

"It is quite natural that enzyme chemists have, thus far, been occupied with the discovery of many kinds of enzymes, the ingenious methods of preparing them, and the measurement of their activity. But at this point we must inquire into the chemical mechanism by which they work."

L. Michaelis 1946

PREFACE

Monoamine oxidase plays an important role in the physiological processes of animals and humans, carrying out oxidative deaminations of a variety of biogenic amines. Some of these amines have been implicated in mental disorders. Due to the availability of potent inhibitors of the enzyme, a number of applications in the field of the chemotherapy of mental diseases have been derived in recent years.

Although much has been said about the physiological effects of such inhibitors, our knowledge of the nature and mechanism of action of this enzyme is still extremely limited. It is largely confined to the recognition of general types of structures which can act as either substrates or inhibitors. No adequate rationalizations have been put forth as to structure-activity relationships amongst substrates or inhibitors although speculations have been advanced by various groups of investigators.

The enzyme is strongly bound to the particulate fraction of cells and has defied numerous attempts at purification. As a consequence, we lack such basic information as to the nature, composition and cofactor requirements

of the enzyme. Therefore, it appeared that new approaches were necessary if any progress was to be made in the interpretation of the mode of action of inhibitors, a field rich in practical applications.

Since nothing was known about the rate limiting step in the oxidation of amines to aldehydes by monoamine oxidase, it was decided to investigate the possibility that substitution of the alpha-hydrogens for deuterium might lead to the observation of a kinetic isotope effect. If present, the information gained would be pertinent to the nature of the transition state and Michaelis complex involved as intermediates in the breakdown of the amine. The possibility also arose that kinetic isotope effects, if present, might be stereospecific and thus pertinent to the mechanism of action of the enzyme.

A simple chemical model was examined and confirmed the possibility of an isotope effect with labelled amines. These studies were then extended to the enzyme system and along with inhibitor studies furnished an insight into the relationship between the enzyme, its inhibitors and substrates.

The introduction consists of a brief historical review followed by a more detailed critical account of past and current investigations which are pertinent to the

problem set forth.

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TABLE OF CONTENTS

	<u>Page</u>
I. INTRODUCTION	
A. Review of Monoamine Oxidase	10
1. Definition	10
2. Historical	11
3. Occurrence	14
4. Properties	16
5. Co-Factor Requirements	18
6. Assay Procedures for MAO Activity	21
7. Substrate Specificity	22
8. Absolute Stereospecificity of MAO	27
9. Inhibitors	28
10. Physiological Role of Monoamine Oxidase ..	31
B. Deuterium Isotope Effects in Biochemistry	36
C. Nature and Scope of This Investigation	38
II. EXPERIMENTAL	
A. Materials	39
1. Syntheses	39
a. Preparation of α -d ₂ -Mescaline	39
b. Preparation of α -d ₂ -Benzylamine	40
c. Preparation of α -d ₂ -Tryptamine	40
d. Preparation of α -d ₂ -Kynuramine	40
e. Preparation of β -d ₂ -Kynuramine	41
f. Preparation of 2-Benzylseridine	42
g. Preparation of 2-Phenylseridine	42
2. Preparation of Mitochondrial Monoamine Oxidase	42
B. Methods	43
1. Assay Using Tyramine As Substrate	43
2. Assay Using Kynuramine As Substrate	45

	<u>Page</u>
3. Model Studies	46
C. Results	47
1. Model Studies	47
2. Oxidation of Deuterium and Protium Substrates	53
a. Tyramine and α - d_2 -Tyramine	53
b. Stereospecifically Labelled Tyramine	56
c. Michaelis Constants	58
d. Competition Between Tyramine and α - d_2 -Tyramine for the Enzyme	58
e. Isotope Effects with α - d_2 - and β - d_2 - Kynuramine	62
3. MAO Inhibitor Studies	65
a. Determination of pI 50 for a Series of Phenylalkylamines	65
b. Type of Inhibition	68
c. Reaction of Ninhydrin with d_1 -trans- 2-Phenylcyclopropylamine	70
d. Incubation of l -trans-2-Phenylcyclo- propylamine for 24 Hours	70
III. DISCUSSION	71
A. Model Studies	73
B. <u>Alpha-Deuterium</u> Isotope Effects	75
C. Absolute Optical Stereospecificity	78
D. <u>Beta-Deuterium</u> Isotope Effects	80
E. The Nature of the Transition State	83
F. Correlation of Substrate Structure with Relative Rates of Oxidation by MAO	84
G. Interpretation of Structure-Activity Relation- ships Amongst Inhibitors of the Phenylalkyl- amine Series	86
IV. CLAIMS TO ORIGINAL RESEARCH	96
V. BIBLIOGRAPHY	98

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1.	Relative rates of reaction of tyramine and α - d_2 -tyramine with ninhydrin in butanol.	48
2.	Standard curve for percent as α - d_2 -tyramine vs. optical density.	51
3.	Relative rates of reaction of tyramine and α - d_2 -tyramine in butanol - 3% pyridine.	52
4.	Relative rates of oxidation by monoamine oxidase for tyramine and α - d_2 -tyramine at a substrate concentration of $1.15 \times 10^{-3}M$.	54
5.	Relative rates of oxidation by monoamine oxidase for tyramine and α - d_2 -tyramine at a substrate concentration of $3.4 \times 10^{-3}M$.	55
6.	Relative rates of oxidation by monoamine oxidase for tyramine, α - d_2 -tyramine, R- and S- α - d -tyramine.	57
7.	Michaelis-Menten constants for tyramine and α - d_2 -tyramine.	59
8.	Relative rates of oxidation by monoamine oxidase for kynuramine, α - d_2 -kynuramine and β - d_2 -kynuramine.	63
9.	Michaelis-Menten constants for kynuramine and α - d_2 -kynuramine.	64
10.	Percent inhibition vs. log concentration of phenylaziridine.	66
11.	Competitive inhibition of monoamine oxidase by amphetamine and d -trans-2-phenylcyclopropylamine.	69
12.	Interactions in the transition state for substrate oxidation by monoamine oxidase.	92
13.	Mode of interaction between 2-phenylcyclopropylamine and monoamine oxidase.	92

LIST OF TABLES

<u>Table</u>		<u>Page</u>
I	Kinetic isotope effects with a series of phenylalkylamines.	47
II	Analysis of a mixture containing 70% α - d_2 -tyramine and 30% tyramine.	49
III	Rate ratios for oxidation of tyramine and α - d_2 -tyramine at various substrate concentrations.	53
IV	Ratios of maximum velocity and Michaelis constants for tyramine and α - d_2 -tyramine.	58
V	Analysis of swine mixture isolated from incubation of α - d_2 -tyramine and tyramine with monoamine oxidase.	60
VI	Composition of swine mixture after incubation of tyramine and α - d_2 -tyramine with monoamine oxidase.	61
VII	pI 50 values for a series of phenylalkylamines and oxiridines.	67

ABSTRACT

In order to test the possibility of the operation of deuterium isotope effects when an alpha-carbon-hydrogen bond is broken to liberate a proton, a simple chemical model is examined. This consists of the reaction between alpha-bis-deuterated amines and ninhydrin. The large isotope effect observed suggested a method for the quantitative determination of the composition of mixtures of deuterated amines and their hydrogen isomers.

Extending the use of deuterium labelled substrates, such as alpha-bis-deuterio-tyramine and kynurexine to monoamine oxidase, it is shown that a fair degree of bond weakening in the transition state accompanies the oxidation of these substrates by the enzyme. Relative affinities of the labelled vs. non-labelled tyramine for the enzyme are determined by the Michaelis constants and confirmed by a special application of the ninhydrin method. The establishment of an absolute stereospecificity of MAO towards the alpha-hydrogens of tyramine is described.

In order to gain deeper insight into the nature of the transition state, studies of the occurrence of isotope effects with beta-deuterated amines are described. It is found that such labelling leads to rate retarding effects in the oxidation reaction, thus implying some bond weakening of

the beta-carbon-hydrogen bonds in the transition state.

Concurrent investigations with a series of inhibitors, including the extraordinarily potent 2-phenyl-cyclopropylamine, lead to a rationalization of the mechanism of action of some of these inhibitors as well as generalizations in the field of substrate-activity relationships.

INTRODUCTION

A. REVIEW OF MONOAMINE OXIDASE

1. DEFINITION

Classification of the amine oxidases is at best beset with difficulties and it seems advisable to define these enzymes in such a way that misunderstandings are reduced to a minimum. This group of enzymes has been subdivided according to the substrates which they oxidize, i.e., monoamines by monoamine oxidase, diamines by diamine oxidase, benzylamine by benzylamine oxidase and spermine by spermine oxidase. This classification has been shown to be arbitrary by Fouts, Blankens, Carbon and Zeller (1) since there is a profound lack of substrate specificity displayed by each group. In spite of this inappropriate nomenclature, it is convenient to use the terms monoamine oxidase (MAO) and diamine oxidase (DO) since each enzyme can be distinguished by other criteria.

Monoamine oxidase may be defined as the enzyme which is responsible for the oxidative deamination of such amines as tyramine, 3,4-dihydroxyphenethylamine, serotonin, adrenaline and isocamylamine. Monoamine oxidase is not inhibited by semicarbazide, whereas, DO is completely blocked (2), as are the other amine oxidases (3, 4, 5). In this definition semicarbazide cannot be replaced by the term

carbonyl reagent, since many monosubstituted alkyl- and arylhydrazines block both DO and MAO (6) while monosubstituted acylhydrazines and hydrazine itself, inhibit DO (7) exclusively.

Early reviews on monoamine oxidase have appeared by Zeller (8) and Blaschko (9). Davison (10) and Sourkes (11) have published extensive reviews which cover the literature up to 1958.

2. HISTORICAL

In 1877 Schmiedeberg (9) discovered that benzylamine given by mouth to dogs was excreted as benzylglycine. He recognized that this involved the intermediate formation of benzoic acid and thus furnished the first indication that amines could be degraded in vivo by decamination. It has since been demonstrated that benzylamine oxidase is a distinct enzyme belonging to the semicarbazide-sensitive group of oxidases.

Evins and Laidlaw (12), as early as 1910, were able to show that tyramine could be decaminated in the isolated liver of the cat or rabbit. In perfusion experiments they demonstrated that tyramine was broken down to p-hydroxyphenylacetic acid in 70% yield. Later (13) they isolated indoleacetic acid as a breakdown product of tryptamine. Eighteen years later, Hare (14) was able to demonstrate the

presence of an enzyme which she called "tyramine oxidase" in liver. Pugh and Quastel (15) found an "aliphatic amine oxidase" in various mammalian tissues. Blaschko, Richter and Schloesman (16) described an enzyme capable of oxidising adrenaline.

"Tyramine oxidase", "adrenaline oxidase" and "aliphatic amine oxidase" were shown to be identical independently by Blaschko's group (16), Pugh and Quastel (17) and Kohn (18). Zeller (8) later classified this enzyme as monoamine oxidase on the basis of what was then known about its substrate specificity.

In the older work it was assumed that the deamination process was a result of hydrolytic action



after which the alcohol was further oxidized to the corresponding acid (19). The possibility of an oxidative pathway was first suggested by Bernheim (20), who formulated the reaction as



The above equations give a true picture of the reaction when catalase is absent. However, in crude tissue preparations

catalase is usually present and the hydrogen peroxide is broken down as follows



which leaves the overall reaction catalyzed by MO to be



in which one atom of oxygen is absorbed for each molecule of substrate oxidized. Bernheim (20) has shown that the oxygen consumption varies from one to four atoms per mole of substrate depending on the pH of the medium, age and concentration of the enzyme preparation. However, in the presence of cyanide only one atom of oxygen per mole of substrate is taken up which has led many workers (21,22,23) to add cyanide to the incubation mixture. Creasey (24), using a preparation of washed rat liver mitochondria, has shown the difference in oxygen consumption to be due largely to further oxidation of the primary deamination products. Many of these aldehydes undergo spontaneous oxidation in the presence of air. He concluded that a buffered solution containing MAO, substrate, semicarbazide and cyanide would eliminate the endogenous oxygen uptake of crude MAO preparations and thus put manometric measurements on a truly quantitative basis.

Davison (25) has recently pointed out that shaking

monoamine oxidase in oxygen with added cyanide sensitizes the enzyme to certain inhibitors, a factor which must be considered in interpreting results of a number of investigations by Randall (23), and others (21,26).

Richter (27) demonstrated the formation of aldehydes by the chemical and crystallographic analysis of the 2,4-dinitrophenylhydrazones. Without special precautions, such as trapping the aldehyde as the semicarbazide, further oxidation to the corresponding acid occurs as was the case in Ewin's and Laidlaw's work mentioned earlier. Richter, using tyramine, repeated these authors' work and recovered the aldehyde quantitatively as the semicarbazide. In quantitative experiments he demonstrated that one mole of ammonia is liberated for each atom of oxygen consumed, or in the case of secondary and tertiary amines, one mole of a volatile alkylated amine.

3. OCCURRENCE

Monoamine oxidase has been found in all classes of vertebrates studied and in many invertebrate phyla (16) as well as in plants. However, it would appear that the semicarbazide-sensitive plant monoamine oxidase is not identical to animal MAO. Earlier studies on MAO activity in various tissues were accomplished by grinding the tissue with sand followed by centrifugation which resulted in an

unknown loss of activity. Present techniques of homogenizing the tissue yield a more accurate picture of enzymatic activity although difficulties are encountered with tough fibrous tissue. These factors must be borne in mind when comparing data on amine oxidase activity.

Bhagvat, Blaschko and Richter (26) found very high activity in the livers of rats, pigs, sheep and oxen. Kidney levels were also high in all species with the exception of rats. High activity has also been found in the intestine, stomach, and blood vessels of various species. The heart also contains MAO activity although there are marked species differences. Adrenals, thyroid, uterus, pancreas, placenta, veins, lung and spleen all have moderate monoamine oxidase activity in the mammalian species studied. Enzyme activity is very weak or absent in plasma, skeletal muscle and blood.

Pugh and Questel (15), who first studied MAO activity in nervous tissue, found the brain to be active in this respect. MAO is also present in the stellate ganglia of the dog (9). This has led investigators to search for the enzyme in other structures with adrenergic innervation. It has been shown that MAO occurs in the nictitating membrane and iris of the cats and rabbits (29). This was confirmed by Koelle and his associates (30) and is in agreement with the assumption that substances such as tyramine are protected from degradation by the blocking of MAO by

iproniazid. It is interesting in this connection that Bellesu, Burba, Pindell and Reiffenstein (31) were able to demonstrate in vivo that the MAO in the nictitating membrane shows the same absolute optical stereospecificity as does the enzyme in rat liver, thus suggesting some important degree of similarity between these enzymes from different sources.

Monamine oxidase is usually located where true cholinesterase levels are low (32,33). For example, nervous tissue contains low levels of MAO but high levels of cholinesterase, whereas intestine contains large amounts of MAO and much less cholinesterase. It has long been thought possible that just as cholinesterase is responsible for destroying acetylcholine, monamine oxidase may in a like fashion inactivate an excess of a neurohormonal transmitter such as serotonin or dopamine. At the present time, there is little evidence for any parallelism although the possibility has been investigated by Koelle and Valk (30).

4. PROPERTIES

The most characteristic property of monamine oxidase is its localization in the insoluble cell constituents. Hawkins (34) has determined the enzymatic activity of various fractions of rat liver homogenates and found the enzymatic activity to be present in only the

particulate matter. She found that two-thirds of the activity was present in the mitochondrial fraction while the remainder was associated with the microsomes. Cotzias (35) arrived at essentially the same conclusions. It is currently accepted that the microsomes and mitochondria account for the total activity of liver homogenates. These observations may make one ask; can monoamine oxidase be considered as a single entity?

Worle and Keover (36) considered that there is more than one type of MAO. These workers were able to separate enzymes from animal sources capable of oxidizing only aliphatic monoamines or only aromatic amines. Allen and Hoegard (37) tested a large series of typical substrates with liver extracts from several species and found marked species differences as judged by the relative rates of oxidation. Setake (38) has suggested that MAO may be a mixture of enzymes which have different substrate specificity and each tissue may contain a distinct composition of the enzyme system. This brings to mind the recently described isozymes of lactic dehydrogenase (39). That is, MAO could be a mixture of enzymes which are electrophoretically distinct.

Two sets of observations can be quoted (9) as supporting the homogeneity of MAO: 1) oxidation of representative substrates occurs in all organs in which amines

are oxidized; 2) two substrates incubated together at the same time are oxidized at a rate intermediate between the rates of oxidation of the two amines when tested separately. These criteria can be misleading and further purification of the enzyme or enzymes will have to be carried out before the question of homogeneity can be answered.

Mitochondrial preparations can be subjected to lyophilization and stored for several months without loss of activity (40) as can acetone-dried powders from some species (9). Preparations in isotonic sucrose (0.25M) can be stored at room temperature for a day without loss of activity and at zero degrees for several weeks. Heating for 10 minutes at 50°C. destroys half of the activity of the enzyme. The enzyme is inactivated at pH's below 6 and above pH 9-10. The optimum pH for tyramine oxidation is 7.4 (10).

5. CO-FACTOR REQUIREMENTS

There are no known co-enzymes or prosthetic groups associated with MAO. The insensitivity of the enzyme to most carbonyl reagents would seem to eliminate pyridoxal phosphate as a co-factor. In 1942, Friedenwald and Herrmann (41) suggested that monoamine oxidase possesses a sulfhydryl group which is essential for activity. The enzyme was inhibited by organic mercurial compounds. Reversal of

inhibition was obtained by adding glutathione or cysteine in the presence of cyanide. However, it was later shown (42) that cyanide reacted with the organo-metallic reagent used in the above experiments and this accounted for the re-activation. Lagnado and Sourkes (43) found that dimercaprol, thioglycolic acid, cysteine and cystine exert inhibitory effects in vitro. This is in agreement with the view that the sulfhydryl group functions in the oxidation of the substrate and that an excess of some -SH compound or of a disulfide could inhibit MAO at one phase of the catalytic process.

