When upon you I gaze sweet maid,
Efforts unnatural must prevail,
That appraisal scientific can be made,
Of your structure and composition chemical.

Still a mystery it must remain,
How proteins, fats and carbohydrates,
All alone and ever so plain,
Could in unison give heartaches.

J.V. Burba

PREFACE

The oxidation of an amine to an aldehyde or acid, the decarboxylation of an acid, and the methylation of a phenolic hydroxyl to produce a methyl ether are reactions well known to the organic chemist, and they are carried out routinely in the laboratory. However, when an enzyme produces the same reactions and with much more efficiency, the biochemist sees beyond the simple reaction itself, and wonders about the mechanisms by which these reactions are brought about. The above reactions occur in most living species, and are essential to life. Hence, any further knowledge gained is of paramount importance in understanding certain disease states that occur due to a dysfunction of the enzymes that bring about these processes.

The mechanism of the decarboxylation of amino acids was carried to the point where it could be shown whether the decarboxylation occurred with retention or inversion of configuration. This would have been difficult to do if advantage had not been taken of the kinetic isotope effects produced by asymmetrically labeled deuterated substrates. The same method was used to determine the absolute optical specificity of rat brain monoamine oxidase, and was applied *in vivo* to gain a better insight into the mode of action of monoamine oxidase at the adrenergic effector cell level.

The second phase of this thesis deals with catechol-O-methyl transferase, and the discovery of a non-competitive inhibitor of this enzyme. Investigations were carried out with this inhibitor to gain a knowledge of the type of Michaelis complex that would be formed with this relatively new enzyme and its substrate.

Acknowledgements

To Dr. Bernard Belleau, I would like to express my gratitude. His continued guidance has made this thesis possible. There was an induction period of seven years before I finally decided to continue my studies and enter graduate school. Hence I would like to thank Dr. Belleau most sincerely for the tremendous patience that was necessary for the first year or so.

I would like to thank the Ontario Research Foundation for the necessary financial help in the form of a scholarship.

Last but not least I extend my profound thanks to my wife Charlotte, for moral and financial support, and also for typing this manuscript.

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ABSTRACT

It had been established that enzymic decarboxylation of amino acids proceeded stereospecifically. Advantage was taken of kinetic isotope effects with asymmetrically labeled substrates, and this led to the discovery that enzymic decarboxylation of amino acids proceeds with retention of configuration.

The same tool was used to determine the absolute optical specificity of rat brain monoamine oxidase in vitro, as well as to establish the role and stereospecificity of monoamine oxidase at the adrenergic effector cell level.

It was discovered that tropolones could inhibit catechol-O-methyl transferase non-competitively. This inhibitory power was not altered to any significant extent by a variety of substituent groups on the tropolone nucleus. Further investigation with 4-methyltropolone made it possible to propose a mechanism for Michaelis complex formation with enzyme, metal, and inhibitor. These results also enhanced the credibility of an enzyme, metal, substrate Michaelis complex mechanism already proposed by other investigators.

INTRODUCTION

A. GENERAL INTRODUCTION

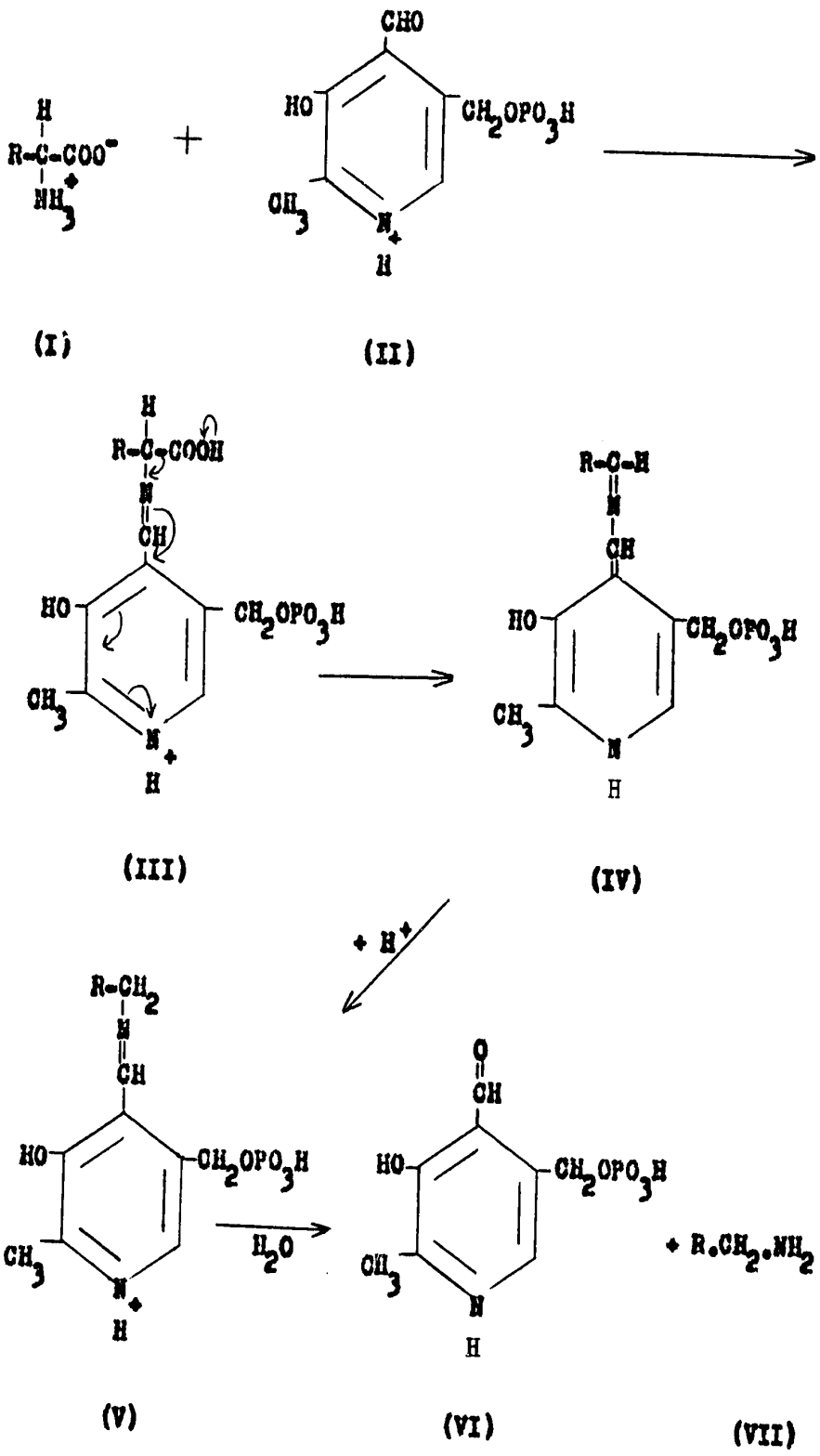
Tyrosine decarboxylase, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) are enzymes which are intimately associated with adrenergic mechanisms.

a) Tyrosine Decarboxylase

Tyrosine decarboxylase is a member of the many amino acid decarboxylases. The enzyme isolated from animal tissues will decarboxylate not only L-tyrosine but also other aromatic amino acids. Hence, it does not possess the marked substrate specificity for L-tyrosine alone as it was once postulated. The decarboxylation reaction can be represented by the following overall equation:

$$\text{L-tyrosine} + H_2O \overset{E}{\rightleftharpoons} \text{tyramine} + H_2CO_3 \qquad (1)$$

Amino acid decarboxylases all require pyridoxal phosphate as a cofactor. Pyridoxal will also catalyze the decarboxylation of amino acids even in the absence of the enzyme. The pyridoxal initially forms a Schiff base with the amino acid, and the decarboxylation would proceed according to the following scheme[1]:


(I)
(II)
(III)
(IV)
(V)
(VI)
(VII)
+ H+
H2O
+ R.CH2.NH2

b) Monoamine Oxidase

Monoamine oxidase (MAO) plays an important part in neuroamine catabolism. It is primarily a mitochondrial enzyme, which so far has not been purified to any great extent. All attempts at purification resulted in concomitant loss of activity. This enzyme lacks substrate specificity, and in general terms it can be said to be responsible for the oxidative deamination of neuroamines like tyramine, 3,4-dihydroxyphenethylamine, serotonin, isoamylamine, metanephrine and normetanephrine. The oxidative reaction can be represented by the following overall equation:

$$R\text{-}CH_2\text{-}NH_2 + 1/2\ O_2 \longrightarrow R\text{-}CHO + NH_3 \qquad (2)$$

The enzyme has no known cofactor requirements.

The importance of this enzyme in homeostatic mechanisms cannot be underestimated. One of its best substrates is 5-hydroxytryptamine (serotonin). The latter contracts smooth muscle in extremely low concentrations and has complex effects on the cardiovascular system both directly and indirectly. It has been shown that serotonin sensitizes pressor receptors in the mucosa of the intestine[2], so that it may play a role in normal peristalsis. It has been shown that high concentrations of serotonin are found in the gut. It is possible that MAO is responsible for controlling the levels

of this amine in the intestine; also it has been suggested that MAO in the intestine may be responsible for the detoxification of amines of bacterial origin in the gut[2].

