d_{6,5}$-norepinephrine, and $d_{7,6,5}$-alpha-methylnorepinephrine) were added to the isolated caudate nucleus (2 nanomole each in a total volume of 0.2 ml of a 0.1N HCl solution) or hypothalamus (1 nanomole each, 0.1 ml of the same solution). Control rats were administered no drugs, and after sacrifice, internal standards were added as in the dosed animals. Homogenization and amine analysis were performed as described under General Procedures (see above). Cims spectra were obtained as described there. For results, see the text (Table VII, Figures 37-38).

Determination of Alpha-methyldopa in the Caudate Nucleus after Administration of ^{12}C-R, ^{12}C-S, and ^{13}C-S/^{12}C-R Alpha-methyldopa.

^{12}C-R, ^{12}C-S, or ^{13}C-S/^{12}C-R alpha-methyldopa (approximately 60 mg each isomer alone, and 60 mg of each isomer in the pseudo-racemic mixture) was administered ip in a volume of 2.0-2.5 ml (0.9% saline-0.1N HCl). The rats were sacrificed 1.0-1.5 hr after administration of the drug. Caudate nuclei from animals receiving the individual unlabeled isomers (^{12}C-S or ^{12}C-R) were homogenized and analyzed for amino acid (as described in General Procedures, see above) after addition of either d_3-alpha-methyldopa (2 nanomoles in 0.2 ml of 0.1N HCl solution) or ^{13}C-alpha-methyldopa (5 nanomoles in 0.5 ml of the 0.1N HCl solution). Caudate nuclei from animals receiving the pseudo-racemic mixture were homogenized and analyzed similarly, but without addition of internal standard amino acid. Control animals received no drugs, and the tissue analyzed after addition of internal standards. Cims spectra were obtained as described above, for results see the text (Figure 39).

Determination of Amine Metabolites after Intraventricular Administration of ^{12}C-R and ^{12}C-S Alpha-methyldopa. For intraventricular drug administration, a 22 gauge stainless steel cannula was stereotaxically implanted into the third ventricle under pentobarbital (Nembutal) anesthesia (50 mg/kg). The following day each rat received two intraventricular injections, 1 hr apart, of either ^{12}C-S or ^{12}C-S alpha-methyldopa (300 microg in 10 microliter solution) under light ether anesthesia. In this experiment only, the drug was administered in 0.9% saline (1 ml) containing 2 drops 2N HCl. The animals were sacrificed 2.5-2.8 hr after administration of the drug. The caudate nuclei were homogenized and analyzed (as described under General Procedures, see above) after addition of the amine internal standard solution (2 nanomoles of each standard, 0.2 ml total volume). Cims spectra were obtained as described above (General Procedures), and the results are described in the text (see Figure 40).

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