Heavy metals such as mercury, arsenic, silver and cadmium (43) inhibit the enzyme although this is not conclusive evidence that the sulfhydryl group combines with one of the heavy metals. The fact that a number of sulfhydryl compounds inhibit the reaction provides a strong basis for the view that an -SH group is necessary in the mechanism of action of the enzyme.

Because of the similarity of MAO to D-amino acid oxidase, a flavin dinucleotide containing enzyme, Richter (28) suggested that MAO may well be a flavoprotein. There is little experimental evidence to justify this suggestion although Mahler (44) has included this enzyme in his classification of yellow enzymes. Hawkins (35), studying MAO activity in riboflavin deficient rats, found a decrease in

MAO activity in the livers of deficient rats and postulated that the vitamin is involved in the synthesis of the apoenzyme.

There has been some interest in determining whether the enzyme contains a heavy metal as do most of the flavo-proteins. Corbin (40) has shown that the MAO of rat liver and brain mitochondria is reversibly inhibited by various chelating reagents such as 8-hydroxyquinoline, cyclohexanediamine tetraacetate, and diethyldithiocarbamate. Partial removal of the inhibition caused by 8-hydroxyquinoline and cyclohexanediamine tetraacetate is achieved by addition of some divalent metals, particularly the ions of zinc and copper. This author concludes that MAO has the properties of a true metalloenzyme.

It is probable that MAO, like other oxidase systems, consists of a dehydrogenase linked to a respiratory chain which may include cytochromes or flavins. Legendre and Sourkes (45) have been successful in coupling amine dehydrogenase directly with tetrazolium salts so that the electron-transporting system is by-passed. They have shown a requirement for a heat stable co-factor present in boiled extracts of rat brain in this system, although its identity remains unknown. Actual characterization of the enzyme will be necessary to show whether a flavin nucleotide acts as the primary electron acceptor.

6. ASSAY PROCEDURES FOR MAO ACTIVITY

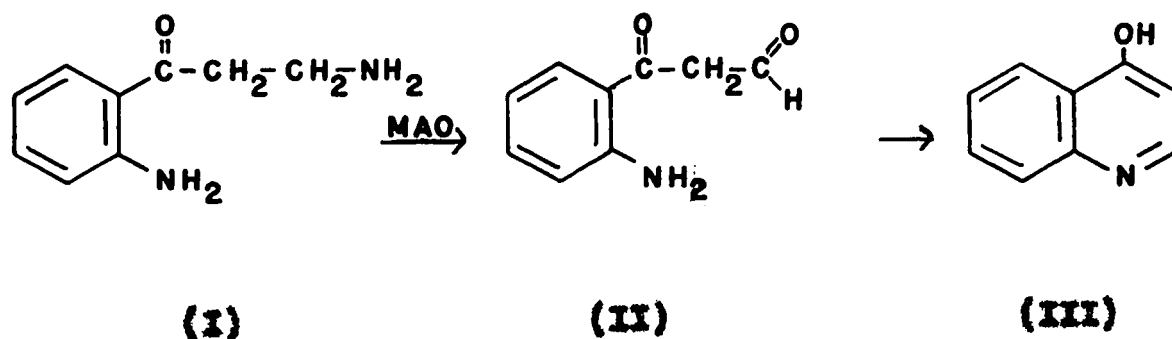
Monocamine oxidase activity is usually measured manometrically because of the convenience of the method and the fact that any amine can be assayed by this procedure. The chief drawbacks to this method are: 1) it lacks sensitivity; 2) unless cyanide is added, it is difficult to determine if the oxygen uptake is due to the MAO-catalyzed reaction alone; 3) added cyanide, which eliminates endogenous oxygen uptake, is unsatisfactory in determining inhibitory powers of compounds in view of Davison's findings (supra vide); 4) Aebi (46) has demonstrated that relative substrate-enzyme affinities can be varied by varying the oxygen tension. Measurement of ammonia liberation is more accurate but is limited by serious technical difficulties.

Green and Haughton (47) developed an assay procedure whereby the enzymatically formed aldehyde is trapped as the semicarbazide followed by conversion to the 2,4-dinitrophenylhydrazone which is then determined quantitatively by spectrophotometric means. The procedure is quite involved and hardly suitable for large scale incubations.

Several authors (48,49) have developed methods for specific substrates, such as tyramine and serotonin, based on the extraction of the amine from alkaline solution and then re-extraction into dilute HCl. The solution is then

assayed spectrophotometrically, colorimetrically, or by spectrofluorometry, depending on the nature of the amine and the sensitivity desired.

Recently, Weissbach, et.al. (50), found that kynurexine (I) is rapidly oxidized by MAO. The enzyme oxidatively deaminates the amine to the aldehyde (II), which undergoes intramolecular (nonenzymatic) condensation to 4-hydroxyquinoline (III).



The overall reaction can be conveniently followed in a recording spectrophotometer. The rapid and exact determination of the disappearance of kynurexine (360 m μ), and/or the appearance of the product, 4-hydroxyquinoline (310 to 335 m μ) can be followed in situ.

7. SUBSTRATE SPECIFICITY

Monooxamine oxidase deaminates a wide variety of amines of the general formula R-CH₂-NH₂ where R may be a substituted aryl or alkyl group or even an amino-alkyl

chain. The amino group may be substituted. Hence, the enzyme displays a fairly poor substrate specificity and it appears that it can even oxidise a greater variety of diamines than diamine oxidase itself.

The presence of a second amino group, as in diamines of the type $\text{NH}_2(\text{CH}_2)_n\text{NH}_2$, interferes with the substrate specificity of MAO by decreasing the affinity of the lower members of the series ($n = 2$ to $n = 6$). Beginning with the member having 7 methylene groups, oxidation then increases with chain length. A maximum rate of oxidation is reached with $n = 13$; beyond this, the rate of oxidation decreases with increasing chain length (51,52,53). The reason for the disturbing influence of the second amino group is not well-known although it has been shown that this interference decreases with increasing chain length and it has been postulated that with an increasing number of methylene groups, the orientation of the diamine at the enzyme surface becomes more like that of the monoamines.

Members of the aliphatic series $\text{CH}_3(\text{CH}_2)_n\text{NH}_2$ are substrates for the enzyme and the rate of oxidation is dependent upon chain length. The lowest member, methylamine, is not attacked at all whereas the homologue, ethylamine, is oxidised slowly by MAO. A maximum rate of oxidation is reached at $n = 4$, $n = 5$ and with a further increase in chain length the rate of oxidation falls off.

Some branched chain aliphatic amines such as isocamylamine are oxidized readily by MAO (16,54).

Aromatic amines such as aniline are not oxidized at all (16). Benzylamine and its p-sulphamido derivative are oxidized very slowly (55) by MAO and the rate of oxidation rises markedly with the homologue, beta-phenethylamine. Beta-phenethylamine and its derivatives have been the classical substrates studied because the group contains many "biogenic amines", e.g., tyramine, dopamine, and sympathin.

Randall (56) conducted a systematic study of progressive N-methylation and found in a series of substituted phenylethylamines that the tertiary amines were always oxidized less rapidly than the primary and secondary amines. It has also been shown that if the N-substituent is large, e.g., ethyl, isopropyl, the rate of deamination is much slower. Quaternary ammonium compounds are not degraded although it was recently shown that an N-oxide can be oxidized (57).

In the case of phenethylamines and tryptamine, aromatic ring substitution to form phenolic derivatives yields compounds which are the best known substrates for MAO. Tyramine, adrenaline, 3-hydroxy-tyramine (dopamine) and serotonin belong to this group. Para-hydroxyl substitution of phenethylamines leads to compounds more readily

oxidisable than the parent compound. The meta-substituted analogues are also good substrates and are better than the ortho-compounds (58). Methylation of the para- and meta-substituted hydroxyl groups results in less active substrates but a better substrate results in the case of ortho-hydroxyl substituted compounds. It has been suggested (56) that in the case of the unsubstituted ortho-compound, chelation with a metal occurs between the amino and ortho-hydroxyl groups thus interfering with complex formation. Methylation would suppress this interaction thus facilitating the formation of a substrate-enzyme complex. On the other hand, methylation of the 3-hydroxyl group in adrenaline or noreadrenaline has no effect since metanephrine and normetanephrine are oxidised at approximately the same rate as their phenolic counterparts (53).

It is interesting to note that para-hydroxylation of benzylamine yields results opposite to those in the phenethylamines series, the compound being inert as a substrate (59). Hydroxylation of tryptamine produces different results. Serotonin (5-hydroxytryptamine) is a good substrate whereas the 7-hydroxy compound is oxidised slower. Substitution in the 6 position produces a substrate which is oxidised 85% slower than serotonin (60).

Substitution in the beta-position of phenethylamine may or may not result in compounds susceptible to

oxidation. If both available hydrogens are replaced by methyl groups, the compound no longer acts as a substrate (58). Beta-mono-methyl or beta-mono-hydroxyl substitution yields compounds which are oxidized slower in most cases although there are some species differences (61). Steric factors are apparently not important in the beta-mono-substituted derivatives since d- and l-adrenaline are oxidized at approximately the same rate (16).

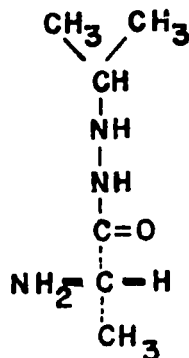
An increase in the length of the side chain of phenethylamine to phenylpropylamine or phenylbutylamine results in compounds having low affinity for the enzyme (62). However, kynuramine is a good substrate but reduction of the keto group to the alcohol results in much slower oxidation. Nor-kynuramine is also slowly oxidized (22).

Zeller has been much concerned with substrate-activity relationships and mechanism studies. He has published many papers (62,63,64,65) emphasizing the importance of a "phenethylamine backbone" and the necessity of the presence of two alpha-hydrogens for amines to act as substrates. From these observations he has drawn three dimensional pictures of the active sites. Considering that isocanlyamine and kynuramine are good substrates whereas nescaline is poor, Zeller's postulate of the "phenethylamine backbone" seems unlikely as a primary requisite for a compound to act as a substrate. It is also objectionable

to postulate that a hydrogen alpha to the amine group of a substrate or inhibitor can enter into "covalent bond formation" with an ill defined active site during the process of binding onto the enzyme (22).

8. STEREOSPECIFICITY OF MAO

In addition to the lack of substrate specificity, monoamine oxidase displays a relative lack of optical specificity. The enzyme does not make an absolute distinction between the inhibitors d- and l-amphetamine (66) or between cis- and trans-2-phenylcyclopropylamine (67). However, in some cases a striking difference is observed; L-alanine isopropyl hydrazide (IV) is a highly active inhibitor



(IV)

whereas the D-form is almost inactive. On the otherhand, this stereospecificity is much less pronounced with the serine derivatives and absent with the leucine and the phenylalanine isopropyl hydrazides (68). It should be

emphasized that the results with amphetamine and the phenyl-cyclopropylamines are based on in vitro studies in contrast to the results reported with the above hydrazides which have been obtained with in vivo preparations. It is possible that the in vivo stereospecificity can be attributed to selective transport mechanisms or more probably to the specific hydrolysis of only one optical form of these derivatives prior to the establishment of the MAO inhibition.

From these considerations, it is obvious that the enzyme has not been shown to display an absolute optical specificity of the type known to exist for alcohol dehydrogenase (69). Preliminary in vivo studies by Belleau, et.al. (31) had demonstrated a marked increase in the potency of sympathomimetic amines produced by stereospecific deuterium substitution on the alpha-carbon of tyramine. This suggested that MAO could have been responsible for these stereospecific effects even though the substrate lacks asymmetry in its natural form.

9. INHIBITORS

Alpha-substituted amines are not oxidized by monamine oxidase. If the alpha substituent is a methyl group, the compound may act as a competitive inhibitor of the enzyme. Such is the case with alpha-methyl-phenethylamine (70), ephedrine (71) and 2-amino-4-methyl-n-butane (9).

Blaschko pointed out that amphetamine was a better inhibitor than ephedrine; the beta-hydroxyl lowering affinity for the enzyme as is the case with substrates.

Blaschko and Dunthie (72) reported that mono and diguanidines, diisothiourea derivatives, and mono and diamidines also inhibit the enzyme. Octanol (73), cocaine and related anesthetics (74), harman, harmine (75), and 2-quinocyclohexyl-p-tolyl ether (9) are representative of the variety of inhibitors. However, most of the above compounds are inactive in vivo (75,76).

The discovery of highly effective MAO inhibitors, such as iproniazid (1-isonicotiny1-2-isopropylhydrazine), by Zeller and his group (77) in 1952 revived interest in monoamine oxidase inhibitors as pharmacological agents. Zeller's group determined that MAO was blocked by compounds with the general structure NH-NHR while those with the structure NH-NH_2 block diamine oxidase (78). A few hydrazines such as phenylhydrazine itself, block both enzymes effectively.

Interest in the series of hydrazine inhibitors was exemplified in a recent symposium (79) where literally hundreds of new MAO inhibitors were discussed. Biel and his group varied the nature of the acyl residue in iproniazid but could not arrive at any generalization. Continuing

their studies, 62 more compounds were synthesized which led them to the conclusion that "the irregularity of the observations makes it difficult to comment on the results obtained". It was observed in these studies that some of the compounds displayed profound organ specificity which lends support to the previously mentioned isoenzyme postulate (cf., p.17). In 1959, Naess and Nimmo (60) reported the far greater in vivo and in vitro inhibitory action of 2-phenylcyclopropylamine first synthesized by Burger and Yost (81). Their studies indicated a non-competitive inhibition of rat liver MAO. Fifty percent inhibition was obtained at a concentration of $2.8 \times 10^{-6}M$, representing a respective increase in potency of one-thousand and one-hundred fold over amphetamine and iproniazid. This has led many investigators to search for new non-hydrazine inhibitors. Zeller (67) reported cyclopropylamine to be inactive in this respect whereas the N-methyl- and N-dimethyl-2-phenylcyclopropylamine are active (82). Some investigators have employed the cyclopropyl ring in combination with hydrazine and in one example, oxalic acid bis [2-(1-cyclopropyl) ethyl] hydrazide, was found to produce fifty percent inhibition at $10^{-3}M$ (27).

It has been reported (83) that N-methyl-N-benzyl-2-propynylamine inhibits MAO at a concentration of $9 \times 10^{-7}M$. However, in the test system used by the authors, iproniazid inhibits at a concentration of $7 \times 10^{-6}M$ which is several

orders of magnitude lower than usually observed (67,75). In another paper (84), these authors state that their compound is seven times as potent as iproniazid whereas 2-phenylcyclopropylamine is 1,000 times as potent as determined by Ozaki (75). This last example emphasizes the possibility of drawing erroneous conclusions when the data was obtained with different in vitro test systems.

Recently Ozaki, et.al. (75) reported a thorough investigation of over 80 compounds for in vitro inhibition of MAO but were unable to establish any structure-activity relationships. These authors demonstrated that adrenalone would inhibit serotonin oxidation whereas Blaschko previously showed this compound to be a substrate (16). This becomes understandable if one considers that a compound with high affinity for the enzyme, but not oxidized at a rapid rate, can nevertheless act as a substrate competitor for another compound whose affinity and oxidation rate are high.

10. PHYSIOLOGICAL ROLE OF MONOAMINE OXIDASE

Monoamine oxidase has been implicated in a number of functions and dysfunctions but its actual role in the mammalian organism is somewhat uncertain and obscure. Discovery of potent inhibitors of the enzyme has enabled many investigators to gain an insight into the role of the enzyme

in physiological processes. Biologically active compounds such as adrenaline, serotonin and dopamine are formed by specific enzymatic reactions and stored in cellular particles in which form they are inactive. Release of these amines into the circulation causes profound pharmacological effects which would be undesirable if the release should become excessive. It is presumably for this reason that a destructive enzyme such as MAO is provided. Probably this is also the reason why one finds the enzyme in the brain, nervous tissue and smooth muscle.

High intestinal levels of MAO has brought forth the suggestion that the enzyme is responsible for the detoxification of amines of bacterial origin (8). Blaschko (9) finds fault with this hypothesis on the grounds that it is unlikely that amine concentrations would be so high as to require the amount of enzyme actually present. Davison (10) points out that serotonin (5-hydroxytryptamine) is also found in high concentration in the gut and because of its implication in intestinal motility, he suggests that MAO is responsible for regulating the concentration of this amine.

Monamine oxidase in the liver could play an important part in the destruction of biologically active amines in the circulation. In this connection, Dawson and Sherlock (85) examined the effect of iproniazid in patients with liver disease characterized by high blood ammonia

levels. They found that this MAO inhibitor was effective in lowering the blood ammonia level.

There has been much controversy as to the role of this enzyme in adrenergic mechanisms. Adrenaline and noradrenaline are good substrates for MAO in vitro; however, the role of the enzyme in in vivo metabolism has been the subject of many investigations. Schayer (86,87) and his colleagues demonstrated that as much as fifty percent of injected adrenaline is metabolized by MAO in rats. Greissamer (88) found that inhibition of MAO by iproniazid potentiated the response of the nictitating membrane to tyramine but not to adrenaline. Using the nictitating membrane, Belleau, et.al. (31) have shown that noreadrenaline is not a substrate for MAO at the adrenergic effector cell level and they suggest that the role of the enzyme in adrenergic mechanisms can best be described as a protective device for the inactivation of circulating endogenous non-transmitter substances.