Iproniazid, a potent MAO inhibitor, when administered to patients with a liver disease characterized by high blood levels of ammonia, was shown to cause a fall in the arterial ammonia levels. These observations suggest that some of this blood ammonia must be derived from amine degradation by MAO.

The absence or dysfunction of MAO in various parts of the brain could conceivably lead to other pathways for the metabolism of physiologically active amines[2]. For example, adrenaline or serotonin could be oxidised to hallucinogens; e.g. adrenochrome. Hoffer et al[3] suggest that substances which block MAO in vivo would make adrenaline more available for oxidation to adrenochrome. However, this suggestion would now have to include substances that also block catechol-O-methyl transferase, since it has been shown by Axelrod et al[4] that this enzyme is responsible for the primary deactivation mechanism of adrenaline and noradrenaline. These hormones are converted by COMT to the relatively inactive metabolites, 3-methoxy-4-hydroxy phenyl ethanolamine, and its N-methyl analogue.

There is a possibility that some forms of hypertension may be associated with increased levels of pressor

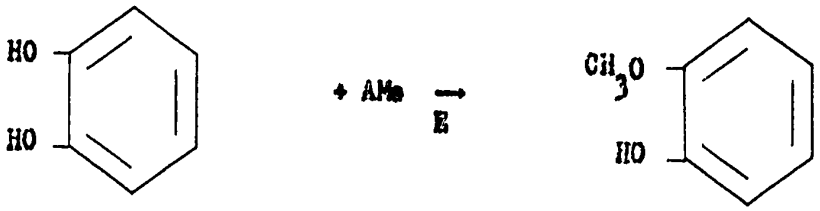
amines in the blood. Injection of a purified MAO preparation into hypertensive rats resulted in a prolonged lowering of the blood pressure[2].

Recently, Barondes[5] has suggested a non-degradative role for MAO. He discovered that phenylethylamine derivatives like tryptamine, benzylamine, tyramine, and isoamylamine share with epinephrine, norepinephrine, and serotonin, the ability to stimulate glucose-1-C^{14} oxidation to $C^{14}O_2$ by beef anterior pituitary slices. α-Methylated amines which are not substrates for MAO did not share this property, and the stimulation induced by the active amines could be blocked by MAO inhibitors. A number of aldehydes, like indoleacetaldehyde, phenylacetaldehyde, benzaldehyde and propionaldehyde were also found to stimulate glucose 1-C^{14} oxidation by beef anterior pituitary slices, and this effect was not blocked by MAO inhibitors. Related acids and alcohols were found to be inactive.

c) Catechol-O-Methyl Transferase

Catechol-O-methyl transferase (COMT) catalyses the transfer of the methyl group of adenosyl methionine (AMe) to the 3-hydroxyl groups of noradrenaline, and other polyphenols[6,7]. According to Axelrod[4], this seems to represent the initial deactivation reaction for catecholamines as well as other endogeneous and exogeneous polyphenols. COMT is found

in the soluble fraction of mammalian liver and in many other organs and was shown to require the presence of a divalent metal ion such as magnesium. The initial detection of normetanephrine and 3-methoxy-4-hydroxymandelic acid in urine by Axelrod[8] has stimulated much research on the role of this enzyme. The general reaction of COMT with a polyphenol substrate can be summarized as follows:



(VIII) (IX)

The enzyme has been purified about 30 fold but has not been crystallized as yet.

B. STEREOCHEMISTRY OF SOME ENZYMATIC REACTIONS

Monoamine oxidase will oxidatively deaminate a large variety of monoamines as well as some diamines; it displays poor substrate specificity towards the latter. Also, the enzyme appeared to lack optical stereospecificity[9]. Most enzymes are specific for either the D or L form of a substrate. Since α-bisdeuterio-tyramine, which is a good subs-

trate for the enzyme, was shown to be much more active than tyramine *in vivo* and since these isotope effects could be reproduced *in vitro* with MAO preparations[9], the logical sequel was to ascertain whether the observed isotope effects might not be dependent on the configuration of the deuterium atom in the corresponding mono-α-labeled tyramine.

a) Alcohol Dehydrogenase System

An analogy can be drawn here, with regard to the elegant work of Vennesland and Westheimer[10], who proved the absolute optical specificity of alcohol dehydrogenase using stereospecifically deuterium labeled ethanol. These authors have recorded fundamental information about the stereospecificity of DPN*-linked dehydrogenation reactions. They prepared DPND** by reducing DPN* with sodium hydrosulfite in D_2O. The deuterium of DPND was found to be non-exchangeable in water solution. DPND, prepared in this way, was subjected to enzymic oxidation by many dehydrogenases with the appropriate substrates, and it was found that a little over half of the deuterium was removed from the coenzyme. However, if DPN* is reduced by alcohol dehydrogenase, (ADH) in the presence of

---

* Diphosphopyridine nucleotide.

** Diphosphopyridine nucleotide, reduced form (deuterium replaces hydrogen).

excess CH_3CD_2OH, only one of the two positions for H on the methylene group at position 4 of nicotinamide is occupied by D. On enzymic re-oxidation of this enzymatically reduced DPND, all the deuterium was removed from the coenzyme. This shows that alcohol dehydrogenase discriminates between the two apparently equivalent hydrogens on the α-carbon of ethanol and also between the two hydrogens protruding on each side of position 4 of reduced DPN. Experiments with many dehydrogenases including several similar types from different sources showed that such stereospecificity was a general phenomenon.

It can be said therefore that alcohol dehydrogenase is stereospecific for one of the α-hydrogens of ethanol, a substrate which has no plane of asymmetry. The two enantiomorphs of ethanol-1-d were obtained[11] by the following procedure:

$$CH_3COH + DPND + H^+ \overset{ADH}{\rightleftharpoons} CH_3CHDOH + DPN^+ \qquad (3)$$

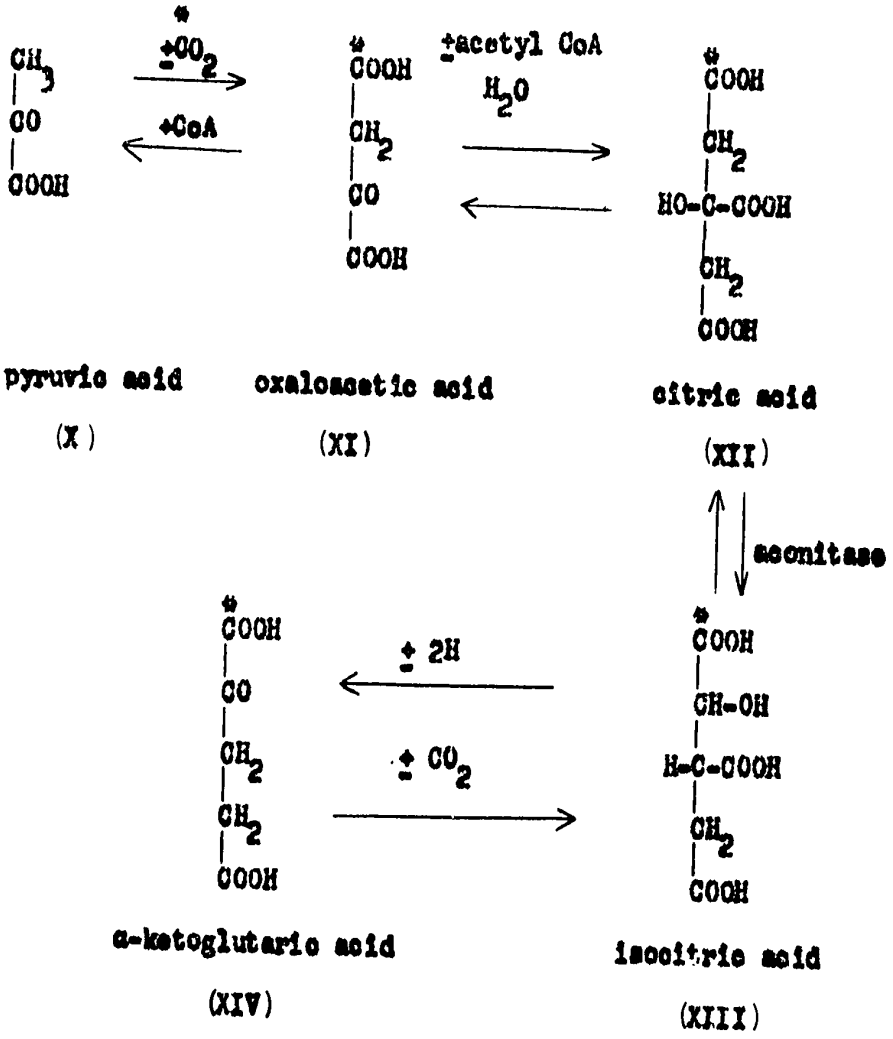
$$CH_3COD + DPNH + H^+ \overset{ADH}{\rightleftharpoons} CH_3CHDOH + DPN^+ \qquad (4)$$

Only the ethanol-1-d prepared by method (3) above led to deuterated reduced DPN on reversal of the reaction.