We now know that adrenaline, noreadrenaline and dopamine (89,90) can be metabolized by O-methylation and recently Kopin, Axelrod and Gordon (91) demonstrated that this pathway represents the major route of adrenaline metabolism whereas MAO would play a major role in the destruction of the O-methylated metabolites.

Serotonin is an excellent substrate for MAO both

in vivo and in vitro (92). It is of interest to note that Bogdanski and Udenfriend (93) have shown that the distribution of serotonin parallels the distribution of MAO in the dog and cat brain. Release of bound serotonin can be brought about by the administration of reserpine thereby making the amine available to MAO. The release of serotonin is followed by a rapid depletion of the "active" amine and this has led Brodie, et.al. (94) to postulate that the normal role of serotonin is akin to that of acetylcholine. In other words, just as cholinesterase is responsible for destroying acetylcholine, monoamine oxidase may in a similar way inactivate an excess of a neurohormonal transmitter such as serotonin.

Walessek and Abood (95) have pointed out that in a case of lack or dysfunction of MAO, alternate pathways of amine metabolism could lead to physiologically active substances capable of causing effects such as mental abnormalities. This is borne out by experimental evidence obtained with MAO inhibitors mentioned in the previous section.

Recently Gertner (96) has demonstrated that representative MAO inhibitors have the ability to block ganglionic transmission in the isolated superior cervical ganglion of the dog. He presents correlations which suggest that the blocking activity is directly related to the MAO inhibitory activity of these compounds. Possible explanations are that an active amine is accumulating at the ganglionic

synapse or that there is interference with the normal function of the neurotransmitter at the ganglionic synapse. In either case, factors other than acetylcholine are implicated in ganglionic transmission.

Recently it has been demonstrated that the deamination products of some neurotransmitters activate the oxidation of glucose in the anterior pituitary (97). Thus, it would appear that MAO could be responsible for the formation of physiologically active aldehydes.

It is clear from the above discussion, that the role of MAO in physiological processes is as yet ill-defined. At the same time, it is apparent that further investigations will be most profitable towards a better understanding of the role of this enzyme in metabolic processes.

B. DEUTERIUM ISOTOPE EFFECTS IN BIOCHEMISTRY

Substitution of deuterium for hydrogen causes a number of observable effects which can be useful in studying reaction mechanisms. Since the zero point energy is greater for the C-H bond than for the C-D bond, the activation energy is lower for the rupture of the C-H bond. Thus, the rate of reaction is correspondingly less for the deuterium containing compound if the cleavage of the bond is rate determining (98). A number of reviews of this subject have been presented (99,100,101).

Kriensmeyer, et.al. (102) and Thorn (103) have described a reduction in the rate of oxidation of partially deuterated succinate. Mahler and Douglas (104) subsequently reported the effect of deuterium on the binding of nicotinamide adenine dinucleotide to lactic dehydrogenase and on its subsequent dehydrogenation. Concurrent with preliminary reports of work presented in this thesis (105), Abeles and his group (108) reported a kinetic isotope effect with deuteromethyl sarcosine. These investigators also reported effects of deuteration on the Michaelis constants although no interpretation of their results was offered. There had been no studies published relating to the effect of deuteration on biologically active amines prior to our reports (105, 106, 107).

Deuterium isotope effects should constitute a powerful tool in this field since such effects are independent of the purity or composition of the enzymes and are theoretically applicable to in vivo studies.

C. NATURE AND SCOPE OF THIS INVESTIGATION

From the introduction it becomes apparent that there is a profound lack of knowledge as to the chemical mechanism of action of monoamine oxidase. Many investigations have pointed out the critical role which MAO plays in physiological processes deeming it necessary that a fundamental study be undertaken if progress is to be made in the field of the chemotherapy of MAO dysfunctions. Most of these studies have been concerned with structures of a wide and varied composition. These investigations have led to an increase in our general knowledge about the enzyme, but allow no general conclusions as to its mechanism of action.

This investigation comprises studies with substrates, their deuterated counterparts and a series of closely related phenylalkylamine inhibitors. In these cases, the structural changes were small in terms of molecular weight and functional groups and this should minimize effects due to membrane permeability and lipid solubility. These latter factors no doubt play an important role when substrates and inhibitors deviate greatly in structure and molecular weight.

Using these techniques it is hoped that subtle differences will provide an interpretation as to the mechanism of action of this enzyme on which further work in this field can be based.

EXPERIMENTAL

A. MATERIALS

Dr. G. E. Uliyet provided generous samples of racemic and resolved trans-2-phenylcyclopropylamine. Cis- and trans-2-phenylcyclobutylamine and 3-phenylcyclobutylamine were gifts from Dr. A. Burger. Bis- α -deuterio-tyramine, R- and S- α -mono-deuterio-tyramine had been prepared in our laboratory in connection with other studies.

1. SYNTHESIS

a. Preparation of α - d_2 Mescaline Hydrochloride

Trimethoxybenzonnitrile was prepared according to Tsao (109) and reduced with $LiAlD_4$ by the following procedure. The nitrile (0.013 moles) in 100 ml of freshly distilled anhydrous tetrahydrofuran is added slowly to a solution of $LiAlD_4$ (0.020 moles) in 50 ml anhydrous diethyl ether with cooling and stirring. After addition is complete, the mixture is refluxed overnight and decomposed with 0.8 ml H_2O , 0.65 ml 20% $NaOH$ and 2.9 ml H_2O . The solvents are removed in vacuo and the hydroxides washed twice with 30 ml portions of ether. The ether solutions are combined, dried over Na_2SO_4 and dry HCl is bubbled through the solution. Recrystallization from isopropyl alcohol-ethylacetate yielded crystals, m.p. 181° - 182° ; lit (110) 180° - 181° ; 23% yield.

b. Preparation of α - d_2 -benzylamine

Commercial benzonitrile reduced by the above procedure gave α - d_2 -benzylamine hydrochloride in 60% yield; m.p. 255-57°, lit. (111) 258°.

c. Preparation of α - d_2 -tryptamine

Indolescetonitrile prepared by the method of Henbest, et.al. (112) is reduced by the above procedure using diethyl ether in place of tetrahydrofuran. Distillation yielded a middle fraction (b. 159-63°/0.4 mm.) which on standing crystallized; m.p. 116-18°, lit. (113) 115-17°.

All compounds prepared by $LiAlD_4$ reduction of the corresponding nitriles were labelled to the extent of 99% or better on the α -carbon as determined by N.M.R. analysis.

d. Preparation of α - d_2 -kynuramine

The preparation is essentially that of Weissbach, et.al. (50). Slight modifications were necessary as in initial attempts the product was contaminated with a substance which was nearly identical in chromatographic behavior. This contaminant had intense absorption in the 4-hydroxyquinoline region of the UV spectra. α - d_2 -N-carbobenzoxytryptamine (2 ϵ), prepared by the Schotten-Baumann method (114), is dissolved in 50 ml of acetic acid and subjected to ozonolysis. The resulting yellow-brown solution is concentrated in vacuo

to about 5 ml and then cooled while 12 ml of 38% HBr in acetic acid is added. After standing at 0° for 5 hours, the solution is concentrated to dryness in vacuo followed by addition of water and extraction with ether. The aqueous layer is passed over Dowex 1-X8 resin (hydroxide form). The column of resin was approximately 2 x 15 cm. The eluant containing the free base is treated with a saturated solution of picric acid, warmed and filtered while still hot. This apparently removed the impurity which caused difficulty in other attempts. After standing for several days in the cold, the red-orange picrate (0.7 g) was collected; m.p. 174-75°. The picrate was dissolved in 50% aqueous methanol (v/v) and passed over Dowex 1-X8 resin (hydroxide form) as above. To the eluant is added an excess of alcoholic HBr and the solution is concentrated in vacuo. After treatment with Norite the residue crystallized from isopropyl alcohol yielding 0.3 g of α -d₂-kynuramine dihydrobromide; m.p. 214-15° with decomposition, lit. (50) for kynuramine dihydrobromide, 214-16° (decomposes).

e. Preparation of β -d₂-kynuramine

Kynuramine dihydrobromide (0.1 g) and D₂O (1 ml) are placed in a sealed tube and heated in an oven at 110° for 8 hours. This process is repeated three times. After solvent removal under nitrogen, the residue is crystallized from isopropanol-ether. Recrystallization from isopropanol yielded 0.08 g of the dihydrobromide, m.p. 214-215° (decomposes).

N.M.R. spectral analysis showed the hydrogens on the beta-carbon to be exchanged to the extent of 95% or more.

f. Preparation of 2-benzylasiridine

Kashlikar's (115) procedure was followed yielding on distillation, a colorless liquid, b. 48-52°/0.1 mm., n_D^{26} 1.5409; lit. (115) b. 73-75°/1 mm., n_D^{20} 1.5431.

g. Preparation of 2-phenylasiridine

2-chloro-2-phenylethylamine was prepared according to Wolfheim (116). Steam distillation of the halogenoamine from KOH by his procedure did not yield any of the desired product. Therefore, the following procedure was used. 2-chloro-2-phenethylamine hydrochloride (3 g) is added slowly to 2.8 gm of KOH in 100 ml H₂O and stirred for 2 hours after which time the solution is saturated with K₂CO₃. Three 75 ml extractions with ether followed by solvent removal in vacuo and distillation gave a middle fraction (1.5 g), b. 57-60°/0.1 mm.; the properties agreed with those reported in the literature.

2. PREPARATION OF MITOCHONDRIAL MONOAMINE OXIDASE FROM RAT LIVER

The procedure followed is a modification of that of Hawkins (34). All operations were carried out at 0°C. with previously cooled reagents and apparatus.

Freshly excised livers from decapitated rats are freed of connective tissue and cut into pieces about 1 cm. square after which they are minced in a Latapie mincer with 0.25 M. sucrose as the perfusing medium. The resulting suspension is brought up to a volume 10 times (w/v) that of the original tissue wet weight. The solution is then homogenized in a glass-teslon homogenizer (#4288B, size C, Arthur H. Thomas Co.) for 40 seconds at 600 RPM allowing for intermittent periods of cooling. The homogenate is centrifuged in an International Refrigerated Centrifuge (0°C, Head #860) for 5 minutes at 1,000 RPM after which the supernatant is removed and the sedimented cell debris discarded. The supernatant is recentrifuged in a Spinco Ultracentrifuge (Rotor #40) for 45 minutes at 23,000 RPM and the supernatant discarded. The precipitated pellet is resuspended in 0.25 M. sucrose so that the final volume is 5 times the original wet weight of the tissue and stored at 0°.

B. METHODS

1. ASSAY USING TYRAMINE AS SUBSTRATE

The procedure used is a modification of that of Udenfriend and Cooper (50).

Reagents:

Borate Buffer: 165 ml. of NaOH is added to 94.2 g. of boric acid in 3 liters of water. The solution is then

saturated with n-butanol and sodium chloride. The final pH is approximately 10.

n-Butanol and Petroleum Ether (80°-100°): Reagent grade solvents were distilled and then shaken with equal volumes of 0.1N NaOH, water, 0.1N HCl and finally twice with distilled water.

1-Nitroso-2-naphthol: 0.1% 1-nitroso-2-naphthol in 95% ethanol made up just prior to use. Nitrous acid reagent: To 5 ml nitric acid is added 5 mg NaNO_2 just prior to use.

Incubations

Enzyme, equivalent to 300 mg liver wet weight, 1.0 ml phosphate buffer (pH 7.4), and tyramine (5-40 μ moles) are brought to a total volume of 5 cc in 30 ml beakers. The mixture is incubated in a Dubnoff metabolic shaker at 37°C in air. Aliquots containing 1-8 μ moles of substrate are transferred at various intervals.

Extraction

Aliquots are transferred to 50 ml flasks containing 2 ml of 20% Na_2CO_3 . To each flask is added 1 ml borate buffer, 3 g NaCl and 20 ml of butanol. The flasks are shaken for $\frac{1}{2}$ hour and an aliquot of the organic phase is transferred to another flask containing an equal volume of petroleum ether and 4 ml 2N HCl. These flasks are shaken for another $\frac{1}{2}$ hour after which time the acid layer is removed.

Colorimetric Assay

To 2 ml of the acid extract containing 0.1-0.8 μ moles of tyramine are added 1 ml each of the nitroso-naphthol and nitrous acid reagents. The tube is stoppered, shaken and put in a water bath at 55°C for 30 minutes. Ten ml of CHCl_3 is added to extract the unchanged nitroso-naphthol and after centrifugation at low speed the acidic layer is transferred to a cuvette. When measured in a Bausch and Lomb Spectronic 20, the optical density is proportional to concentration up to 0.8 μ moles. Enzyme blanks and standards are carried through the same procedure.

2. ASSAY USING KYNURAMINE AS SUBSTRATE

The procedure is essentially that of Weissbach, et.al. (57).

Incubations are carried out in a Beckman 3 ml cuvette. The experimental cuvette contains enzyme equivalent to 40 mg of liver wet weight, kynuramine (0.1-1.0 μ moles), 0.7 ml of 0.5 M phosphate buffer at pH 7.4 and water to a total volume of 3 ml. In all cases, a solution containing enzyme, buffer and water was made up so that in one aliquot all the reagents could be added, followed by the addition of substrate. A blank cuvette was prepared in which the kynuramine was replaced with water. After the final addition, the mixing is achieved by inversion and an initial reading is

made at 360 $m\mu$ or at 329 $m\mu$. The assay was run at room temperature in a Beckman DK-2 with a time drive attachment.

3. MODEL STUDIES

The reaction of unlabelled and α - d_2 -amines is carried out as follows. To 1 ml of the desired solvent containing 2.9 μ moles of the amine is added 0.2 ml of 1.5% (w/v) ninhydrin in ethanol. The reaction is carried out in cuvettes (minimal volume 1 ml) designed for the Coleman Junior Spectrophotometer at the desired temperature in a constant temperature bath controlled to $\pm 0.05^\circ$. After completing the solvent volume to original levels, the optical density at 550 $m\mu$ is determined at appropriate time intervals.

C. RESULTS

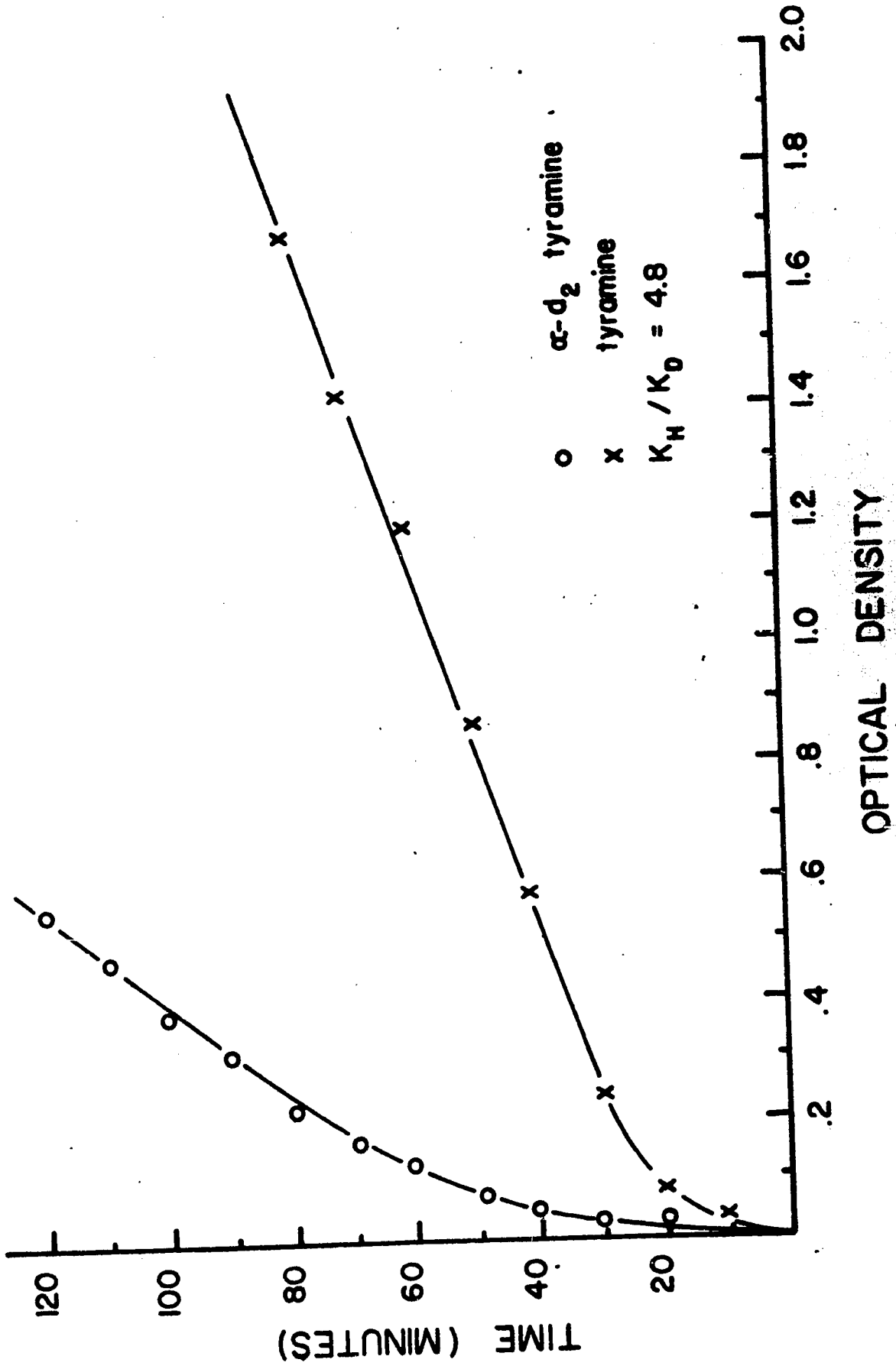
1. MODEL STUDIES

The reaction of α - d_2 -amines with ninhydrin was carried out as described and comparison of the rate of reaction with that of the unlabelled amine demonstrated the presence of an isotope effect. Initial observations made it apparent that the rate of reaction was dependent upon the basicity of the solvent, the rate being qualitatively proportional to the relative basicity of the solvents. That is, the rate of reaction increased with solvent in the following order: $CHCl_3$, C_4H_9OH , C_3H_7OH , C_2H_5OH , CH_3OH , H_2O . Experimental conditions (solvent and temperature) were chosen so that the reaction could be followed over a convenient period of time. Typical rate determinations are shown in Figure 1 and summarized in Table I.

TABLE I. Kinetic isotope effects with a series of phenylalkyl amines.

Amine	Solvent	Temp.	Initial Slope Ratios k_H/k_D
Benzylamine	H_2O	25°	Too Fast
Benzylamine	$CHCl_3$	40°	1.6
Mescaline	$CHCl_3$	50°	4.5
Tyramine	$CHCl_3$	50°	4.5
Tyramine	C_4H_9OH	50°	4.8

Fig. 1. - Relative rates of reaction of tyramine and α - d_2 -tyramine with ninhydrin in butanol. The reaction was carried out as described in methods at 50°C.



It was then realized that this method could serve as a quantitative method for the determination of the percentage composition of deuterated amines admixed with unlabelled amines. Mixtures of the two amines (α - d_2 -tyramine and tyramine) in varied composition were made up and the reaction carried out as before with the exception that the optical density was determined for all samples after 75 minutes of incubation at 50°C. A plot of optical density vs. percent α - d_2 -tyramine is reproduced in Figure 2. In order to determine the accuracy of the method, a mixture of known composition was analyzed by the same procedure and the percent α - d_2 -tyramine determined from the standard curve (Figure 2). Results of this experiment are summarized in Table II.

TABLE II. - Analysis of a mixture containing 70% α - d_2 -tyramine and 30% tyramine

Trial	O.D.	% as d_2	
1	0.67	68	Average 70.3 \pm 1.3%
2	0.65	70	
3	0.63	72	
4	0.64	71.5	

In future work, small samples of the amines as their hydrochlorides were to be analyzed by this method and it became necessary to have a solvent system which would neutralize the

hydrochloride thus eliminating extraction operations necessary to obtain the free base.

Using butanol-3% pyridine as solvent for the reaction, an isotope effect of 6.9 was observed (Figure 3). Because of the greater accuracy using this solvent system, it was adopted for all determinations. Analysis of a known mixture containing 55% α -d₂-tyramine gave the following results representing an average of 4 determinations; 54.9% \pm 0.31 as α -d₂-tyramine.

Fig. 2. - Standard curve for percent α -D₂-tyramine versus optical density. Mixtures of tyramine and α -D₂-tyramine of known composition were allowed to react with ninhydrin as described at 90°C. The optical density was determined after 75 minutes at 550 m μ .

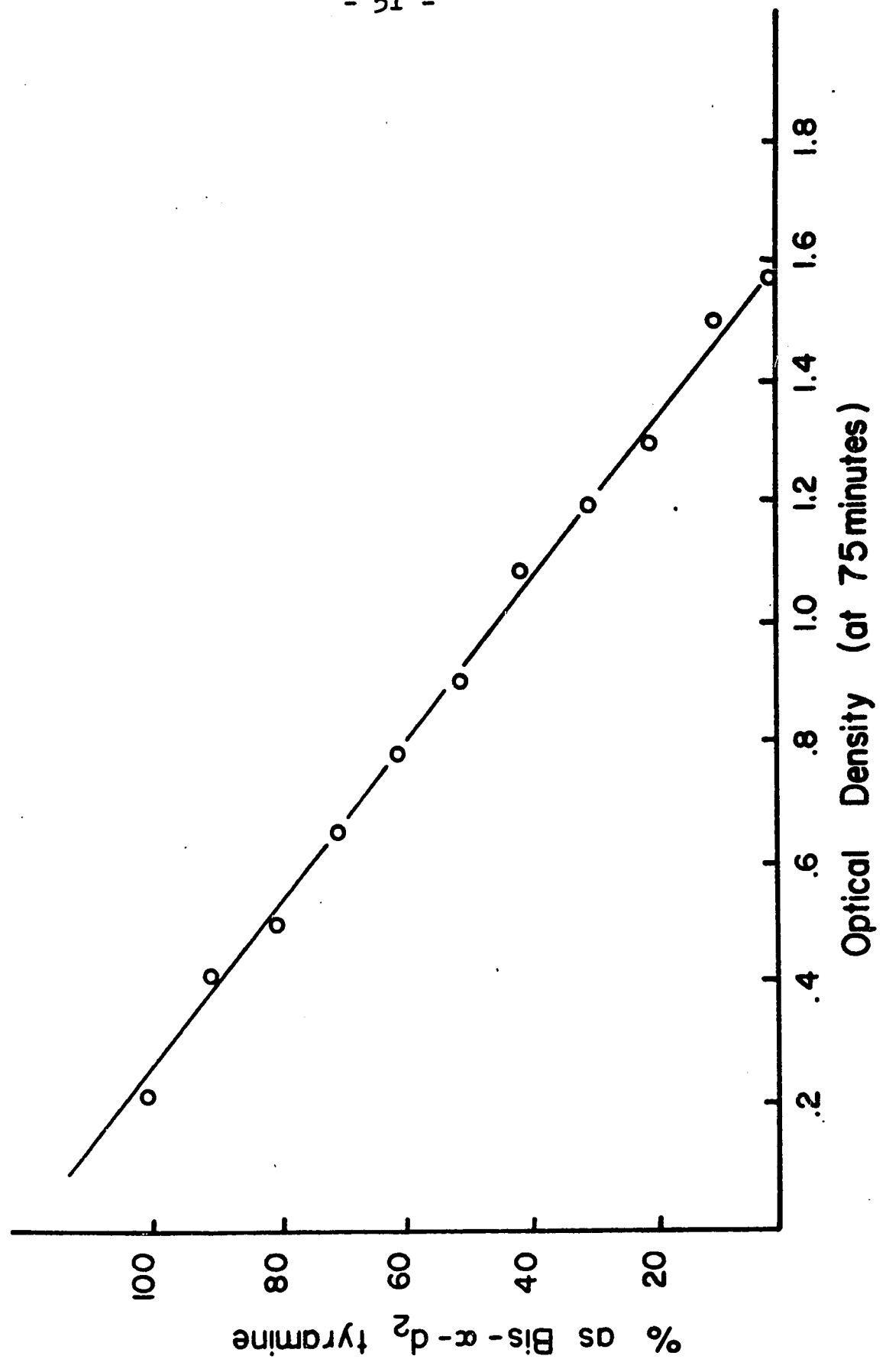
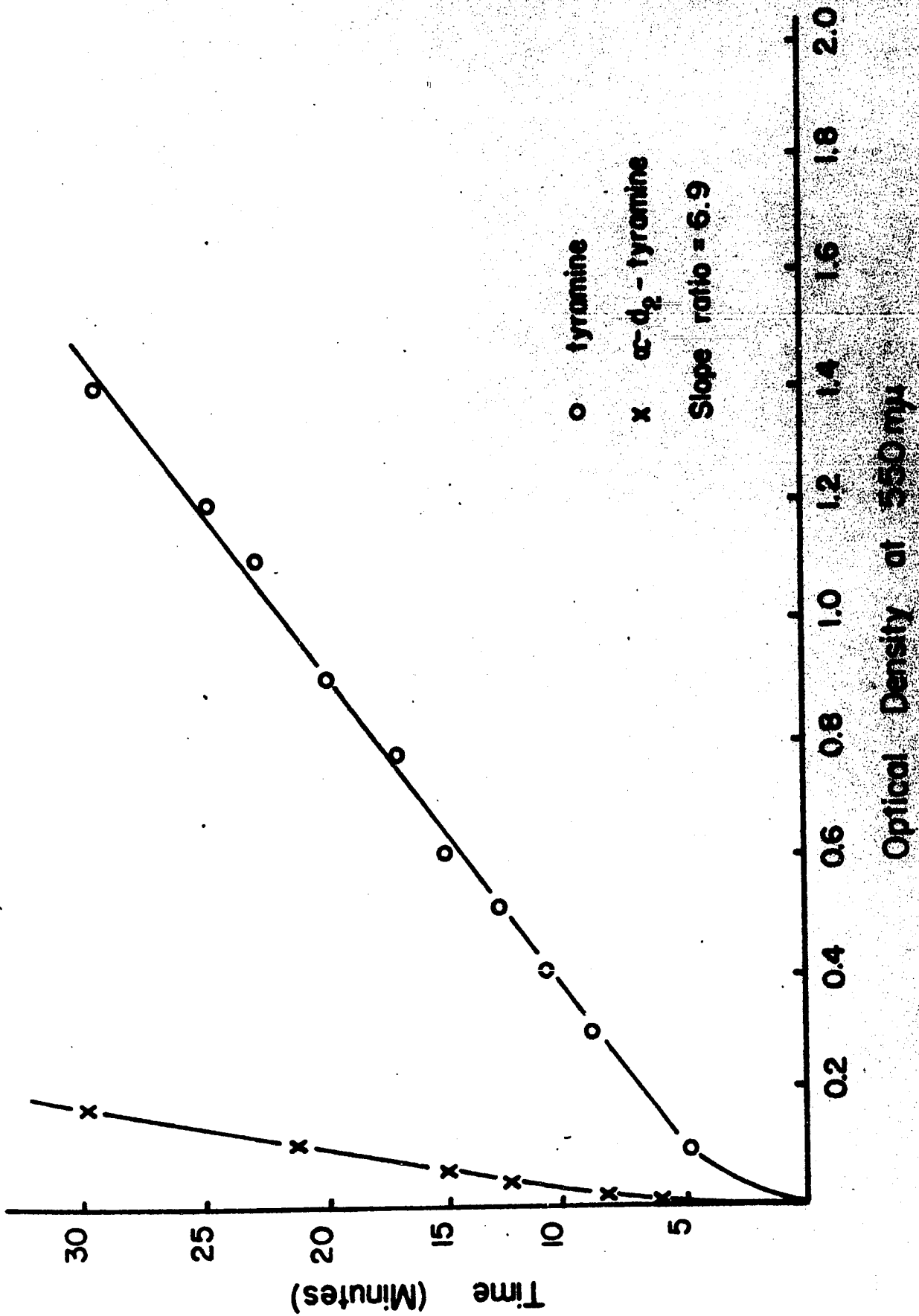


Fig. 3. - Relative rates of reaction of tyrosine and α -D₂-tyrosine with ninhydrin in butanol-3% pyridine. The reaction was carried out as described at 30°C.



2. OXIDATION OF DEUTERIUM AND PROTIUM SUBSTRATES

a. Tyramine and α - d_2 -tyramine

When tyramine was incubated with the enzyme as described in Methods, its initial rate of oxidation was found to be twice that of α - d_2 -tyramine. Further experiments revealed that this isotope effect was dependent upon the initial substrate concentration as shown in Figures 4 and 5. Results of these experiments are summarized in Table III, the rate ratio being the ratio of the slopes.

TABLE III. Rate ratios between tyramine and α - d_2 -tyramine at various substrate concentrations.

Substrate Concentration moles/liter $\times 10^{-3}$	Rate Ratio k_H/k_D
1.15	2.3
2.3	1.8
3.4	1.7
4.6	1.5
5.7	1.4

Fig. 4. - Relative rates of oxidation by monooxygenase for tyramine and α - α -tyramine at a substrate concentration of 1.15×10^{-3} moles/liter. Values obtained by the 1-nitroso-2-naphthol procedure as described in methods.

x tyramine
o α -d₂ - tyramine
 $K_H / K_0 \approx 2.3$

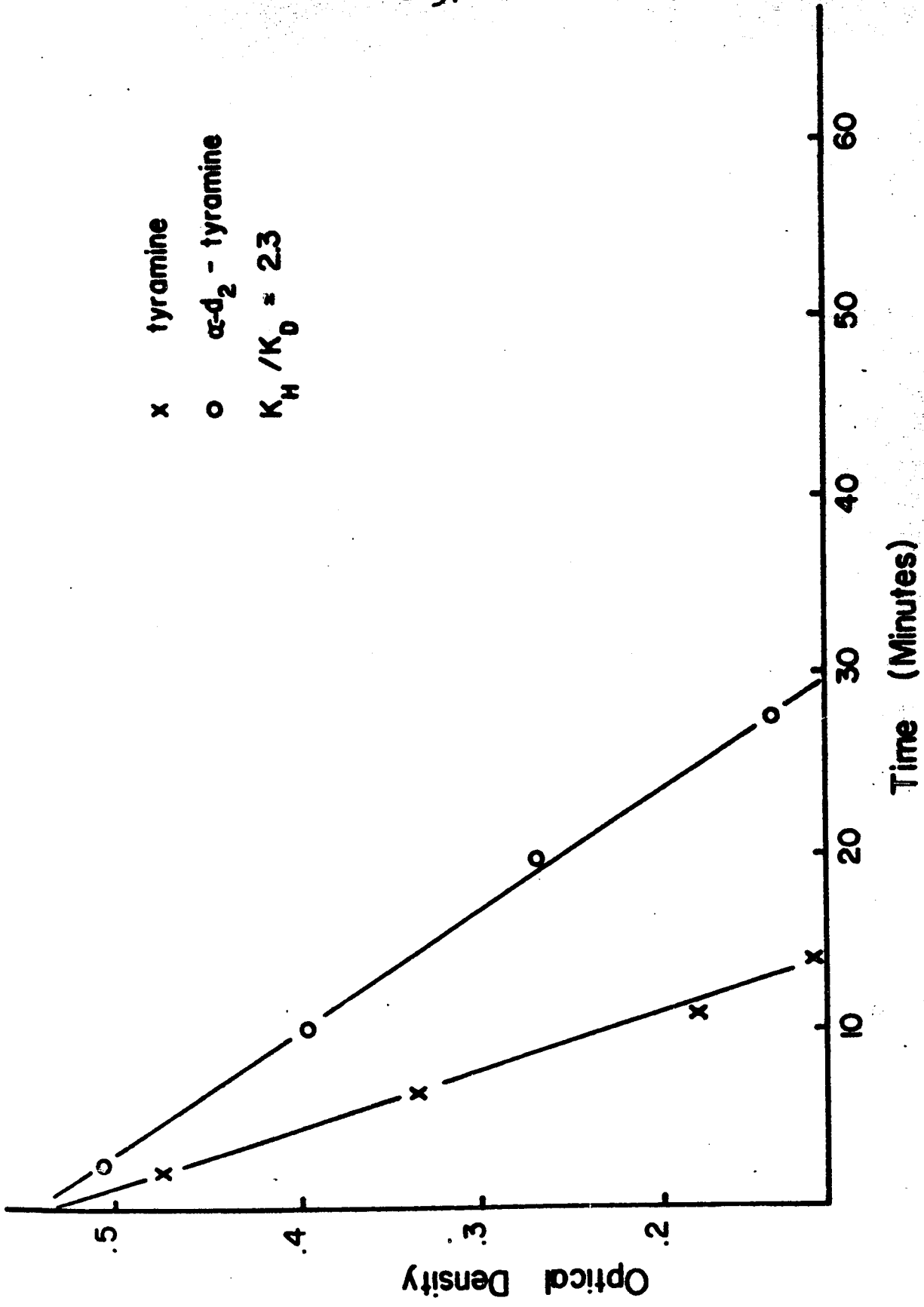
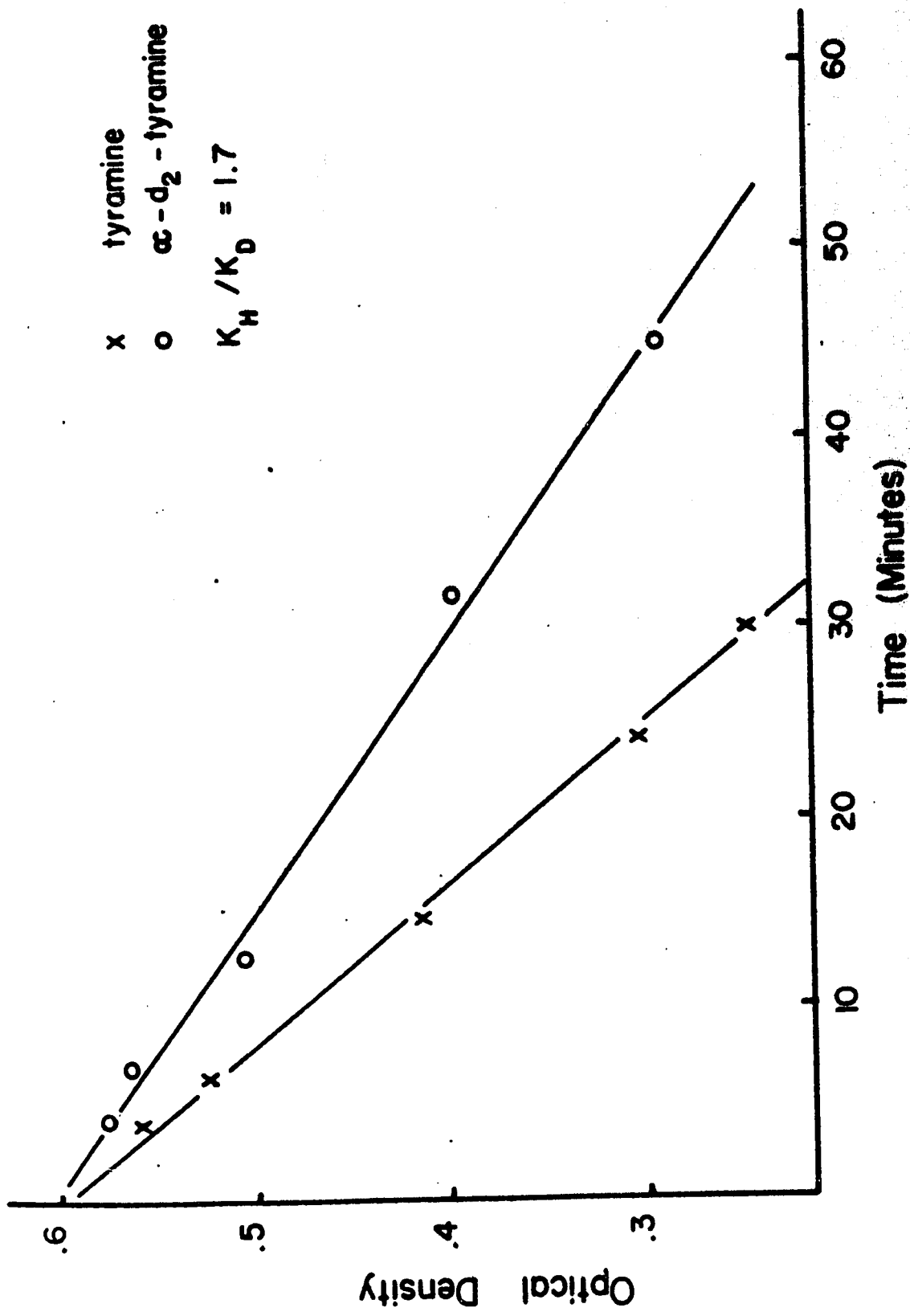


Fig. 5. - Relative rates of oxidation by monooxygenase for tyramine and α - d_2 -tyramine at a substrate concentration of 3.4×10^{-5} mole/liter. Values obtained by the 1-nitroso-2-naphthol procedure as described in methods.