b) <u>Citric Acid-Aconitase System</u>

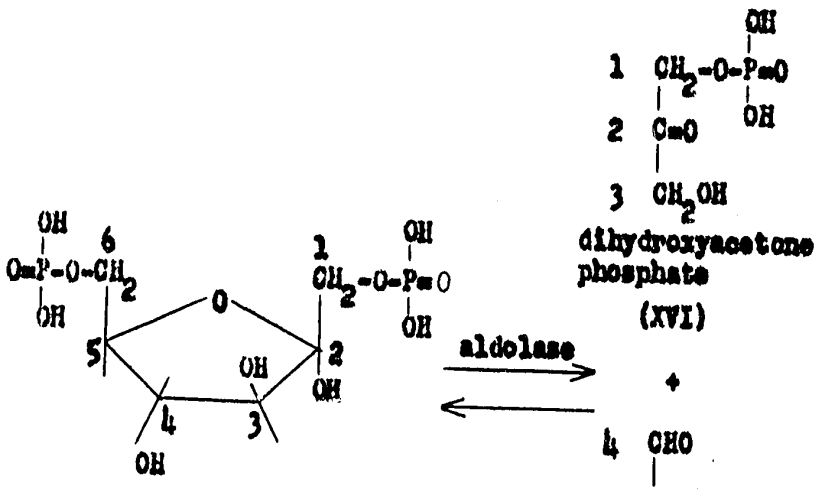
Ogston was one of the first to point out that enzymes should theoretically distinguish between two identical

groups or atoms in symmetrical substrates[12]. This idea stemmed from difficulties in the interpretation of tracer experiments with enzymes of the Krebs cycle. It was thought for a time that, since pyruvic acid after incubation with isotopic CO_2 and pigeon liver preparation produced α-ketoglutaric acid which contained the isotope in the carboxyl group adjacent to the carbonyl group, the production of α-ketoglutaric acid could not involve any symmetrical intermediates such as citric acid, the role of which had nevertheless been clearly established[13]. Ogston, however, called attention to the fact that although citric acid is a symmetrical compound, the enzyme which causes its metabolic transformation is not. Hence, the enzyme-catalyzed conversion of a symmetrical molecule such as citric acid but labeled asymmetrically with an isotope would be expected to take an asymmetric course, provided the substrate has a specific spatial relationship to the enzyme at three points in the enzyme-substrate complex. Experimental evidence for the formation of such isotopically asymmetric citric acid by rat liver homogenates was provided by Potter and Heidelberger[14]. The two acetic acid moieties of citric acid are not identical for aconitase; this is illustrated by the following equations:

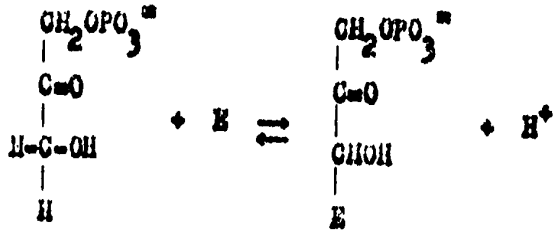


c) Dihydroxyacetone Phosphate-Aldolase System

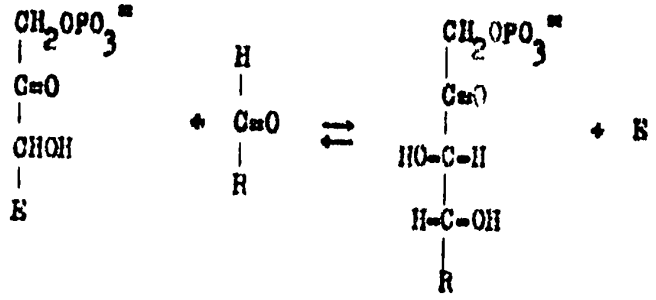
Another classic example of the ability of an enzyme to distinguish between two identical atoms on the same carbon atom is provided by the system dihydroxyacetone phosphate-aldolase. The enzyme catalyzes the formation of fructose-1,6-diphosphate from D-glyceraldehyde 3-phosphate. The equilibrium reaction can be represented as follows:



Tracer experiments suggested that the enzyme combines with dihydroxyacetone phosphate in such a way as to labilise only one of the two hydrogen atoms on carbon 3, and this in a stereospecific manner[15]. The condensation reaction has been shown to proceed via the following pathways:



(XVIII) (XIX)

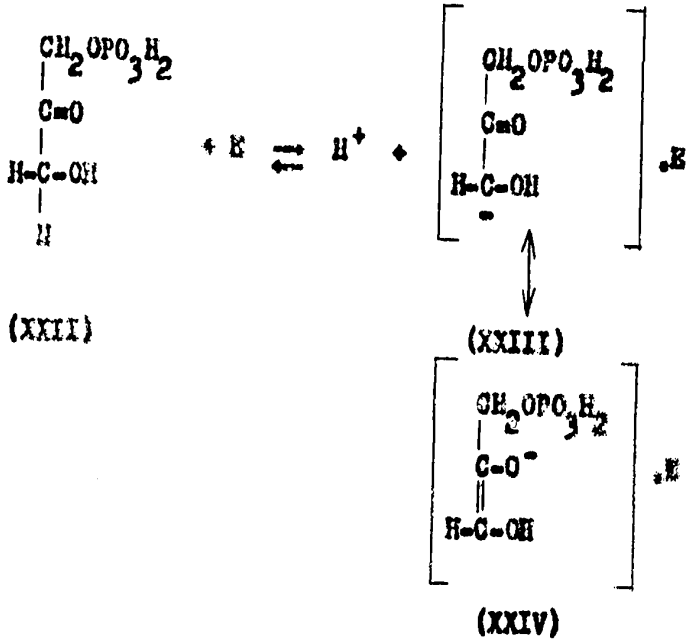


(XIX) (XX) (XXI)

It was shown that dihydroxyacetone phosphate became radioactive upon incubation with aldolase alone, in the presence of tritiated water. No isotope was incorporated in the presence of heat-inactivated enzyme. The labeling was found to be approximately equal to 1 μ mole of exchangeable hydrogen. Also, it was found that fructose-1,6-diphosphate was <u>non-isotopic</u> regardless of the presence or absence of dihydroxyacetone phosphate. This provides strong evidence for the fact that the enzyme can discriminate between the two hydrogens on the carbinol carbon of dihydroxyacetone phosphate.

Bloom and Topper[16] further substantiated this work of Rose and Rieder with respect to the stereospecificity of aldolase. Not only did they confirm that approximately one atom of carbon-bound hydrogen per molecule of dihydroxyacetone phosphase exchanges with the aqueous solvent on incubation with aldolase in the absence of acceptor aldehyde, but they could also propose a mechanism for the activation and sub-

sequent labilisation of one hydrogen atom on the primary carbinol function of dihydroxyacetone phosphate.

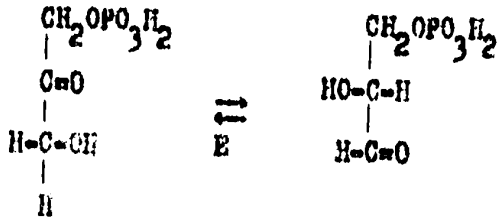


(XXII) (XXIII) (XXIV)

The activation would then consist in the formation of a resonance system consisting of carbanion and enol.

d) Dihydroxyacetonephosphate-Phosphotriose Isomerase System

Another metabolic reaction of dihydroxyacetone-phosphate consists in its transformation into D-glyceraldehyde-3-phosphate as catalysed by phosphotriose isomerase;



(XXII) (XXV)

Further experiments[16] demonstrated that approximately one atom of tritium is incorporated per molecule of dihydroxyacetone phosphate incubated with phosphotriose isomerase. This suggests that both enzymes are capable of distinguishing between the two hydrogens atoms on the primary carbinol. They further determined that the hydrogen which is labilised by aldolase is not the same as that which is labilised by phosphotriose isomerase. This was done by incubating dihydroxyacetone phosphate containing enzymatically introduced tritium through the action of aldolase. Whereas complete loss of the isotope to the solvent occurred when the substrate was incubated with aldolase in water, no such exchange was observed upon incubation with phosphotriose isomerase. These results show that not only is each enzyme stereospecific for one hydrogen on the primary carbinol, but each display a different stereospecificity. The foregoing then, are additional interesting illustrations of the validity of the Ogston concept which is a refinement of the polyaffinity theory of

Fruton and Bergmann[17]. Other enzyme catalyzed asymmetric reactions with symmetric substrates are also well known[18].

These precedents suggested to us that MAO might display a hitherto unrecognized type of stereospecificity. This was only a matter for speculation since as pointed out above, MAO displays no optical specificity. An unambiguous method of approach to this problem would involve the use of both the R and S forms of mono-α-deuterated tyramine; through the application of kinetic isotope effects in the oxidation of either isomer one might be able to detect rate differences that should be related to the stereochemistry of the substrates.

Deuterium isotope effects in biochemistry are well documented, and some of the more pertinent literature has been briefly reviewed in another thesis[9]. Some of the salient points may be recalled.