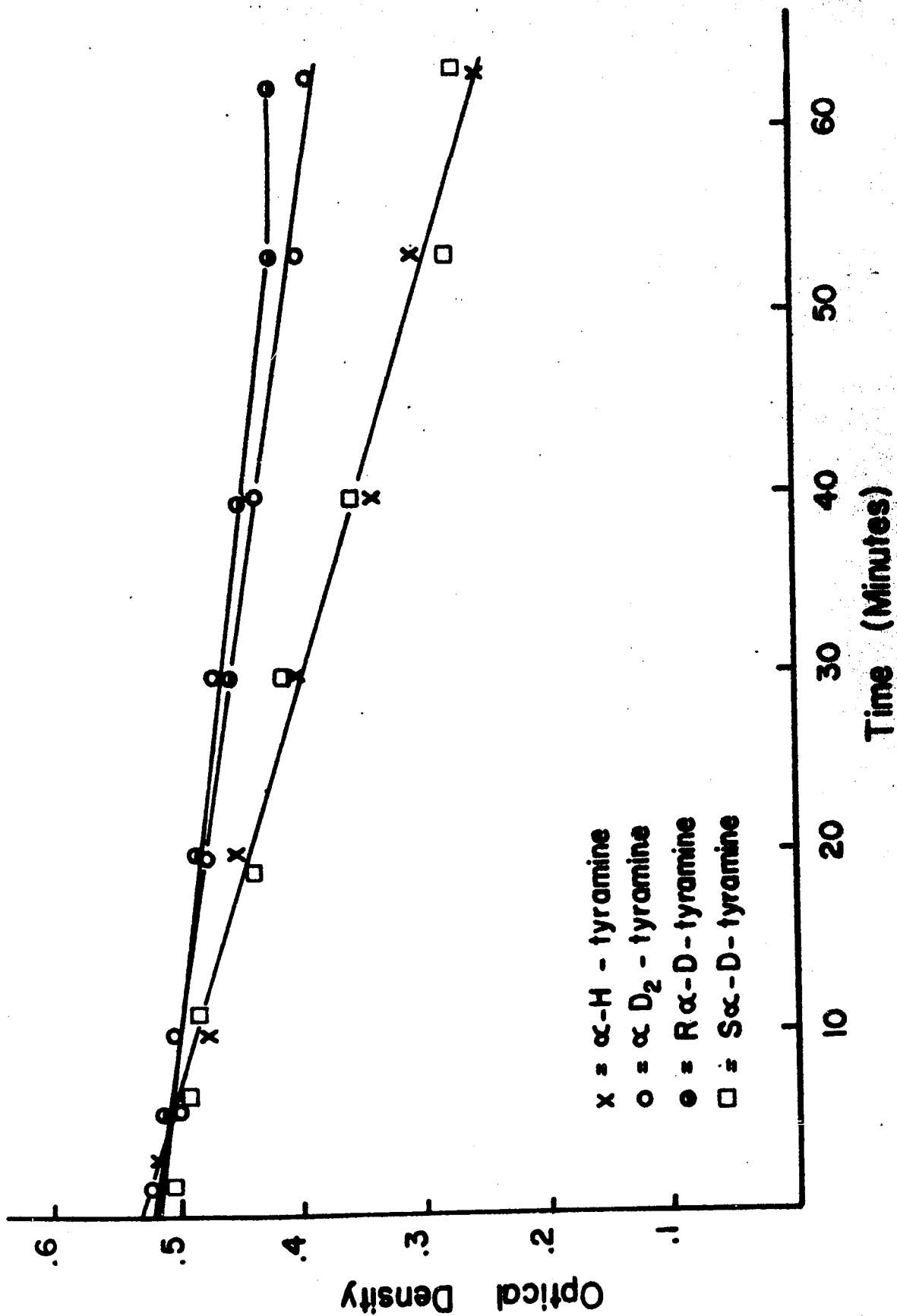


b. Stereospecifically labelled tyramine

Enzymically prepared R- and S- α -d-tyramine* were incubated with MAO according to the standard procedure described. When the two optically pure enantiomers were incubated under identical conditions, the results shown in Figure 6 were obtained. It can be seen that tyramine and S- α -d-tyramine were oxidized at the same rate whereas R- α -d-tyramine and α -d₂-tyramine were degraded at a rate 2.3 times slower.

* The optical isomers were prepared by J.V. Burke. Specification of asymmetric configuration according to Cahn, Ingold and Prelog (117).

Fig. 6. - Relative rates of oxidation by mononitric oxides for tyramine, α - β -tyramine, *N*- and *S*- α - β -tyramine followed by the 1-nitroso-2-naphthol procedure. In all cases the substrate concentration was 4.6×10^{-4} mole/liter.



x = α -H - tyramine
o = α -D₂ - tyramine
● = R- α -D - tyramine
□ = S- α -D - tyramine

c. Michaelis Constants

Results in the previous section made it pertinent to examine the effect of deuteration on binding of the enzyme with substrate. The Michaelis constants were determined in the usual manner by measuring the velocities at various substrate concentrations and the data treated according to the method of Lineweaver and Burk (118) (Figure 7). Kinetic isotope effects determined in these studies are summarized in Table IV.

TABLE IV. Ratios of maximum velocity and Michaelis constants for tyramine and α - d_2 -tyramine

Experiment	$\frac{V_{maxH}}{V_{maxD}}$	$\frac{K_{mD}}{K_{mH}}$
1	1.2	2.55
2	1.25	2.25

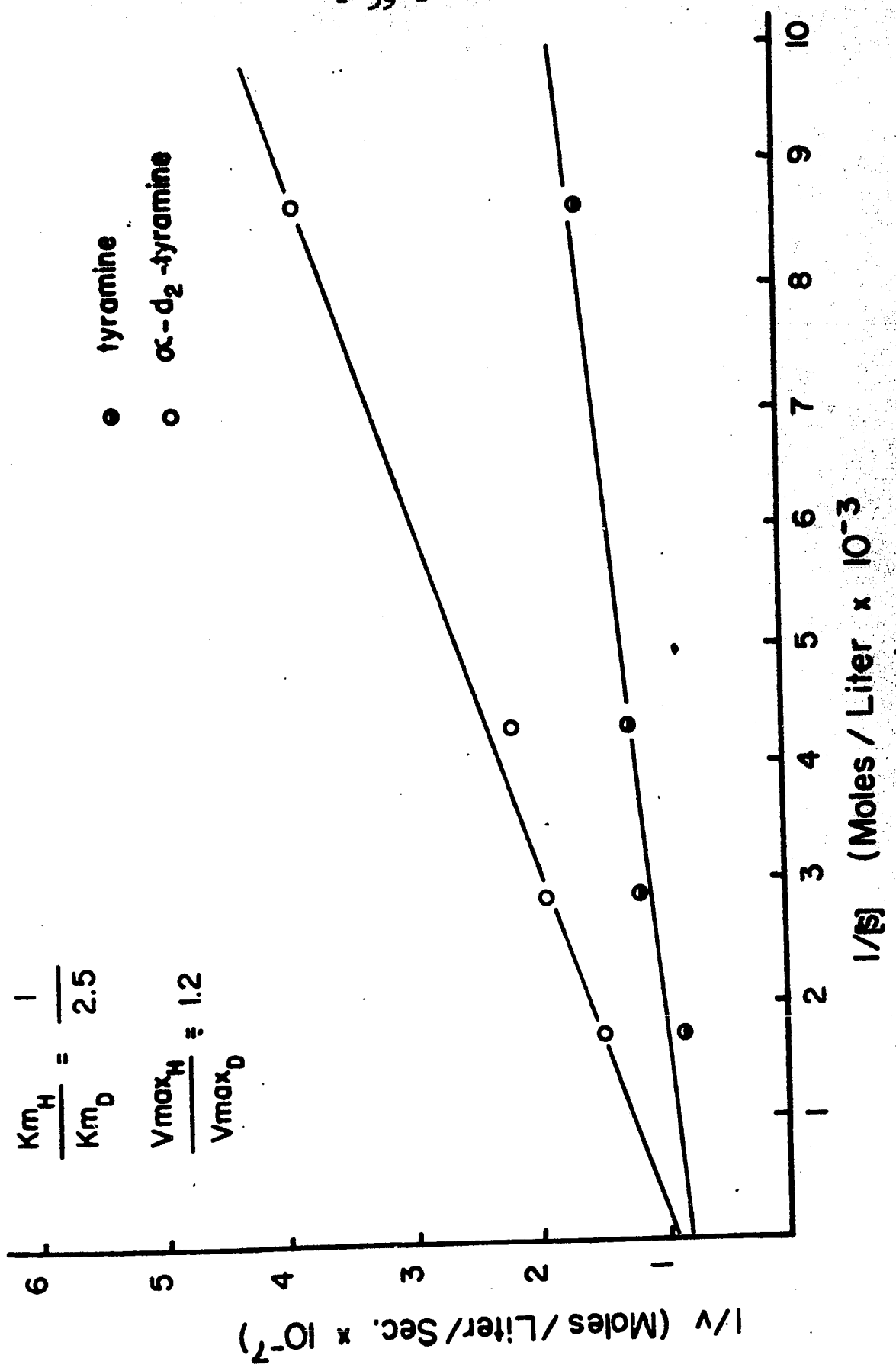
d. Competition between tyramine and α - d_2 -tyramine for the enzyme

In order to ascertain whether the Michaelis constant, K_m , approximates the true equilibrium binding constants (keeping in mind that the steady state treatment would be more accurate), direct measurement of the amount of α - d_2 -tyramine and tyramine left after incubation of an equimolar mixture

**Fig. 7. - Michaelis-Menten constants
and maximum velocity ratios for
tyrosine and α -D₂-tyrosine.**

$$\frac{K_{m_H}}{K_{m_D}} = \frac{1}{2.5}$$

$$\frac{V_{max_H}}{V_{max_D}} \approx 1.2$$



of the two species was carried out as follows: tyramine, α - d_2 -tyramine and an equimolar mixture of the two amines were each incubated with the enzyme as described, with the exception that the quantities of all reagents were increased 5 fold in the case where the two species were incubated as an equimolar mixture. The rates of the three reactions were followed in the usual manner for 30 minutes after which time the incubation mixture was quenched by heating to 100° for 10 minutes. After centrifugation, the supernatant was passed over an Amberlite CB-50 column (3.5 x 10 cm), the column washed with 2 liters of distilled water and the amines eluted with 4N acetic acid according to the procedure of Davis, et.al. (119). The tyramine was isolated as the hydrochloride and recrystallized three times. This purified sample was then analysed for its content in deuterium according to the above described method using butanol - 3% pyridine as the solvent.

TABLE V. Analysis of the amine mixture isolated from incubation in which the molar ratio of tyramine and α - d_2 -tyramine was initially 1:1.

Sample	O.D.	% as α - d_2 -Tyramine
1	.50	69
2	.51	68.5
3	.49	70
4	.50	69
Average 69.15 ± 0.4		

Using this figure (69% as α -d₂-tyramine), the total amount of each amine left unoxidized at the end of the incubation could easily be estimated by the application of the 1-nitroso-2-naphthol procedure described earlier. Results are summarized in Table VI.

TABLE VI. Composition of the amine mixture after incubation of tyramine, α -d₂-tyramine in a 1:1 molar ratio of the two species. Tyramine was determined by the 1-nitroso-2-naphthol method and the content in α -d₂-tyramine by the ninhydrin method.

Moles Tyramine $\times 10^{-3}$ in 5 cc Incubation Mixture					
Initial Concentration		Final Concentration (After 30 minutes)		Oxidized by MAO	
H ₂	5.75	3.4		2.35	
D ₂	5.75	3.9		1.85	
D ₂	2.875	5.75	3.56 \times 69%	2.45	.43
H ₂	2.875				1.10

e. Isotope effects with α - d_2 and β - d_2 kynuramine

Incubations were carried out as described in the case of the assay for kynuramine following the rate of disappearance of substrate at 360 m μ using the time drive attachment. When the rate of disappearance of α - d_2 -kynuramine was compared to kynuramine, an isotope effect of 2.1 was observed at a molar concentration of $1.2 \times 10^{-4}M$. At the same concentration, β - d_2 -kynuramine showed an isotope effect of 1.18 (Figure 8). Lineweaver-Burk plots were constructed and led to the observation that the ratio of the Michaelis constants for α - d_2 -kynuramine ($K_m D/K_m H$) was equal to 3 (Figure 9). Although β - d_2 -kynuramine showed a consistent isotope effect of 1.15 - 1.20 over the same range of concentrations, no detectable effect on the Michaelis constants could be observed.

Fig. 8. - Rates of oxidation of kynurexine, α - d_2 -kynurexine and β - d_2 -kynurexine followed in situ with a recording Beckman DU-2 spectrophotometer with time drive attachment. In all cases the substrate concentration was 1.2×10^{-4} mole/liter.