C. DEUTERIUM ISOTOPE EFFECTS IN BIOCHEMISTRY

Substitution of deuterium for hydrogen in an organic molecule causes a number of observable effects which can be useful in studying reaction mechanisms. Since the zero point energy of the C-D bond is greater than that of the C-H bond, the activation energy is lower for the rupture of the C-H bond. Thus, the observed rate of a reaction will be correspondingly less for the deuterium containing compound

if the cleavage of the C-H bond is rate determining[19]. A number of reviews on this subject have been presented[20,21,22].

Erlenmeyer, et al[23] and Thorn[24] have described a reduction in the rate of oxidation of partially deuterated succinate. Mahler and Douglas[25] subsequently reported the effect of deuterium on the binding of DPN to lactic dehydrogenase and on its subsequent dehydrogenation. Concurrent with preliminary reports of work presented in this thesis[26], Abeles and his group[27] reported a kinetic isotope effect in the oxidation of deuteriomethyl sarcosine by sarcosine oxidase. These investigators also reported an effect of deuteration on the Michaelis constants although no interpretation of the results was offered. There has been no published studies relating to the effect of deuteration on biologically active amines prior to our reports[26,28,29].

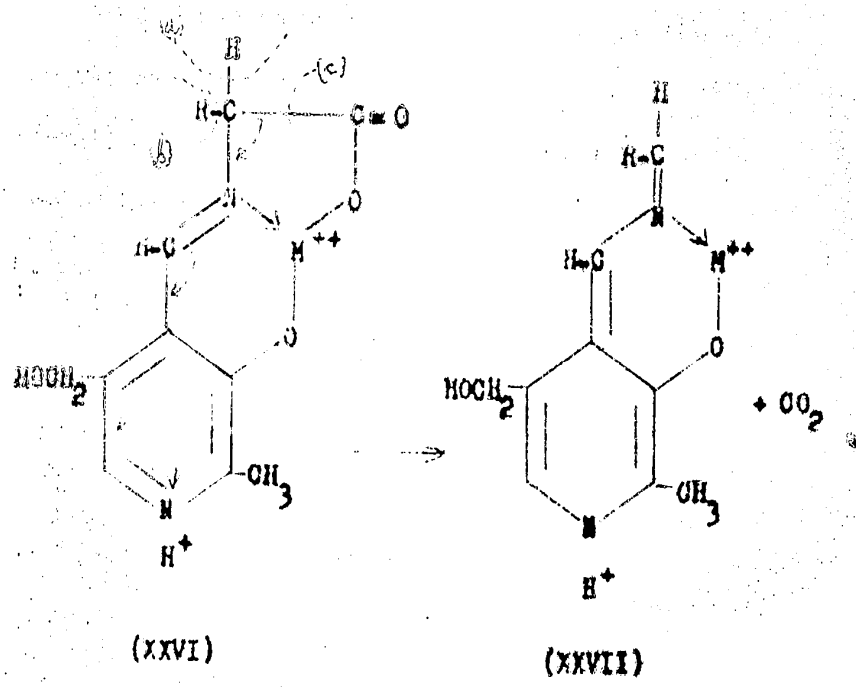
Deuterium isotope effects should constitute a powerful tool in this field since such effects are independent of the purity or composition of the enzymes and are theoretically applicable to <u>in vivo</u> studies.

The method used to prepare the two asymmetrically deuterated tyramine isomers was to decarboxylate tyrosine enzymatically in D_2O [30], a reaction known to be stereospecific; then DL-α-deuterated tyrosine was in turn decarboxylated in ordinary water to give the deuterated enantiomorph. Proof that the two α-deuterio enantiomers of tyramine had been

obtained was gained by measuring their respective rate of oxidation by MAO. This observation of a configuration dependent isotope effect raised the question of the absolute stereochemistry of the enzymic decarboxylation of amino acids. This biochemical reaction, which is known to be pyridoxal phosphate (PPal) dependent, as pointed out above, is of practical value in the preparation of optically pure α-deuterated amines.

a) Mechanism of Enzymic Decarboxylations

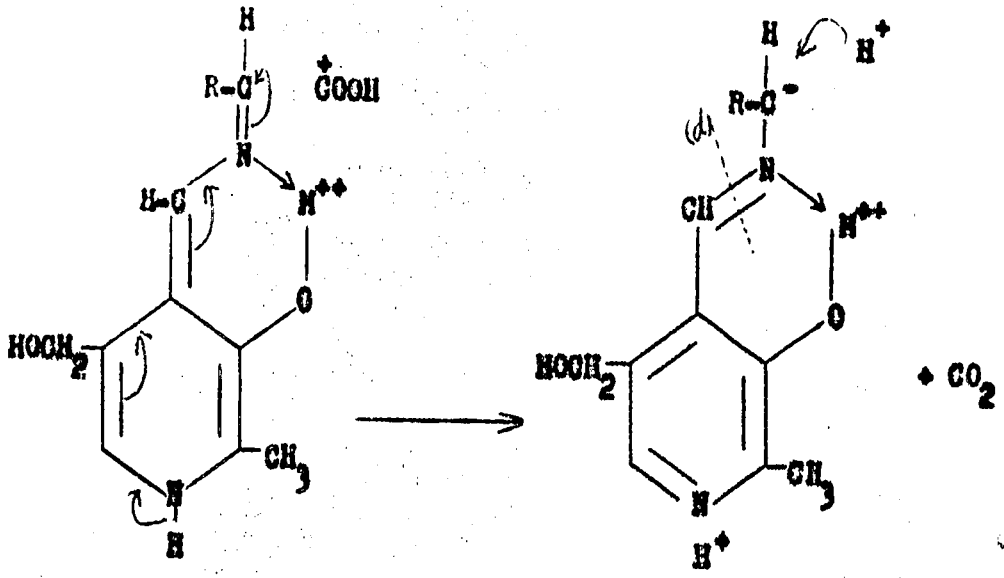
Metzler, Ikawa and Snell[31] while investigating non-enzymatic transaminations, racemizations, decarboxylations etc., which are pyridoxal catalysed, arrived at a mechanism that would explain all of the various catalytic properties of this cofactor. They proposed that a metal ion which greatly enhances the rate of reaction, functions in promoting the formation of the Schiff base and in maintaining planarity of the conjugated system through chelate ring formation. This chelated metal ion would also provide an additional electron-attracting group that operates in the same direction as the heterocyclic nitrogen atom, thus facilitating electron displacements from the α-carbon atom:



(XXVI) (XXVII)

An electromeric displacement of electrons from bonds a, b, or c as shown in the above scheme would result in the release of a cation (H^+, R^+, or $COOH^+$). The wide variety of reactions catalysed by pyridoxal would be accounted for on the basis of that mechanism.

With regard to the release of CO_2 or $COOH^+$, an intermediate (XXVIII) would be formed which on protonation at the α-carbon would lead to the formation of the corresponding amine (intermediate (XXIX)).



(XXVIII) (XXIX)

If as is the case, (XXVIII) is in an asymmetric environment then the protonation step might be expected to be stereospecific. Experimental evidence has been provided in support of this view. One difficulty with this mechanism is that the involvement of metals in the action of PPal dependent enzymes is controversial[32,33].

Recent evidence has shown that some non-enzymatic transaminations and decarboxylations which are pyridoxal-catalysed can occur in the complete absence of any metal ion.

It was observed[32] that a weak base alone such as imidazole can catalyse the non-enzymatic transamination of phenylglycine with pyridoxal. Moreover, Kalyankar and Snell[33], recently used amino acids in which the α-hydrogen was substituted for a methyl or phenyl group and showed that decarboxylation occurred readily upon heating with pyridoxal alone in dilute aqueous solution. Two closely related but independent reactions were suggested to occur as follows:

$$RR'CNH_2COOH \xrightarrow{\text{Pyridoxal}} RR'CHNH_2 + CO_2 \qquad (5)$$

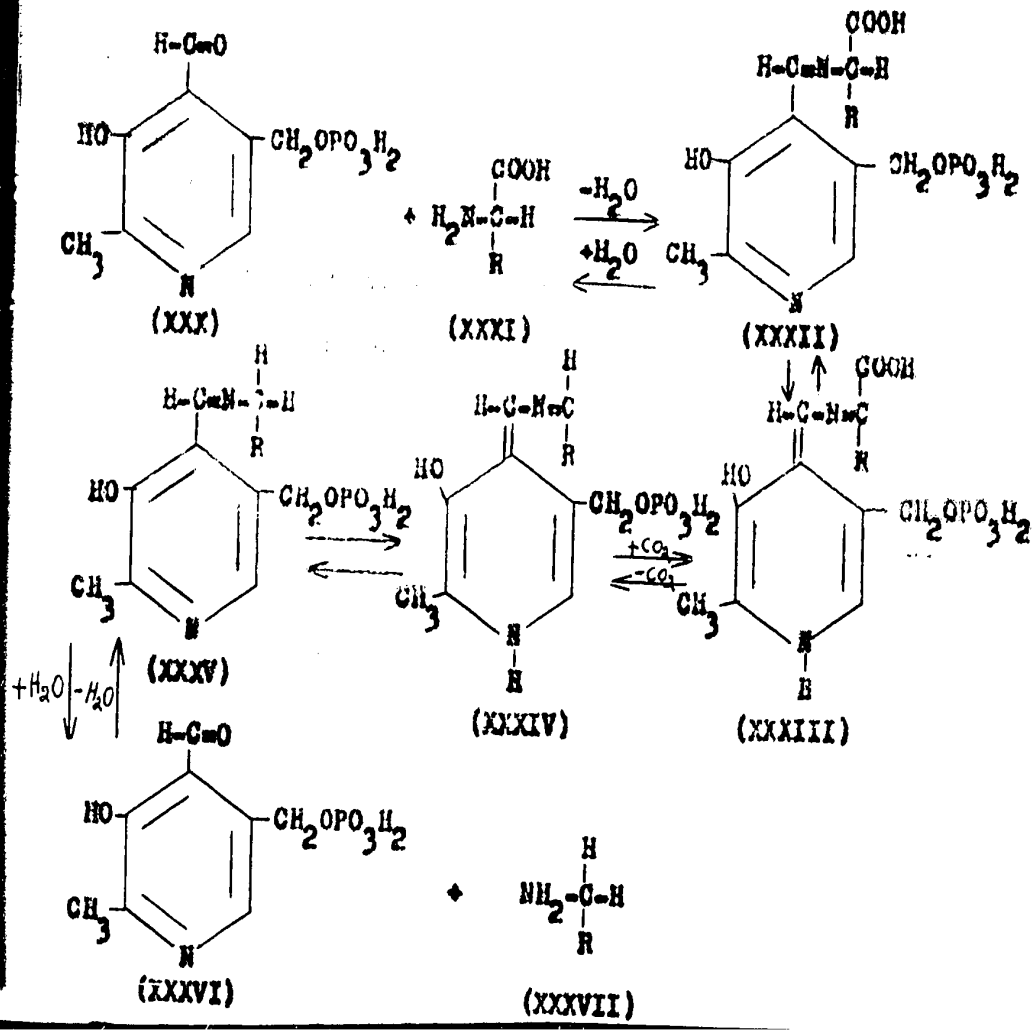
$$RR'CNH_2COOH + \text{pyridoxal} \longrightarrow RR'C=O + CO_2 + \underset{\text{pyridoxamine}}{} \qquad (6)$$

Reaction (5) is analogous to the decarboxylation of amino acids by PPal enzymes. Reaction (6) is a decarboxylation-dependent transamination reaction for which no enzymatic analogy is yet known. Reactions (5) and (6) were both partially inhibited by those metal ions which catalyse the previously studied transamination reactions between pyridoxal and amino acids. The suggested mechanisms appear quite plausible.

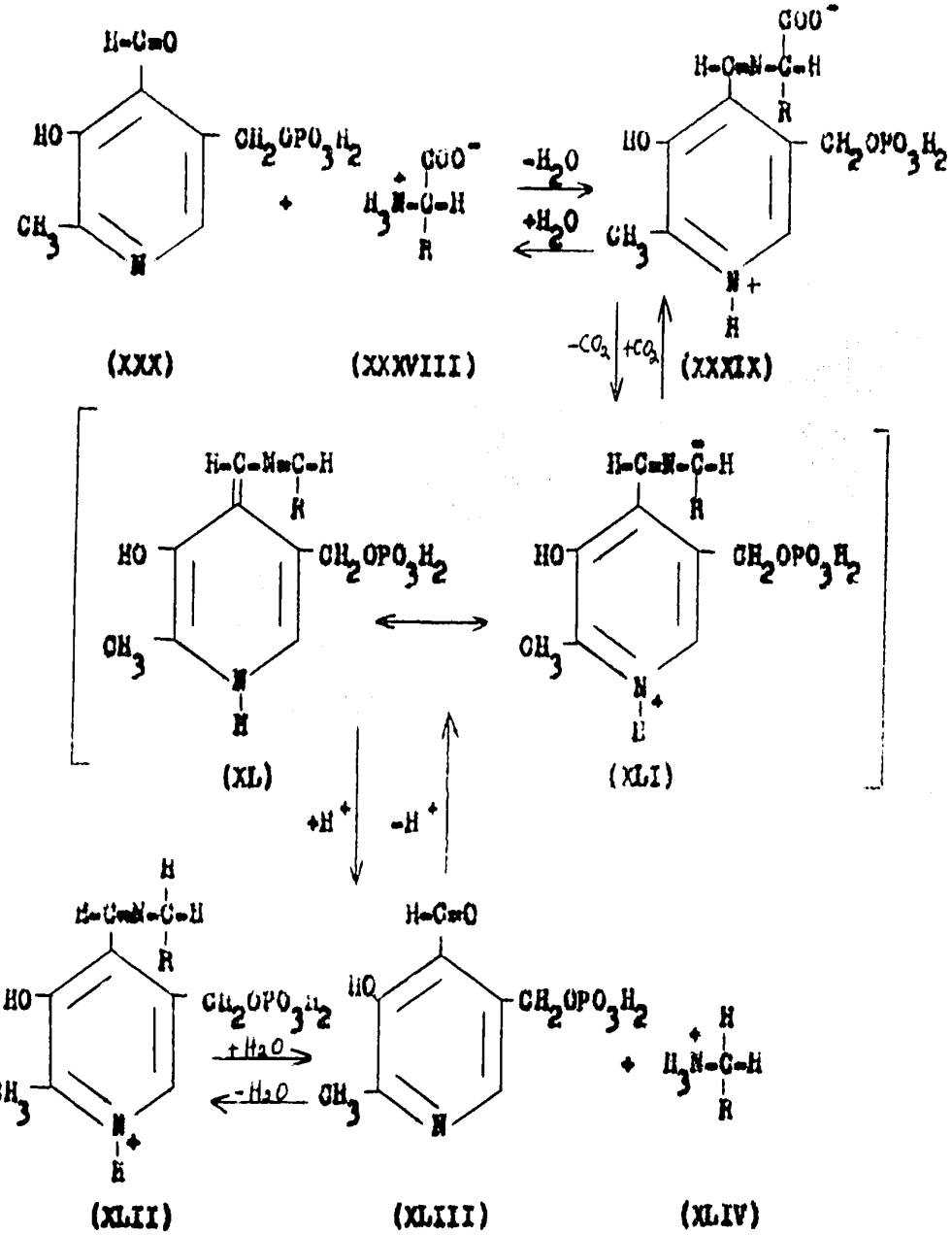
The stabilisation thought to be necessary and specific for the activation of the intermediary Schiff bases, (initially postulated to be achieved by metal chelation), may be ensured by direct selective formation of hydrogen bonds or other links between suitable groups on the enzyme

protein itself, and the nitrogen atom and phenolic hydroxyl of the coenzyme-substrate complex azomethines.

Unambiguous proof that the decarboxylation of amino acids proceeds stereospecifically, was provided by Mandeles, Koppelman and Hanke[30]. At that time two schemes were put forward for the mechanism of the decarboxylation reaction. The one which was proposed by Werle involved the removal of the hydrogen atom from the α-amino carbon before decarboxylation as shown below. This mechanism is now invalid because tyramine would exchange two deuteriums on the α-carbon atom, when deuterium oxide is used as the solvent.

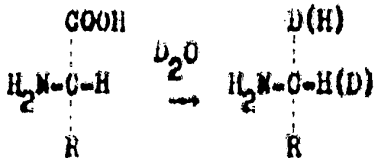


Westheimer's alternative proposal, requires the α-hydrogen of the amino acid to be non-exchangeable during the course of the reactions.



As is evident from the above schemes, the former requires the addition of two hydrogen atoms from the solvent to the α-carbon, while the latter requires that only one hydrogen should be incorporated. If enzymatic decarboxylation of an amino acid is carried out in deuterium oxide, the deuterium content of the resultant amine clearly allows a decision between the two mechanisms. This is exactly what Mandeles et al have shown experimentally, the amines formed by enzymatic decarboxylation of lysine, tyrosine, and glutamic acid in 99.8% D_2O contained no more than one atom of deuterium per molecule. There is no reason why one could not extend this argument to encompass all the PPal dependent amino acid decarboxylases, as they should proceed essentially by the same mechanism.

Although, there is little doubt now that decarboxylation of amino acids proceeds stereospecifically, it was not known whether the overall reaction proceeds with retention or inversion of configuration ((XLV) → (XLVI))

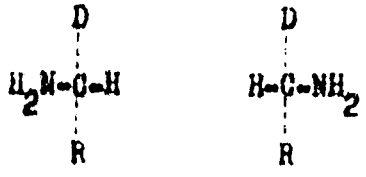


(XLV) (XLVI)

It might be expected, however, that the transition state for

the release of carbon dioxide should resemble that for protonation of the α-carbon, since, in all probability the same active site accommodates the R group in both transition states. Accordingly, over-all retention of configuration may be expected in the enzymic decarboxylation of amino acids (XLV) → (XLVI). Retention of configuration is also a characteristic of electrophilic displacement reactions. Experiments were performed in this laboratory to elucidate this point.

The first problem was to prepare chemically the two enantiomers ((XLVII) and (XLVIII)) of α-deutero tyramine using asymmetric intermediates of known absolute configuration, and reactions whose mechanisms are known. In this way, the R and S-α-d-tyramine ((XLVII) and (XLVIII) respectively) of established configurations would be produced.



(XLVII) (XLVIII)

The relative rates of their oxidation by monoamine oxidase would then allow assignments of absolute configurations to the enzymically prepared α-d-tyramine. The synthesis was accomplished by what appears to be well-established unambi-

guous procedures, (see discussion).

One of the first to demonstrate the principle of asymmetric syntheses was McKenzie[34], who while experimenting on the reduction of esters of pyruvic acid had discovered that when the acid is esterified with optically active l-menthol, the reduction of the α-keto group led to optically active (levo) lactic acid. Since then a vast number of stereospecific syntheses have been accomplished, using a variety of techniques. Streitwieser[35,36,37,38,39] has published a series of classical papers on the stereochemistry of the primary carbon, and has described the preparation of deuterated compounds of high optical purity starting from optically pure intermediates.

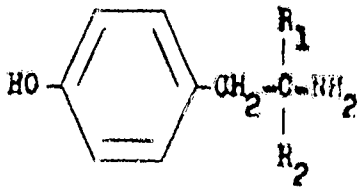
b) Isotope Effects and Monoamine Oxidase

A configuration dependent deuterium isotope effect in the enzymatic oxidation of asymmetrically labeled tyramine by rat liver monoamine oxidase has been reported previously[9]. It was revealed that rat liver monoamine oxidase is an enzyme exhibiting a degree of stereospecificity paralleling that of alcohol dehydrogenase. These results could not be generalised to include brain monoamine oxidase, due to the fact that there is a marked difference in the activity of some natural and synthetic compounds in the brain as compared to the somatic system. (e.g. d-amphetamine is more

active in the brain than l-amphetamine, and l-amphetamine is more active in the periphery). This problem was of fundamental interest since nothing was known as yet as to whether the brain enzyme acts by a mechanism similar to that of the liver enzyme.

The basic principles of the above experiments were followed through to include in vivo work. The physiological role of monoamine oxidase in adrenergic mechanisms, i.e. at the effector cell level, has been quantitatively and unambiguously assessed. It was reasoned that, in order to evaluate monoamine oxidase activity in effector cells and other tissues, it would be essential to compare two adrenergic amines stereochemically indistinguishable by monoamine oxidase but differing in susceptibility to degradation by it. It may be questioned whether the α-deuterated and the non-deuterated adrenergic amines are actually identical stereochemically. If $(-CD_2-)$ has a larger van der Waals radius than $(-CH_2-)$, then it becomes possible that the binding constants and the rates of reaction may be altered independently of the zero-point energy difference between a C-H and a C-D bond. However, Mislow et al[40] using the optical rotatory dispersion technique have evaluated the relative bulks of $(-CD_3)$ as compared to $(-CH_3)$ in the Meerwein-Pondorf reduction. It was observed that $(-CD_3)$ and $(-CH_3)$ have the same volume kinetically. Such requirements are not met by the usual series of analogs,

isologs, or isosteres because of distinct stereochemical differences between any two compounds. However, the substitution of deuterium for hydrogen atoms in a molecule does fulfill the above requirements, and differences between the rate of degradation of a substrate and its deuterium labeled counterpart can be ascribed to zero point energy differences as long as the rate-limiting step of the degradation involves breaking of a carbon-hydrogen bond. For example, should the adrenergic amine tyramine (which is a good substrate for monoamine oxidase) be labeled with deuterium on the α-carbon ((XLIX), R_1=R_2=D), the rate of its oxidation by the enzyme would be expected to be decreased as long as the rate-determining step involves breaking of a carbon-deuterium bond. Now, if monoamine oxidase is involved as a limiting



(XLIX)

factor in the response of let us say a nictitating membrane, the substitution of deuterium for hydrogen in tyramine should lead to quantitative differences in the pattern of membrane contraction. Since the labeled tyramine is stereochemically identical to normal tyramine (see above), any difference in

in response can be ascribed to variations in binding constants and rate of degradation of the substrate under perfectly controlled physiological conditions.

It was also of interest to examine the possibility that any in vivo isotope effects might be stereospecific. This was suggested by our previous in vitro results with monoamine oxidase and the two stereoisomers of α-d-tyramine obtained enzymatically.

Recently it has been postulated that tyramine triggers the release of catecholamines[41], which then produces the observed responses. It was found that tyramine, phenylethylamine, ephedrine, and d-amphetamine will not produce a pressor action when catecholamine stores are depleted. This would delegate a secondary role to tyramine. However, if this were so, the mediated responses to tyramine should still be sensitive to isotope effects as long as MAO limits the sojourn of tyramine at the level of the storage sites for catecholamines.

Similar reasoning as above also applied to noradrenaline which in a similarly labeled form (α-d_2-noradrenaline) might lead to valuable information about the nature of the receptors. The administration of iproniazid, a powerful MAO inhibitor, could be shown[42,43] to potentiate the action of various adrenergic amines (such as tyramine) on the nictitating membrane of the cat. However, no potentiation of the action

of the normal transmitter noradrenaline could be demonstrated[42,43]. This would seem to indicate that termination of the action of noradrenaline and adrenaline at the periphery does not involve breakdown through attack by monoamine oxidase. It has been shown by Axelrod and his collaborators that O-methylation is in all probability an important mechanism of inactivation of catecholamines[44], although according to Brodie and his group[45], monoamine oxidase would be involved in the disposition of catecholamines and serotonin in the brain. However, the demonstration by Carlson[46] of the presence of appreciable concentrations of dopamine in the brain, provides another possibility that some of the effects of iproniazid in the CNS could be related in some way to increased availability of dopamine which can act as an excellent substrate for MAO.

D. CATECHOL O-METHYL TRANSFERASE

a) Historical

Catecholamine hormones like adrenaline and noradrenaline play a dominant role in adrenergic mechanisms and their actions on various tissues and the volume of literature on this subject is phenomenal. The question now arises; which mechanism or what enzyme or enzymes is (are) responsible for their inactivation. Cytochrome oxidase, was believed for a time to be the inactivating enzyme for catechol-

amines, leading to the formation of adrenochrome. However other workers could not substantiate these findings. Axelrod[47] has observed that cytochrome oxidase metabolizes catecholamines *in vitro* more rapidly than any known enzyme, but *in vivo*, catecholamines were attacked only to a negligible extent. There are many factors to be considered with respect to *in vivo* metabolism, as opposed to *in vitro* systems such as, localisation and degree of binding of substrates, affinity of substrate for the enzyme, naturally occurring inhibitors, and passage of the compound across cellular membranes and access to the intracellular site of metabolism. An enzyme, adrenaline dehydrogenase present in rabbit plasma as has been described by Inaisumi et al[48], reversibly oxidises adrenaline to adrenalone. It was postulated that this conversion would constitute the first inactivation step in the metabolism of adrenaline and noradrenaline. However, this could not be substantiated by other workers[49] who not only could not detect this enzyme (using βH^3 adrenaline) in rabbit plasma, but also could find no trace of it in human plasma and various tissues of the rat.

Conjugation, mostly in the form of sulfo- and glucosiduronic acid conjugates, was thought to be the major pathway of disposition. However, later experiments showed that physiological doses of catecholamines administered

intravenously led to the excretion of only small amounts of conjugated catecholamines[47].

An attractive hypothesis that was advanced not too long ago consists in that MAO would be responsible for the inactivation of catecholamines in the intact organism using as a parallel the system acetylcholinesterase-acetylcholine which operates at the parasympathetic level. However, with the advent of powerful MAO inhibitors like iproniazid, it was soon discovered that blockade of MAO did not alter the concentration of catecholamines in many tissues, nor did it alter or potentiate the action of catecholamines at the adrenergic effector cell level; e.g. the nictitating membrane of the cat[47]. There is still some discussion with regard to the participation of MAO in the metabolism of noradrenaline in the central nervous system[47].