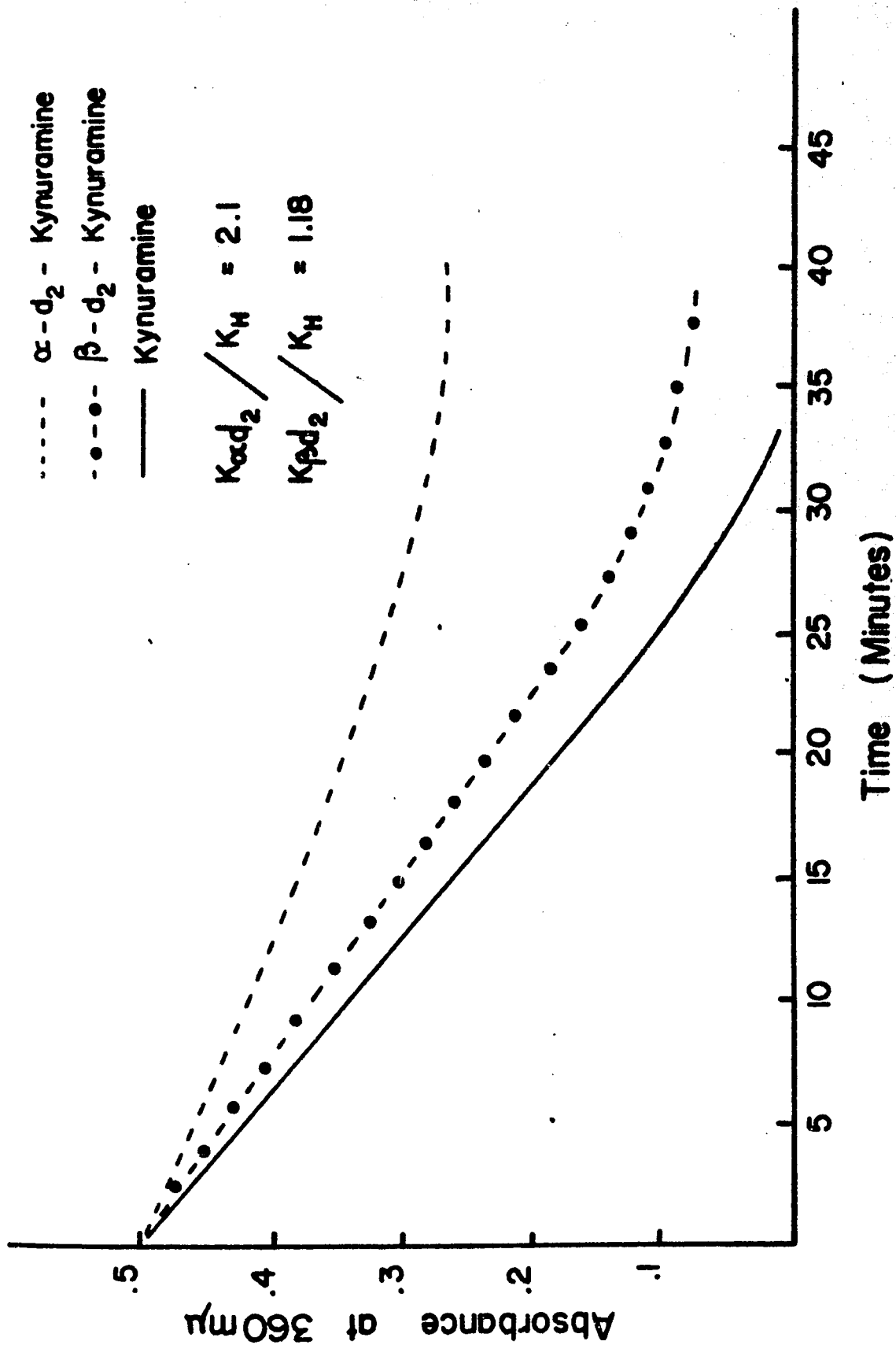
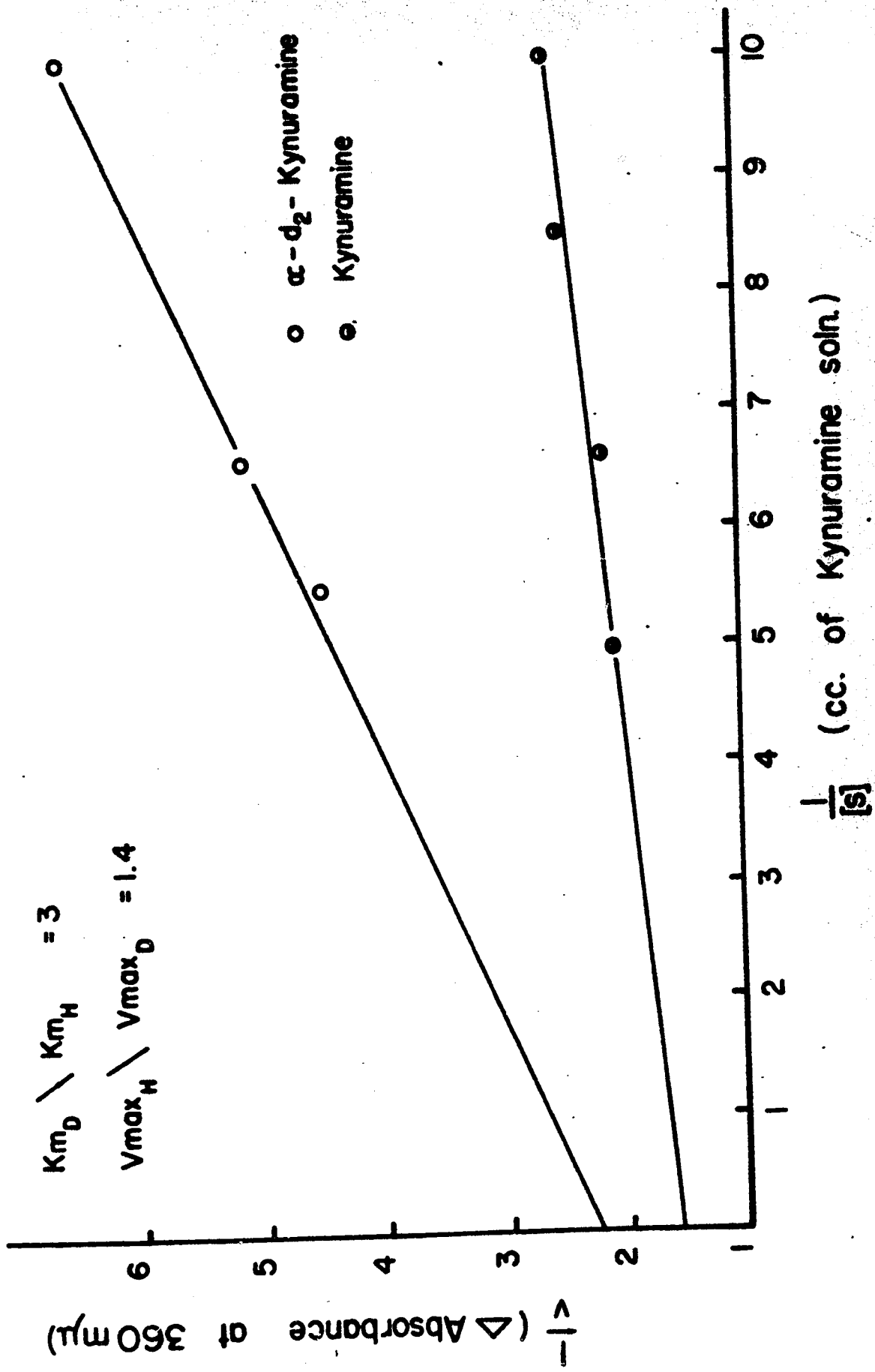


Fig. 9. - Michaelis-Menten constants and maximum velocity ratios for kynurexine and α - d_2 -kynurexine. Various levels of the enzyme incubated as described in experimental. V = change in absorbancy at $360 m\mu$ per 30 minutes; (S) = cc of 2×10^{-5} molar kynurexine solution per 3 ml.



3. MAO INHIBITOR STUDIES

a. Determination of PI_{50} for a series of phenylalkylamines

Incubations were carried out as described for the assay using kynuremine with the exception that the inhibitor solution was replaced by an equal volume of water. The inhibitors were preincubated with the enzyme for twenty minutes as it has been demonstrated that this is necessary for maximum inhibition with some compounds (75). After the addition of substrate, appearance of the absorbancy due to 4-hydroxyquinoline at $329m\mu$ was followed for thirty minutes. The following equation was used to calculate the percentage inhibition:

$$\% \text{ inhibition} = \left(1 - \frac{\text{inhibited rate}}{\text{non-inhibited rate}} \right) \times 100$$

When inhibitors showing an activity greater than 50% at $10^{-3}M$ were found, the PI_{50} determinations were carried out by testing them at various concentrations. In this way, concentrations producing inhibition larger and smaller than 50% were determined. Results were plotted on semi-log paper (Figure 10) and the concentration producing 50% inhibition was determined. The PI_{50} is defined as the negative logarithm of the concentration producing 50% inhibition. A summary of the results obtained with the series of inhibitors used in these studies is presented in Table VII.

Fig. 10. - Percent inhibition versus log concentration of phenylsulfidine using the kynurexine assay. The substrate concentration was 1.2×10^{-4} mole/liter.

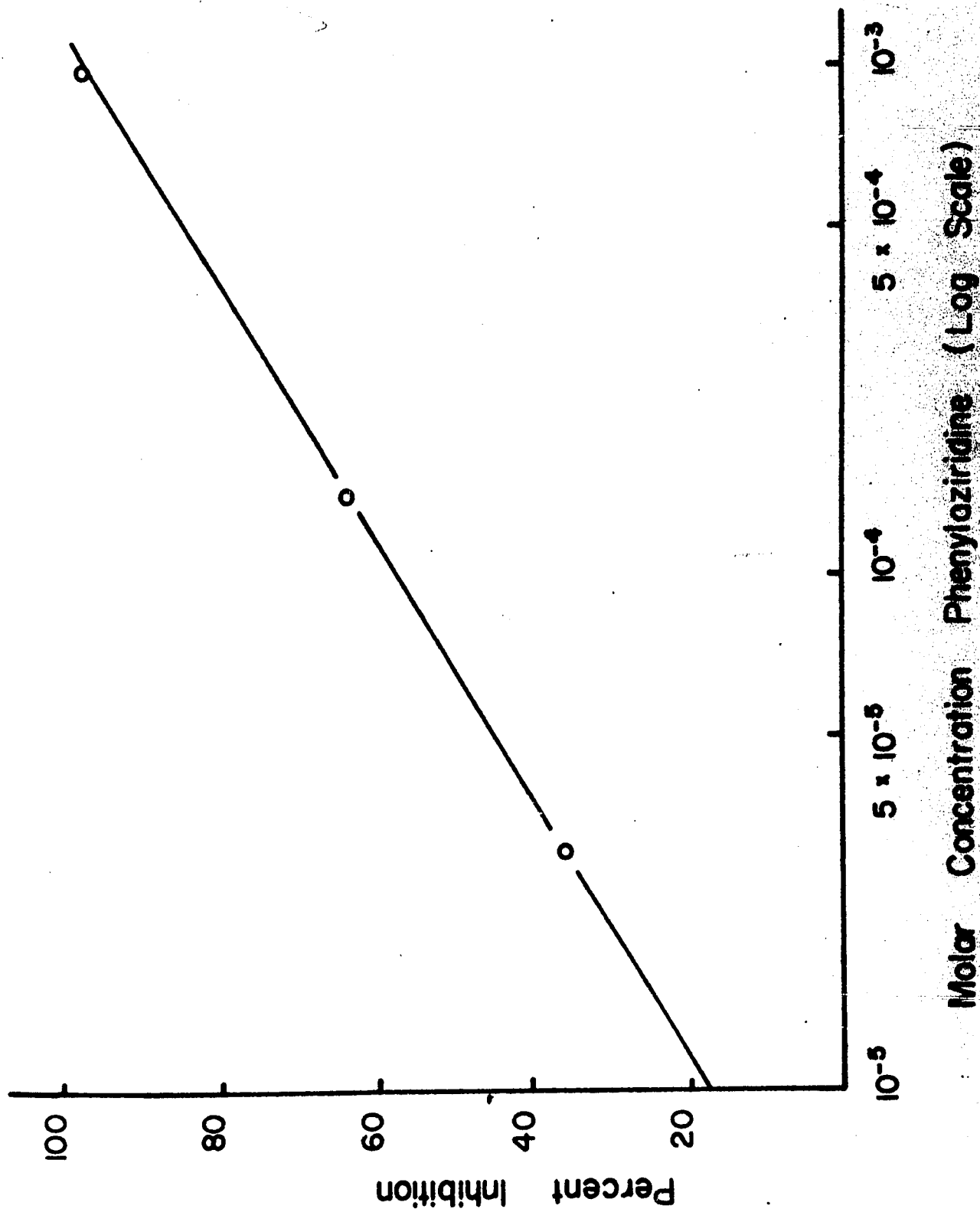


TABLE VIIPI₅₀'s For a Series of Moncamine Oxidase Inhibitors

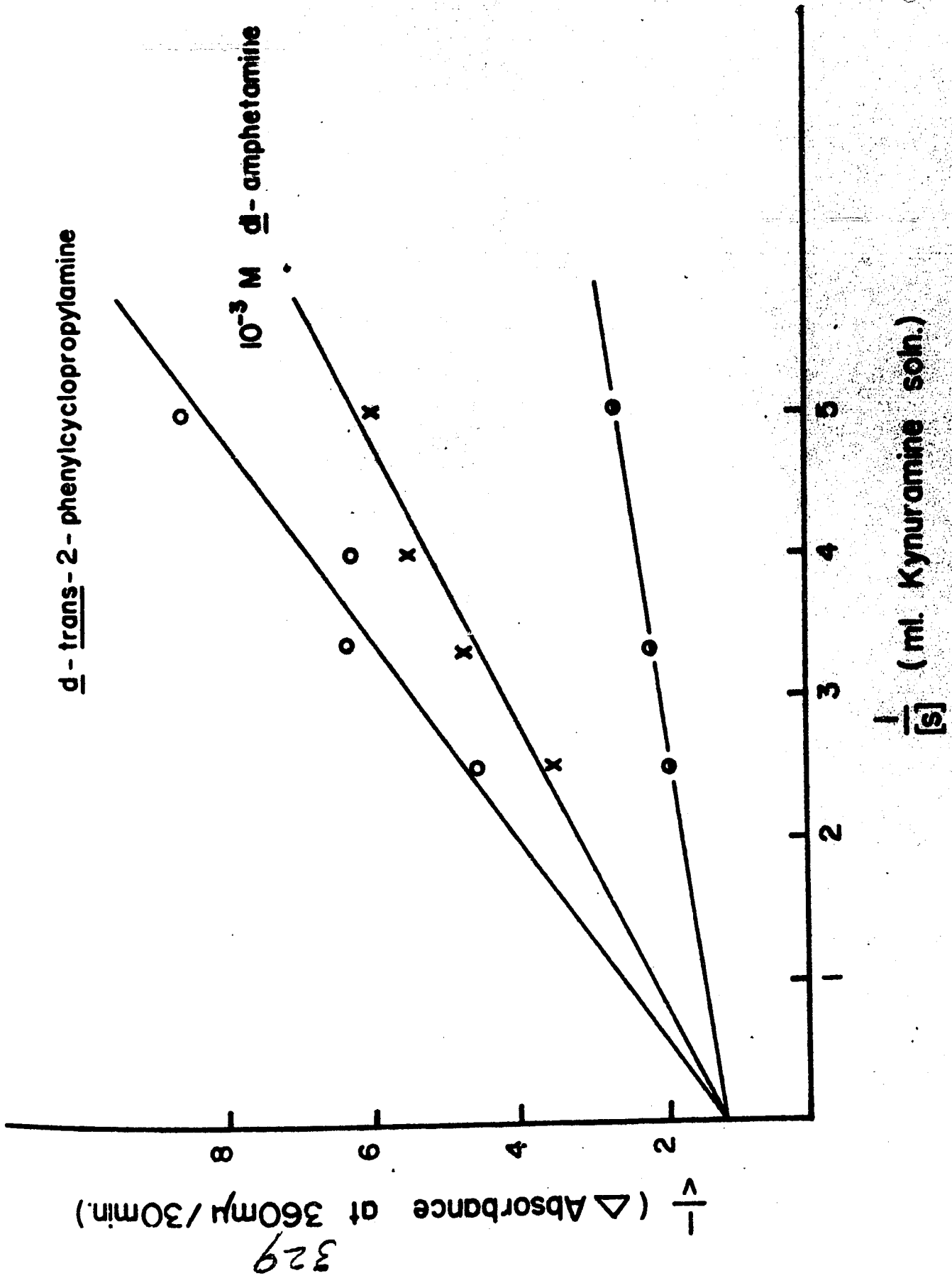
INHIBITOR	CONCENTRATION INHIBITING 50%	PI ₅₀
$\begin{array}{c} \text{CH}_3 \\ \\ \text{C}_6\text{H}_5\text{CH}_2\text{CH} \text{NH}_2 \end{array}$ dl-phenylisopropylamine	$1 \times 10^{-3} \text{ M}$	3
$\text{C}_6\text{H}_5 \text{---} \triangle \text{N-H}$ dl-phenylaziridine	$7 \times 10^{-5} \text{ M}$	4.1
$\text{C}_6\text{H}_5\text{CH}_2 \text{---} \triangle \text{N-H}$ dl-benzylaziridine	$8 \times 10^{-4} \text{ M}$	3.1
$\text{C}_6\text{H}_5 \text{---} \triangle \text{NH}_2$ d-trans-2-phenylcyclopropylamine	$3 \times 10^{-7} \text{ M}$	6.5
$\text{C}_6\text{H}_5 \text{---} \triangle \text{NH}_2$ l-trans-2-phenylcyclopropylamine	$5 \times 10^{-6} \text{ M}$	5.3
$\text{C}_6\text{H}_5 \text{---} \square \text{NH}_2$ dl-cis-2-phenylcyclobutylamine	$5 \times 10^{-4} \text{ M}$	3.3
$\text{C}_6\text{H}_5 \text{---} \square \text{NH}_2$ dl-trans-2-phenylcyclobutylamine	$1 \times 10^{-3} \text{ M}$	3
$\text{C}_6\text{H}_5 \text{---} \square \text{NH}_2$ 3-phenylcyclobutylamine	$6 \times 10^{-5} \text{ M}$	4.2

b. Type of Inhibition

The difference in the nature of the Lineweaver-Burk plot for competitive and non-competitive inhibition provides a quantitative means of distinguishing between the two types of inhibition (118). Amphetamine, a known inhibitor (9), and trans-2-phenylcyclopropylamine were used at 10^{-3} and $10^{-5}M$ respectively in the kynuramine assay and the rates of kynuramine oxidation measured at increasing substrate concentrations ($6-20 \times 10^{-5}M$). Lineweaver-Burk treatment of the data is given in Figure 11.

Fig. 11. - Competitive inhibition of monoamine oxidase by amphetamines and d-trans-2-phenylethylpropylamine. Various levels of the amine were incubated as described in the experimental. The inhibitor concentration was held constant. V = change in absorbance at 360 m per 30 minutes; (S) = ml of 2×10^{-3} molar kynuremine solution per 3 ml.

Fig. 11. - Competitive inhibition of monoamine oxidase by amphetamine and *d*-trans-2-phenylethylpropylamine. Various levels of the amine were incubated as described in the experimental. The inhibitor concentration was held constant. V = change in absorbance at 360 m μ per 30 minutes; (S) = μ l of 2×10^{-3} molar kynuramine solution per 3 ml.



c. Reaction of ninhydrin with dl-trans-2-phenylcyclopropylamine

Ninhydrin (0.002 moles) and dl-trans-2-phenylcyclopropylamine were allowed to react in water at 100°C for 20 minutes after which the aqueous solution was extracted with ether. After solvent evaporation, the residue was treated with 2,4-dinitrophenylhydrazine and the hydrazone subjected to descending chromatography on Whatman No. 1 according to Rice (120) using 5% ether-petroleum ether (80-100°) as the mobile phase. The 2,4-dinitrophenylhydrazone of the reaction product behaved identically to an authentic sample of cinnamaldehyde 2,4-dinitrophenylhydrazone.