b) <u>O-Methylation as a Route of Inactivation</u>

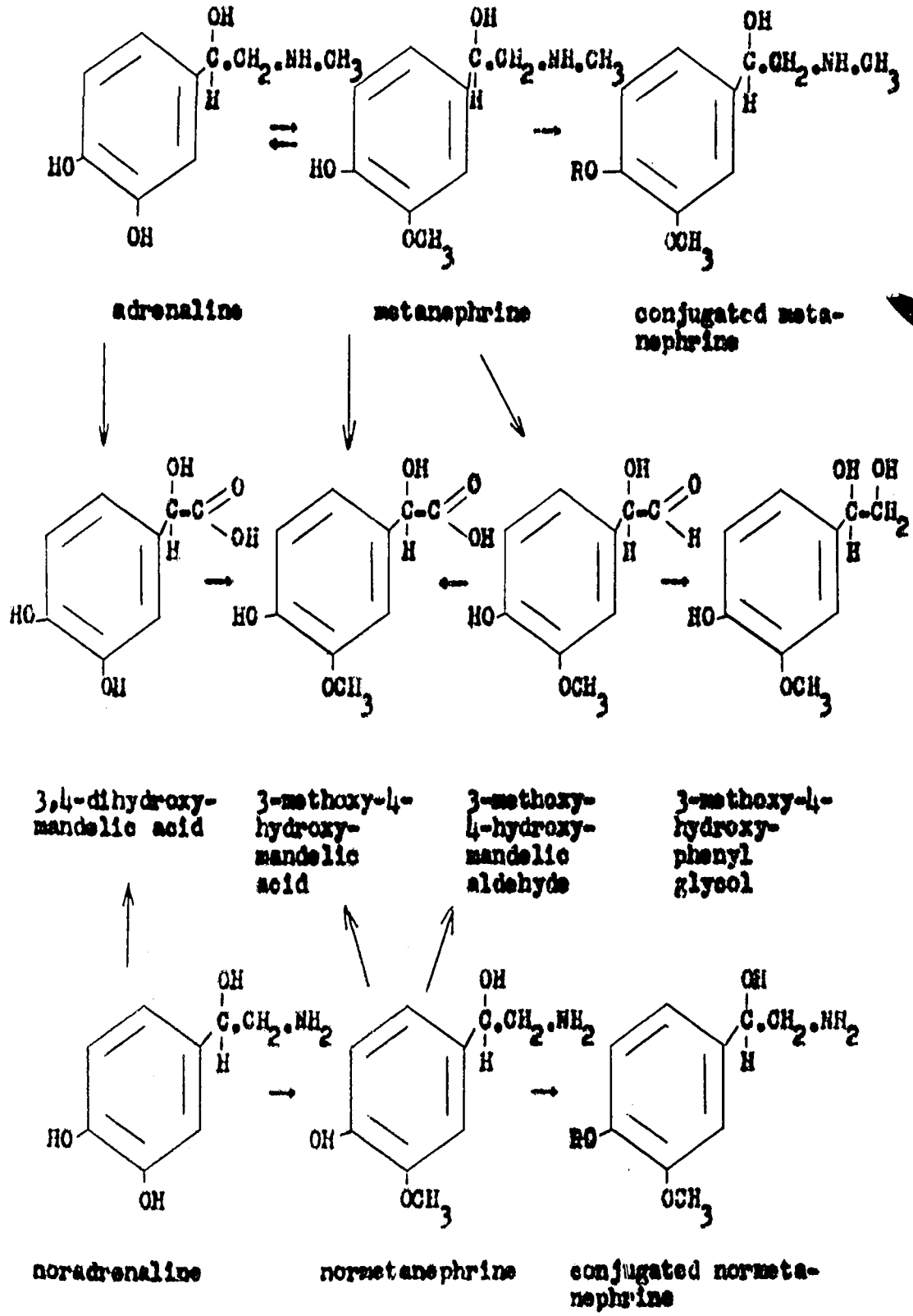
Observations such as the following: phenolic compounds are O-methylated in the body[50], injected catechol flavanoids are excreted as 3-methoxy-4-hydroxyphenylacetic acid[50], and the discovery by D'Iorio and Pellerin[51] (simultaneously with Axelrod) that catechol derivatives were methylated in the meta position by rat liver and kidney preparations, led to the discovery of a new metabolic pathway for catecholamines. New metabolites of adrenaline and noradrenaline,

carrying an O-methyl group in the meta position, (such as 3-methoxy-4-hydroxy-mandelic acid[50], metanephrine, normetanephrine[50], and 3-methoxy-4-hydroxyphenylglycol[50]), were identified. The use of radioactive [βH^3] adrenaline and [βH^3] noradrenaline gave a metabolic picture of the origin and fate of these compounds in man and other species[50]. It was found that only small amounts of free catechols are excreted in the urine[50]. More than 80 per cent of administered adrenaline is excreted as the O-methylated metabolites metanephrine (free and conjugated), 3-methoxy-4-hydroxy-mandelic acid and 3-methoxy-4-hydroxyphenylglycol sulphate[52]. The presence of large amounts of 3-methoxy-4-hydroxymandelic acid[47] and normetanephrine[47] in the urine of patients with phaeochromocytomas suggests that O-methylation is an important route of metabolism of catecholamines in man. The excretion of a large percentage of the O-methylated metabolites in the deaminated form, raised the question as to whether oxidative deamination either preceded or followed O-methylation. Studies of the excretion pattern after administration of metanephrine and adrenaline showed that both led to the same proportion of O-methylated metabolites. The degree of O-methylation as compared to deamination, was determined unambiguously by Kopin[50] who administered simultaneously, [βH^3] adrenaline and metanephrine in which the methoxy group was labeled with O^{14}, and assayed the ratios of the isotopes found in each of

the excreted metabolites. The ratios of H^3/C^{14} and the total radioactivity of each metabolite established the relative importance of the two pathways. Calculations based on the observed ratios showed that approximately 68 per cent of the circulating adrenaline is O-methylated to metanephrine, and 23 per cent directly deaminated, followed by O-methylation. The remainder is excreted as unchanged and conjugated catecholamines. Studies with noradrenaline, show it to be metabolised in essentialy the same way. From these and other results, Axelrod[50] has proposed the following metabolic pathways for adrenaline and noradrenaline, (see page 34).

The enzyme (COMT) responsible for the O-methylation of catecholamines has been described by Axelrod and Tomchick[6] and its properties will be discussed later. It catalyses the transfer of the active methyl group of S-adenosylmethionine to the m-hydroxyl group of catecholamines in the presence of a divalent cation such as Mg^{++}.

The foregoing data cannot be generalised to include the metabolism of catecholamines in all tissues, but is physiologically applicable to amines which are normally released into the bloodstream or from the adrenal gland. Most of the noradrenaline is released directly into individual tissues from the sympathetic nerve endings, and may undergo metabolic changes before it even reaches the circulation. Carlsson and Hillarp[53], gave intravenous injections of 3,4-dihydroxy-


adrenaline
metanephrine
conjugated meta-nephrine
3,4-dihydroxy-mandelic acid
3-methoxy-4-hydroxy-mandelic acid
3-methoxy-4-hydroxy-mandelic aldehyde
3-methoxy-4-hydroxy-phenyl glycol
noradrenaline
normetanephrine
conjugated normeta-nephrine

phenylalanine (DOPA) to rabbits and have obtained data which suggests that the 3,4-dihydroxyphenethylamine (dopamine) formed in the brain from the administered DOPA first reaches sites of MAO. The 3,4-dihydroxyphenylacetic acid (DOPAC) thus formed then reaches sites of COMT. Crout et al[54] determined the COMT and MAO activities in the brain, heart and liver of the rat. Then they administered inhibitors of COMT and MAO separately and together in order to determine which enzyme activity predominates in relation to the accumulation of endogenously formed catecholamines in the brain and heart. Finally, the in vivo accumulation of injected noradrenaline in the rat heart was studied in control animals and in animals treated with the two types of enzyme inhibitors in order to determine the relative ability of each enzyme to degrade exogenous noradrenaline in the myocardium. In vitro MAO activity was found to be respectively 3,6 and 5 times greater than COMT activity in the brain and the heart. However, in rat liver, COMT activity was 15 times greater than MAO activity. Also, the affinity of noradrenaline for either enzyme was roughly the same as indicated by K_m values. The results of these experiments suggest that MAO plays a greater role in the initial metabolism of noradrenaline in the brain and heart of the rat while COMT is of greater significance in the liver. These findings may not apply to other species, and the results of other workers[55] indicate indeed that these

metabolic pathways may vary from organ to organ and from species to species.

c) Other Routes of Metabolism

Kirshner and Schnepdryver[56] carried out metabolic studies on cats in which dl-adrenaline-2-C^{14} was infused. Their results indicate that blockade of either MAO or COMT is largely compensated for by the activity of the enzyme left intact. Combined inhibition of both enzymes resulted in the formation of a new adrenaline metabolite which was not identified, and also in the increased production of acidic and mainly conjugated metabolites, the identity of which was also unestablished. Recently it was shown that rats treated with iproniazid, produce a new urinary metabolite of noradrenaline[57], which was identified as N-acetyl-normetanephrine. This seems to be an alternative pathway when MAO activity is blocked. To further substantiate this observation, Weissbach et al[58] described a cell free system which can acetylate a wide variety of biogenic amines. They stress that acetylation takes place primarily when MAO activity is suppressed.

There has also been some convincing evidence that a fair percentage of catecholamines are taken up by tissue constituents[59,60], and bound in a way which makes them inaccessible for enzymatic destruction. Hence, part of the

exogenous and endogeneous circulating catecholamines may be temporarily inactivated by this mechanism.

On the basis of experiments with H^3-adrenaline, as well as other work, Axelrod[47,50] proposed a chronological sequence of events in the elimination of circulating adrenaline. The first phase (lasting 0-5 minutes), involves rapid dilution by redistribution into tissues, then O-methylation by COMT and specific binding into certain tissues; the second phase (5-180 minutes or longer), consists in slow release of adrenaline from the binding sites, and then attack by COMT.

d) Activity of Metanephrine and Normetanephrine

The enzyme catechol O-methyl transferase has been unambiguously implicated in the O-methylation reaction of catecholamines; but does it actually terminate the action of these hormones? It has been demonstrated by several investigators[61,62,63,64,65,66] that in vivo and in vitro inhibitors of COMT like pyrogallol and quercetin prolong the duration of certain pharmacological responses to adrenaline, and inhibit COMT reversibly. The inhibition of MAO by iproniazid has no effect on these responses[42] as pointed out above.