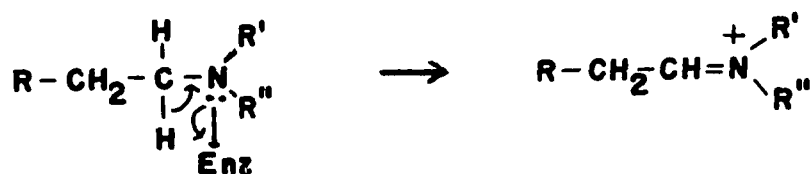
d. Incubation of l-trans-2-phenylcyclopropylamine for 24 hours

A standard incubation mixture having a final concentration of $10^{-4}M$ l-trans-2-phenylcyclopropylamine in place of substrate was incubated for 24 hours. After heat denaturation of the protein (100° for 10 minutes), an aliquot of the supernatant sufficient to give a $10^{-5}M$ final concentration (calculated on the basis of the original solution) was assayed for its content in inhibitor using the kynurexine assay procedure. The supernatant inhibited the reaction to the same extent (69%) as would be expected if none of the inhibitor was changed in the overnight incubation. This experiment shows that it is not a metabolite but the intact molecule of 2-phenylcyclopropylamine which is active as an inhibitor.

DISCUSSION

It is necessary to make one postulate in order to provide a suitable starting point for the interpretation of the results presented in the previous section. It is not critical that this postulate be valid since this would only lead to parallel but not opposite modifications of the interpretive part of this work. As pointed out in the introduction, tertiary amines can be oxidized by MAO so long as the N-substituents are not so bulky as to cause steric interference with the catalytic surface of the enzyme. Also, the presence of a second basic group in close proximity to the amino function undergoing oxidation results in substrates which are less reactive. These considerations make the following postulate appear logical and likely. That is, oxidation of the amine is initiated by binding to the enzyme through the available electron pair on the nitrogen.

We are now left with two mechanisms which result in formation of the desired intermediate. Abstraction of an electron pair by the functional group on the enzyme suggests that one of the alpha-hydrogens may be removed as a proton as shown in (I) \rightarrow (II), rather than as a free radical.



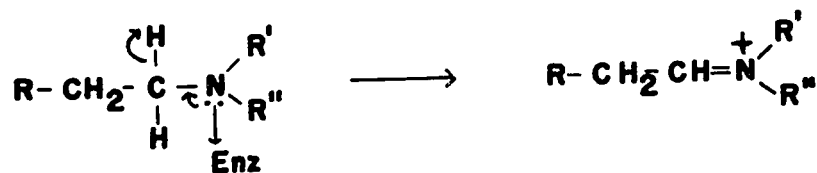
(I)

(II)

(R = aryl or alkyl; R', R'' = H, CH₃, or C₂H₅)

This mechanism finds analogy in the mercuric acetate oxidation of amines as established by Leonard (121). Evidence for the possible participation of a metalloflavin was presented in the introduction (p.18) and the mercuric acetate catalyzed reaction could serve as a chemical model of the enzymic reaction.

Alternatively, it could be envisioned that after initial electrostatic or coordinate binding through the electron pair, the catalytic surface could assist in the removal of an α -hydride ion followed by a shift of the electrons on the nitrogen in a concerted process as in (III) \rightarrow (IV).



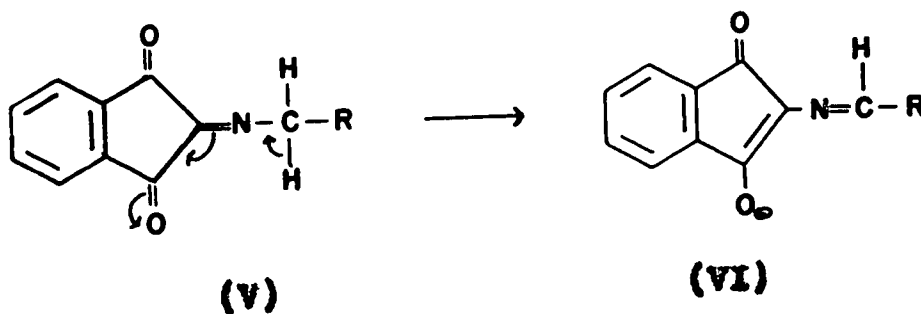
(III)

(IV)

This would lead to the same product, the formulation of which was proposed by Richter (27). This is also in accordance with the mechanism proposed by Mahler (44) for electron transfer catalyzed by known metalloflavin enzymes.

A. MODEL STUDIES

In order to examine the possibility of the operation of deuterium isotope effects when an α -carbon-hydrogen bond is broken to liberate a proton, a simple chemical model was examined which consisted in the reaction of ninhydrin with α -deuterated amines. The reaction can be easily rationalized as shown in equation (V) \rightarrow (VI).



This illustrates an oxidation mechanism involving loss of an α -hydrogen as a proton. When the rate of oxidation of tyramine was compared with that of bis- α -deuterio-tyramine as followed by the appearance of the typical blue-purple color absorbing maximally at 550 m μ , the kH/kD ratio was found to be 4.5 when chloroform was used as the solvent. Mescaline gave similar results whereas with benzylamine the kH/kD ratio

was 1.4. This is understandable when one considers the greater inherent acidity of benzylic hydrogens which would be expected to produce a transition state characterized by a smaller degree of bond-breaking, allowing for a better participation of a proton acceptor.

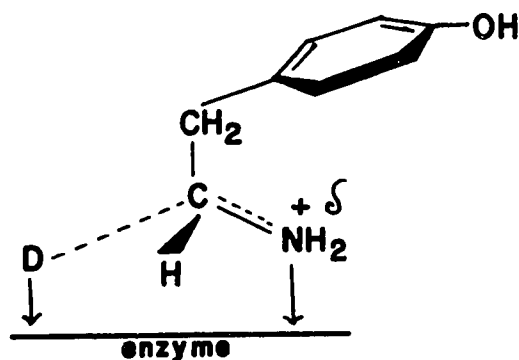
These observations suggested a method for the quantitative determination of mixtures of amines and α -deuterated amines. Results of this phase of the investigation were most rewarding in providing an expedient, efficient and accurate method for analyzing small samples of mixed species. As shown in Table II, the accuracy was within 1.3% with quantities of 2.9 micromoles. N.M.R. analysis proved impractical in this respect because of the large samples that are required and because of the relatively limited accuracy (5-10%).

It was also observed that the rate of reaction increased with increased basicity of the solvent which would be expected if the process is base-catalyzed. In order to eliminate the neutralization operation when amine hydrochlorides were used, which required an additional extraction into the desired solvent, pyridine was added to the reaction mixture. Using butanol-3% pyridine, it was observed that the apparent isotope effect using tyramine and bis- α -deuterio-tyramine was as high as 6.9. Because of this large k_H/k_D ratio, it was to our advantage to use this solvent system in the analysis of mixtures of deuterated and non-deuterated amines since the

sensitivity was proportionately increased (p.50). These rather large isotope effects indicate that there must be a considerable bond-weakening effect in the transition state for removal of an α -proton and therefore suggests that if a similar mechanism applies to MAO, an appreciable kinetic isotope would be expected as long as the removal of an α -hydrogen constitutes the rate limiting step.

B. α -DEUTERIUM ISOTOPE EFFECTS

The ratio of the slopes of the initial velocities for the oxidation of tyramine and α - d_2 -tyramine was found to be 2.3 at a substrate concentration of $1.15 \cdot 10^{-3}M$ (p.53). Hence, the rate limiting step in the oxidation must consist in the removal of an α -hydrogen and the magnitude of the ratio indicates that a fair degree of bond-weakening must occur in the transition state which may tentatively be formulated as in (VII).



(VII)

In a similar fashion, when the rate of oxidation of α - d_2 kynuramine was compared with kynuramine, a k_H/k_D ratio of

2.1 was observed (p.62). It was then demonstrated that these ratios could be varied considerably depending on the initial substrate concentration. The important finding that a rather large isotope effect ($K_{MD}/K_{MH} \approx 2.5$, p.58) operates at the level of the formation of the Michaelis complex supplied the explanation for the variation of the k_H/k_D ratios with substrate concentration. At high substrate concentration (maximum velocity) the k_H/k_D ratio falls to 1.2 and therefore this represents the true kinetic isotope effect operating in the rate-limiting removal of an α -hydrogen in the transition state.

These results were surprising in a number of ways because it means that replacing hydrogen by deuterium causes the substrate to be bound much more loosely. This is the reverse of what one would expect normally if the reactive parts of the substrate form tight bonds with the enzyme active sites. Such tight bonds would raise the vibrational frequencies of the atoms concerned and this would favor the binding of the deuterated amines, the zero-point energy of the C-D bond being lower. Our observation of an opposite effect establishes that a C-H bond is already considerably weakened in the complex. This is the first time that such an insight into the nature of a so-called Michaelis complex is gained. It is of interest that there is little further weakening of the C-H bond when passing from the complex to the transition state and this suggests that complex formation

involves much more than the simple operation of ill-defined van der Waal's forces.

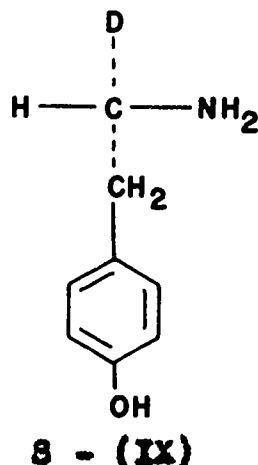
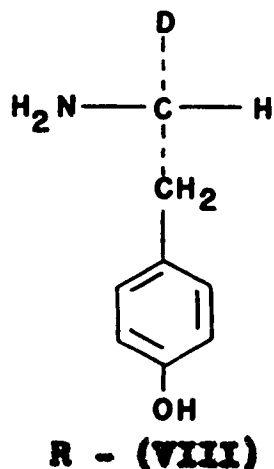
Confirmation of the true differences in binding constants between the two species was sought by studying substrate competition for the enzyme when the latter was saturated simultaneously with α - d_2 -tyramine and α - H_2 -tyramine. Determination of the ratio of d_2 -substrate to H_2 -substrate in the isolated amine after partial oxidation provided a direct measure of the relative affinities of α - d_2 -tyramine and α - H_2 -tyramine for the enzyme. When an equimolar mixture of the two amines was employed, it was found that a 30 minute incubation led to a 38% enrichment in α - d_2 -tyramine. On the basis of a zero-order rate constant ratio of 1.2 (observed at enzyme saturation level), one can estimate that a 14% enrichment would result if the affinities for the enzyme were equal (98). Since the enrichment is from two to three times as high, it is clear that the affinity of the unlabelled tyramine for the enzyme is from two to three times higher than for α - d_2 -tyramine. This is in excellent qualitative agreement with the calculated ratio of the Michaelis constants.

It is clear that the substitution of deuterium for the α -hydrogens of tyramine causes a considerable decrease in the tightness of binding onto the enzyme. Furthermore, the magnitude of the difference indicates that in the complex which must be formed reversibly, a carbon-hydrogen bond has

already begun to suffer fission to a considerable extent. It seems possible that the looser binding of the α -deuterated amines reflects mostly a decrease in bending and stretching vibrations because they occur at sufficiently high frequencies to account for the magnitude of the ratios observed (98). It can be strongly inferred that when tyramine or kynuramine forms a complex with MAO, the bound amine resembles the transition state much more than the ground state of the substrate. After this work had been completed, Abeles and coworkers (108) reported observations on the system deuteromethyl sarcosine and sarcosine oxidase which we interpret in a similar fashion. It seems likely that the above described approach will serve as a powerful tool in the investigation of enzyme mechanisms.

C. ABSOLUTE OPTICAL STEREOSPECIFICITY

The fact that a large isotope effect operates at the level of the Michaelis complex formation demonstrates that at least one alpha-hydrogen must be sterically and electronically accommodated on the enzyme. Further investigation with the enantiomers (VIII) and (IX) of mono- α -d-tyramine clearly demonstrated a configuration dependent isotope effect in the enzymatic oxidation of the asymmetrically labelled substrates. The incubations were carried out under identical conditions and it was found that tyramine and S- α -d-tyramine (VIII) were oxidized at the same rate, whereas R- α -d-tyramine (IX) and α -d₂-tyramine were deaminated at a rate 2.3 times slower (p.56).

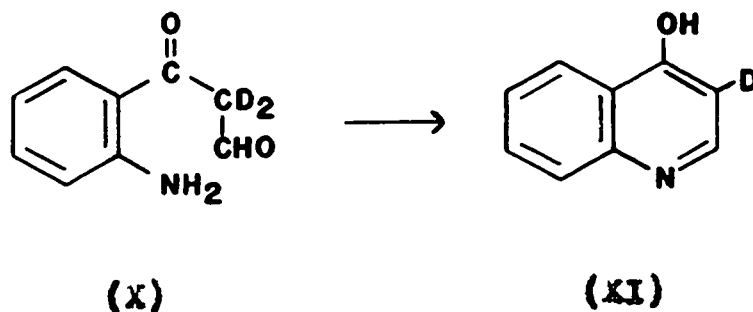


This clearly establishes that the two α -hydrogens are not equivalent for the enzyme. The results can be rationalized by assuming what is loosely termed a "three point contact" (123) between the substrate and enzyme. Obviously, the α -hydrogen corresponding to the α -deuterium of R- α -d-tyrosine is abstracted in a stereospecific manner thus necessitating the "freezing" of the substrate into a single conformation about the α -carbon. Thus, the amino group and one α -hydrogen must be implicated in this "three point attachment" but it does not appear possible at the present time to decide in favor of either the second α -hydrogen or the remaining hydrocarbon moiety as the third group completing the contact or alternatively producing steric repulsions. The possible role of the second α -hydrogen in the binding or in the transition state might be ascertained by determining the Michaelis constants for the enantiomers of mono- α -d-tyrosine. However, time did not permit investigating this problem. The consequences of this optical specificity on

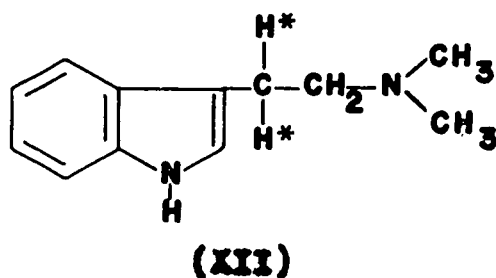
the interpretation of the mechanism of action of inhibitors will be discussed later.

D. β -DEUTERIUM ISOTOPE EFFECTS

After our observations of primary deuterium isotope effects with α - d_2 -kynurenine, it was noted that some of the best substrates for MAO usually have the carbon beta to the amino group unsubstituted (p.22). This led us to postulate that the β -hydrogens may contribute in some specific way to the attainment of the appropriate transition state for the breaking of an α -hydrogen bond. Accordingly, the effect of substitution of the β -hydrogens for deuterium in kynurenine on the course of oxidation by MAO appeared worthwhile investigating and indeed led to the observation of a rate retarding effect. Comparison of β - d_2 -kynurenine with kynurenine in the oxidation by MAO was carried out by measuring the rate of disappearance of the substrate rather than the appearance of the product since the spontaneous formation of the latter (XI) from the intermediate aldehyde (X) could lead to a non-enzymatic isotope effect.



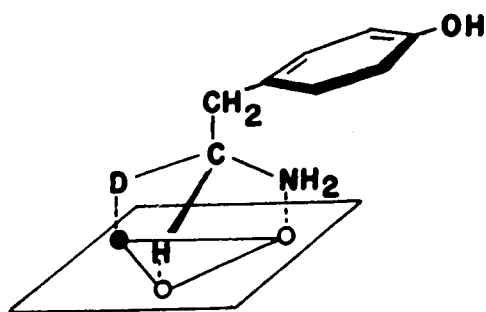
Since it is improbable that a β -deuterium should be abstracted during the formation of (X), only a secondary deuterium isotope effect may be observed. Confirmation of this is provided by results of Smith, et.al. (57) who found there was no loss of tritium in β -labelled N,N-dimethyltryptamine (XII) when



incubated with MAO. These results appeared simultaneously with our publication covering some aspects (107) of the work described herein.

The conclusion is inescapable therefore that the process of the abstraction of an α -hydrogen implies a concomitant weakening of a β -carbon-hydrogen bond in the transition state. As will be seen below, this secondary isotope effect ($k_H/k_D = 1.18$ p.62) can be interpreted as reflecting a contribution of the β -hydrogens to the stabilization of the transition state for oxidation. In contrast to the α -deuterium isotope effect on Michaelis complex formation, the β -deuterio substrate has the same affinity for the enzyme as the non-labelled kynuramine (p.62). This observation immediately suggests that the attachment of the substrate to the

enzyme does not involve the β -hydrogens as otherwise the Michaelis constant would be expected to either increase or decrease depending on whether the β -CH bonds form a strong bond with the enzyme or are weakened by interaction with it. We are left with the possibility that substrates form a complex with the active center of the enzyme through the substituents on the α -carbon, i.e., the amino group and the two α -hydrogens. Other groups such as phenyl in the substrate molecule would not interact with the active centers where the reaction is actually performed but could conceivably interact with some other accessory sites unconcerned in the catalytic process. On the basis of the above results, it is tempting to suggest that the other substituents on the substrate molecule may be primarily concerned in the process of penetration of the mitochondrial structure but otherwise unnecessary for the catalytic surface to achieve complex formation with the substrates. The so-called "three point attachment" onto the enzyme may therefore be loosely rationalized in terms of formulation (XIII) where one site is specific for abstracting the hydrogen corresponding to the α -deuterium of R- α -d-tyramine.

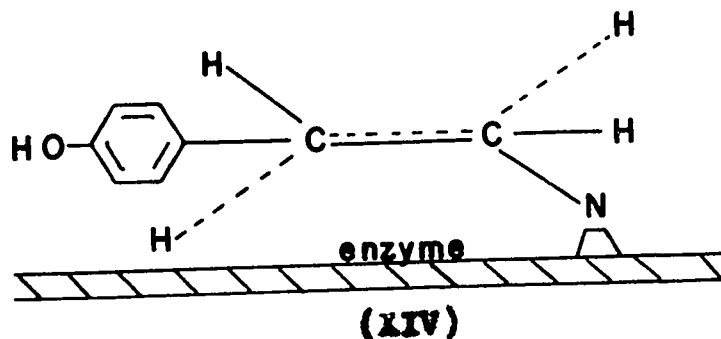


(XIII)

The bearing of these considerations on the mode of action of certain inhibitors is discussed below.

E. THE NATURE OF THE TRANSITION STATE

The above observations on primary and secondary isotope effects allow the conclusion that in the transition state for oxidation of the amine, one α -hydrogen is weakened. From the secondary isotope effect studies, it can be deduced that a β -carbon-hydrogen bond is also weakened in the transition state, a conclusion which might not be valid only if secondary isotope effects cannot be explained by hyperconjugation. However, recent evidence points to the contrary (101,124,125). During the process of the abstraction of an α -hydrogen, one resonance form of the transition state leaves the β -carbon positively charged. Stabilization of the latter through hyperconjugation requires contribution of the resonance species (XIV), the importance of which should markedly



increase when the resulting α, β double bond is conjugated either with a phenyl ring as in tyramine or a carbonyl group as in kynureamine. Shiner (125) has repeatedly demonstrated

that the formation of a carbonium ion or partial carbonium ion is retarded by the substitution of deuterium in a hyperconjugating position.

The possible appearance of double bond (sp^2) character at the site of the α,β -carbon-carbon bond may have an important bearing on the mode of action of the phenylcyclopropyl amine group of inhibitors. For convenience, we may offer the generalization that any factor contributing to an increase in the acidity of the β -hydrogens (thus reflecting in general an increased contribution of hyperconjugation to the stability of the transition state) should facilitate oxidation of the substrate. It is not impossible that hyperconjugation may also be involved to a limited extent in the formation of the Michaelis complex but presently available techniques are not refined enough to allow the detection of such small effects.

F. CORRELATION OF SUBSTRATE STRUCTURE WITH RELATIVE RATES OF OXIDATION BY MAO

Numerous scattered reports on the oxidation of a wide variety of substrates by MAO are available but very few kinetic analyses are to be found. This makes it somewhat difficult to give an exact quantitative evaluation of relative rate data especially since MAO from different sources is not identical. From the qualitative point of view, it nevertheless appears possible to interpret a variety of results on

the basis of the deductions arrived at concerning the properties and nature of the Michaelis complex and the transition state for oxidation. According to the preceding generalizations, whenever increased conjugation in the transition state is possible, the ability of a molecule to act as a substrate for MAO should be increased as long as no other complicating factors such as steric hindrance to complex formation are present. It is therefore necessary to restrict the analysis of substrate-enzyme relationships to strictly homologous and analogous series in seeking evidence for the proposed properties of the complex and the transition state.

It has been observed that phenethylamine is a good substrate for the enzyme whereas benzylamine, phenylpropylamine and phenylbutylamine are uniformly poorer substrates (62). However, kynurexamine, which is not a natural substrate, allows for conjugation in the transition state and is a good substrate. In contrast, nor-kynurexamine and dihydrokynurexamine cannot lead to increased conjugation (22) and consequently are very poor substrates. It is not possible to include in this discussion the use of substituted phenethylamines as there are no kinetic studies available on homologues in this series.

Although these fragmentary correlations appear sufficiently convincing to support our interpretation of the mechanisms involved, it would be desirable to test the theory

through the verification of predictions based on it. To this end, future work should include a study of the relative rates of oxidation of alkylamines and their gamma-keto analogues. It can be predicted that the introduction of a carbonyl group gamma to the amino group should enhance the rate of oxidation appreciably in the aliphatic series.

G. INTERPRETATION OF STRUCTURE-ACTIVITY RELATIONSHIPS AMONGST INHIBITORS OF THE PHENYL ALKYLAMINE SERIES

It was recognized at an early time that α -substituted phenethylamines act as competitive inhibitors of MAO (9). The relatively low affinity of amphetamine and ephedrine for the enzyme parallels their rather low in vivo activity. On the basis of our conclusions concerning the stereospecificity of the catalytic center of the enzyme, it is not surprising that the replacement of one α -hydrogen in a substrate by an alkyl group such as methyl should produce steric interference with the active surface and increase the energy barrier for oxidation to such an extent as to exclude oxidation of the amine. However, complex formation should still occur to some extent but since it has been shown that the weakening of an α -carbon-hydrogen bond is a consequence accompanying the binding of substrate molecules, the steric interference of an α -methyl group would prevent enzymatic alteration of the α -CH bond and would tend to favor only a "two point attachment". Evidence for this interpretation

can be adduced from the relative lack of optical specificity of MAO towards the enantiomorphs of amphetamine. If the presence of an α -methyl group favors a "two point attachment" as opposed to a "three point attachment", which is the case when two α -hydrogens are present, then both optical isomers of amphetamine should be roughly equipotent. Since this is what is observed (66,125), it follows that α -alkylation of amines would lead essentially to a "two point attachment" with the catalytic surface and therefore produce only weak inhibition.

It should be noted that our interpretation of the mechanism by which MAO may initiate the oxidation of an amine, through the initial binding of the electron pair on the nitrogen, does not require the amino group be unsubstituted. In order to achieve complex formation with the enzyme active sites it is only necessary that a free electron pair be present on the nitrogen. Hence, tertiary amines may be expected to be bound by the enzyme but it may be that the presence of alkyl groups on the nitrogen interfere sterically with the attainment of the transition state for oxidation. On this basis, some tertiary amines may act as inhibitors rather than substrates. In fact, a number of such compounds have been reported to block MAO (75,83) and this adds support to our suggestion that Michaelis complex formation primarily requires the presence of a free electron pair on the nitrogen.

The extraordinary potency of the 2-phenylcyclopropylamines must now be considered in relation to the preceding discussion of the mode of action of amphetamine and related compounds. It is a most remarkable phenomenon that the incorporation of the α -methyl group of amphetamine into a cyclopropyl ring should increase the inhibitory potency of the former by a factor of approximately thirty-three hundred (p.67). This fact must be highly significant in relation to the chemical mechanics of MAO especially since the structural change from amphetamine to 2-phenylcyclopropylamine produces stereochemical as well as electronic differences which should bear some relationship to the properties and requirements of the catalytic surface of the enzyme. The structural similarity and equivalence of the molecular weights of the two amines justifies excluding differences in their lipid solubility or partition coefficients at the mitochondrial level as contributing a three thousand fold difference in inhibitory potency. It was essential at first to determine the type of inhibition produced by trans-2-phenylcyclopropylamine in view of the special chemical reactivity of the cyclopropane ring which could conceivably lead to covalent bond formation with the enzyme and produce a non-competitive inhibition. This possibility became more likely when the observation was made that treatment of the drug with ninhydrin led to the formation of cinnamaldehyde. This reaction can be easily rationalized as shown in equation (XV) \rightarrow (XVII).

that both the cis- and trans- isomers of 2-phenylcyclopropylamine display nearly equal potencies (67) confirms our conclusion that the phenyl group of substrates and inhibitors does not interact with the catalytically active surface of the enzyme since the latter displays an optical specificity towards the α -hydrogens of substrates. On the other hand, a "two point attachment" of the inhibitor onto the enzyme would lead to the prediction that the optical isomers of trans-2-phenylcyclopropylamine should be roughly equipotent. Although some difference in the activity of the (+)- and (-)- isomers can be observed (p.67), it is not significant enough to suggest a "three point attachment" onto the enzyme since the operation of this latter type of steric effect usually leads to an "all or none" type of effect. However, it is quite probable that the catalytic surface may show some preference for one orientation of the bulky side of the molecule (carrying the phenyl group) and this can account for the somewhat greater potency of the (+)- isomer of trans-2-phenylcyclopropylamine. Since this preference is far from being absolute, a "two point attachment" is suggested and the high affinity of these inhibitors for the enzyme can be ascribed, at least in part, to the reduced steric interference to complexing produced by an α -methylene as compared to an α -methyl group.

Another factor possibly contributing to the high affinity of phenylcyclopropylamine for the enzyme can be

discerned if our deductions concerning the transition state conducive to oxidation are taken into consideration. This factor which is relevant to the electronic properties of the cyclopropane ring could not have been easily seen if the results of the primary and secondary isotope effects studies had not been available.

The transition state for the oxidation of amines is schematized in Figure 12 where it should be noted that the α - and β -carbons are believed to approach the trigonal state thus necessitating that the α, β -carbon-carbon bond acquire double bond (sp^2) character. These features of the transition state bear a striking electronic analogy to the potent inhibitor 2-phenylcyclopropylamine. In 2-phenylcyclopropylamine, the α - and β -carbons approach the trigonal state in contrast to the tetrahedral geometry of the corresponding phenylalkylamines. Hence, some geometrical features of the transition state are mimicked by the cyclopropyl analogue (Figure 13). It is also well known that the cyclopropane bonds have sp^2 character in the ground state (128), another feature now believed to be a characteristic of the transition state for oxidation. Since steric and electronic complementation between substrate and enzyme must increase as the complex is formed and as the transition state is approached, it seems logical to expect that 2-phenylcyclopropylamine should be tightly bound by MAO, both the ground state steric

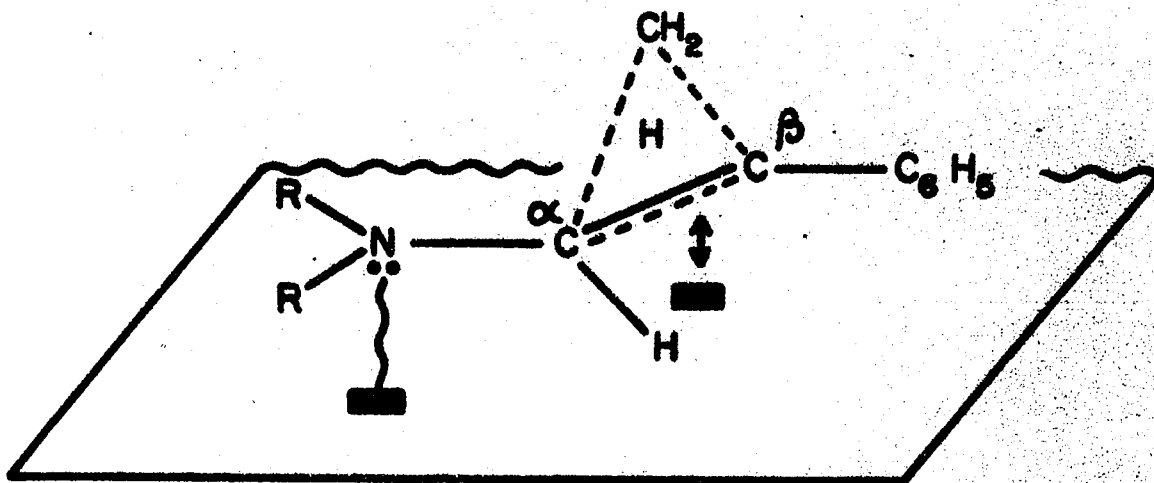


Fig. 13. - Mode of interaction between 2-phenylcyclopropylamine and monoamine oxidase.

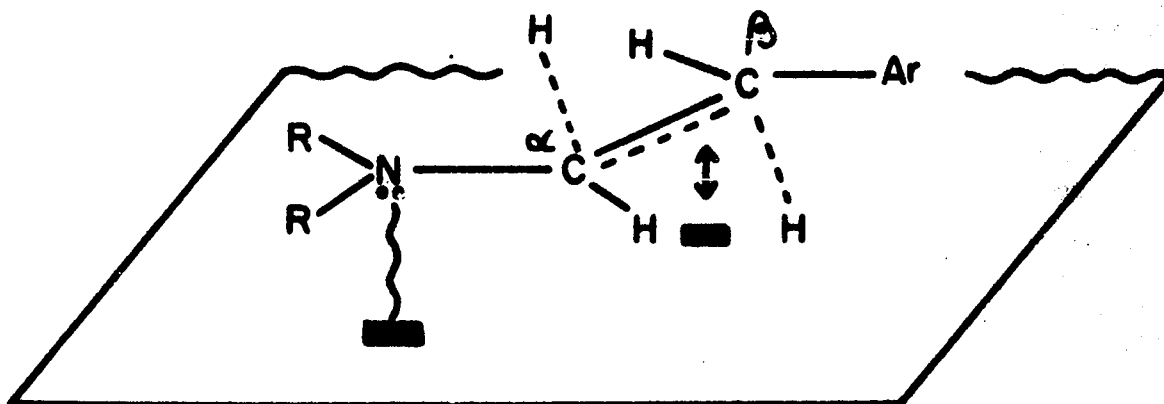


Fig. 12. - Interactions in the transition state for substrate oxidation by monoamine oxidase.

and electronic properties of this inhibitor reproducing some important features of the transition state for oxidation. It can further be inferred that the π electrons generated between the α, β -carbon-carbon bond of substrates may contribute largely together with the amine group to the binding energy in the transition state since at that stage the bond-breaking process at the α -carbon of substrates is already advanced and would make the departing hydrogen a poor anchoring group. If this is true, the high affinity for the enzyme may be ascribed to the high ground state electron density between the α, β -carbon-carbon bond (Figure 13) rather than to a direct interaction with the α -hydrogens. These considerations cannot be reconciled with the speculations of Zeller, et.al. (67) who ascribe a critical role to the α -hydrogen of 2-phenylcyclopropylamine in the binding process. The recent interesting finding of Burger, et.al. (129) on the equipotent inhibitor, 1-methyl-2-phenylcyclopropylamine, strongly supports our interpretation of the factors contributing to the binding energy in the transition state.

Indirect evidence of the validity of this interpretation is supplied by the fact that the cis- and trans-isomers of 2-phenylcyclobutylamine display weak inhibitory properties similar to amphetamine (p.67) in agreement with the complete absence of sp^2 character in the cyclobutane bonds. It is clear however that in the cyclobutylamine

series, greater steric interference with the active surface would occur, the α -methylene now being separated from the β -carbon by an extra methylene. Moreover, the 2-phenyl group is somewhat closer to the catalytic surface in this series than in the case of 2-phenylcyclopropylamine. It is of interest in this connection that moving the phenyl group to the 3-position of the cyclobutylamine ring improves inhibitory activity approximately ten fold (p.67), a result explained by the reduced steric interference between the phenyl ring and the active surface. However, these steric factors alone do not appear sufficient to account for the weak activity of the phenylcyclobutylamines as compared to the phenylcyclopropylamines and for this reason we tend to favor the preceding interpretation based on the electronic properties of the transition state.

This interpretation bears some analogy to that of Bernard and Orgel (130) who suggest that the high affinity of organophosphorus inhibitors towards hydrolytic enzymes is due to the ground state resemblance of the drugs with the transition state leading to acylation of the enzymes. Our interpretation suggests an entirely unexplored approach to the mechanism of enzyme action and to the rational development of enzyme inhibitors or drugs.

Phenyl and benzylaziridine were synthesized and their in vitro inhibition tested (p.67) as it is well-

known (131) that these compounds react readily with thiols and this could lead to non-competitive inhibition. However, their inhibitory potency was only slightly better than amphetazine although with a purified enzyme preparation one might expect greater activity due to the elimination of side reactions with other available functional groups. Further investigation of these compounds would be warranted with purified preparations.

IV. CLAIMS TO ORIGINAL RESEARCH

1. The demonstration that the rate determining step in the oxidation of amines by ninhydrin consists in the abstraction of an α -proton.
2. The elaboration of a method for the quantitative analysis of mixtures of primary amines and their α -deuterated counterparts based on kinetic isotope effects as described in Claim 1. Related to this is the application of this method to substrate competition with monoamine oxidase.
3. The discovery of the absolute stereospecificity of monoamine oxidase towards the α -hydrogens of primary amines. The application of kinetic isotope effects were used for the first time as a tool for the determination of this kind of stereospecificity.
4. The discovery of the operation of large isotope effects at the level of Michaelis complex formation with monoamine oxidase.
5. The discovery of a secondary isotope effect in the oxidation of β -bis-deuterio-kynuramine by monoamine oxidase.
6. The establishment that 2-phenylcyclopropylamine acts competitively as an inhibitor of monoamine oxidase and

that the enzyme does not display appreciable optical specificity towards the various isomers of this inhibitor. The determination of the inhibitory power of a series of phenyl substituted cyclobutylamines and aziridines which had not previously been tested as inhibitors.

7. The suggestion of a novel explanation for the unusual potency of the 2-phenylcyclopropylamines, the rationale stemming from our isotope effect studies.
8. A new theory is offered which explains the mechanism of action of the phenylalkylamine-type inhibitors including the lack of optical specificity of the enzyme towards them.

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