D'Iorio[67] assayed the biological activity of normetanephrine, metanephrine and 3-methoxy-dopamine using rabbit aorta spirals, rabbit duodenum preparations and the

blood pressure response of atropinized cats. In comparing the activities of these compounds to their non-methylated analogues he found that all of the 3-methoxy derivatives are capable of contracting aortic spirals to some degree. Here metanephrine was found to be as potent as adrenaline while normetanephrine and 3-methoxy dopamine showed much less activity. These methylated analogues were far less active on the rabbit duodenum, where metanephrine and normetanephrine were respectively 1/40 and 1/200 as potent as adrenaline. Both metanephrine and 3-methoxy-dopamine showed a significant activity on the blood pressure response. In comparing doses necessary for an equal response, these substances were found to be one-fourth to one-fifth as active as the non-methylated analogues. With regard to normetanephrine, it was only 1/600 as active as noradrenaline. D'Iorio suggested that COMT is not the only enzyme involved in the biological inactivation of catecholamines and also suggested that the enzyme is not equally effective in the inactivation of all catecholamines. In other words, COMT would display some degree of substrate specificity.

Bacq[68] has investigated the activity of normetanephrine and metanephrine at the adrenergic effector cell level; i.e. the nictitating membrane of the cat and has also reported that these metabolites are not totally inactive. They were 40 to 100 times less active than their non-methylated

analogues. It remains that a large part of the sympathomimetic activity is destroyed by O-methylation. Other workers[69,70] obtained similar results with other isolated organs. The methylated and deaminated metabolites such as 3-methoxy-4-hydroxy mandelic acid was totally devoid of activity. The O-methylated catecholamines are not inhibitors of COMT. It is interesting to note that metanephrine has the ability to sensitise peripheral nerve endings to adrenaline. Bacq believes that demethylation of the methylated metabolites by a demethylase first described by Axelrod et al[71] may be of some significance. This would then regenerate the original catecholamine hormones and could account for the peculiar effects of metanephrine and normetanephrine noted above.

Axelrod et al[72] have shown that both meta and para O-methylation of noradrenaline and other catechols occurs <u>in vitro</u>, but could not detect as yet any <u>in vivo</u> para O-methylation of noradrenaline and adrenaline. COMT normally methylates catechols in the position meta to the side chain. A possible dysfunction of COMT in which dopamine is methylated in the para-position has been linked to a disturbance characterised by hypokinetic rigidity in the human and a Parkinsonian-like syndrome in some animals[73]. This does suggest the possibility of the existence of two COMT's: one methylating in the meta and the other in the para-position.

Substrates with meta directing groups in the side chain such as 3,4-dihydroxyacetophenone, arterenone and adrenalone, underwent enzymatic para O-methylation not only *in vitro* (35-45%) but also *in vivo* to the extent of 10-25%. Even apparent methyl migration from para to meta and vice versa has been observed *in vivo*[74].

e) Some Properties of the Purified Enzyme[6]

COMT is a soluble enzyme found in the supernatant fraction of tissue homogenates after high speed centrifugation. Negligible O-methylation occurs when S-adenosylmethionine or Mg^{++} are omitted from the incubation mixture. The rate of O-methylation is not affected by anaerobic conditions. There is no observable stimulation of enzyme activity by glutathione. Experiments in which the pH is varied in the presence of 0.1 M phosphate buffers showed optimal activity for the enzyme between pH 7.5 and 8.2. The rate of methylation shows no further O-methylation after 2 hours of incubation at 37°C.

With regard to substrate specificity, all catechols examined, regardless of the nature of substituents on the aromatic nucleus were O-methylated. Monophenols are not substrates for COMT and no stereospecificity was displayed by the enzyme towards d- or l-adrenaline.

Divalent cations such as Co^{++}, Mn^{++}, Zn^{++}, Cd^{++} Fe^{++} and Ni^{++} could all substitute for Mg^{++} producing varying

degrees of activation.

f) Methods of Assay for COMT Activity

The most often used quantitative method of assay for COMT activity consists in extracting the methylated metabolites followed by their fluorimetric determination at 335 mμ after activation at 285 mμ (an Aminco-Bowman spectrofluorometer is usually recommended). Results were not reproducible in our hands.

There are many pitfalls in the use of the fluorimetric technique for quantitative assay. Braunsberg and James[75] have made a detailed examination of the Aminco-Bowman instrument especially with reference to its variation in sensitivity with wavelength, spectral resolution and stability. Wavelength calibrations showed errors of up to 10 mμ in the range 250-550 mμ with the primary wavelength fixed at a desired value. Attempts to adjust the monochromators were not successful. The calibration experiments which included variation of maximum scatter readings with wavelength, revealed considerable variation of the complete optical system of the instrument with wavelength. This also shows that the over-all sensitivity of the instrument varies considerably with wavelength. For the three most sensitive settings of the recording equipment, readings between 10 and 40 divisions gave standard deviations of 2% of the mean

readings. Braunsberg and James furthermore state that publication of activation and fluorescence spectra without data such as above, limit their credibility.

Strauss and Worm[76], in a critical evaluation of the quantitative methods used for the determination of catecholamines in biological fluids, state from their own experience that there is difficulty in obtaining reliable results using spectrofluorometry as a technique. Among the problems inherent in the basic application of fluorescence for quantitative measurements, they list such factors as reflectance, refraction, absorbance by the cuvets of both excitation and emission energies, the influence of the sample solution with respect to self-absorption, inherent fluorescence of solutes in the sample, possible nephelometric influences, the isolation of the most effective excitation wavelength, and the isolation of the emission wavelengths best suited for quantitation. Udenfriend[77] also reports on similar problems. Due to the high sensitivity of the presently available instruments, extreme care must be taken in the selection and matching of cuvets, precise orientation of cuvets during fluorescence measurements, and prior determination that individual cuvet wells yield consistent galvanometer responses. Galvanometer variations are often associated with reflectance effects of the cuvet, and hence it is desirable to use siliconized, oriented and prematched cuvets.

There are two other points to consider in the development of fluorescence: first, the error due to enhancement, and the other to retardation of fluorescent light. The enhancement stems from the inherent fluorescence capacities of proteins, co-factors, nonspecific chromogens in biological fluids, metabolites closely related to catecholamines and finally the fluorescence contributed by chemical reagents added to the reaction mixtures. On the other hand, these effects are balanced, in part by quenching, which is associated with self-absorption, thermal decay, photochemical changes, solvent density, depth of solution, absorptivity of most materials in solution and the scattering effect of substances in suspension.

Hence, due to the above difficulties it is almost impossible to perform quantitative analyses based upon standards developed in pure solution. It becomes necessary therefore to compensate for the deviations caused by the extraneous biologic inclusions; this can be done by incorporating standards into an aliquot of the sample to be analyzed. Even with this procedure, it is only assumed that the difference between the sample and internal standard readings represents the emission of the standard alone. However, it has been noted[76] that limits of linearity are achieved through a relatively narrow, 5-fold range of concentration. With a 10-fold range, there was a decided breakdown of ideal

linear emission intensity. Experience in our laboratory has shown that with a 10-fold dilution of the samples and readings taken with a 10-fold increase in sensitivity it was not possible to reproduce the original curve. There are many variations of the fluorimetric technique, however, the same basic problems always apply, but this technique has been perfected by numerous investigators and has been applied successfully in the quantitative estimation of many compounds.

The time consuming precautions and the unreliability of the spectrofluorimetric method necessitated the development of a more rapid and reproducible technique. A modification of the colorimetric method of Udenfriend and Cooper[78], wherein tyramine is assayed by reacting it with nitrosonaphthol to form a colored complex which is measured at 450 mμ, was applied successfully to our problem of the quantitative measurement of normetanephrine. This technique was highly reproducible and gave unambiguous results even though the readings were at the low end of the galvanometer scale.

g) Other Quantitative Analytical Methods

There are other methods which could be adapted for the quantitative estimation of metanephrine and normetanephrine and which hold promise of being equally sensitive, rapid and reproducible. Barac[79] reacts quinonechlorimide with metanephrine in alkaline solution, thus producing a blue colored complex which can be measured at 590 mμ with a colorimeter. This method would detect metanephrine in the

10^{-5} M range of concentration.

Another technique which does not present the problem inherent to spectrofluorometry, involves the use of the ultraviolet spectrophotometer. Axelrod et al[74] have used ultraviolet spectrophotometry for the assay of acetovanillone and acetoisovanillone formed enzymatically from 3,4-dihydroxyacetophenone. This method could be adapted to the quantitative estimation of metanephrine and normetanephrine. The spectrophotometric method described by Mahler and Humoller[80] wherein a colored complex is developed with 3-methoxy-4-hydroxy mandelic acid and diazotized p-nitroaniline could also be modified for metanephrine and normetanephrine produced in an incubation mixture.

D'Iorio[81] describes a short method for assaying quantitatively vanillic acid produced from the incubation of protocatechuic acid with COMT. The metabolite is extracted with ethyl acetate and the absorption measured in the ultraviolet spectrophotometer at 279 mμ. Recovery always exceeded 95%. Another recent development was the application of gas chromatography to the separation of many biologically important amines in concentrations as low as 0.05 μg[82].

Other techniques that have been used, such as paper chromatography, absorption on ion exchange resins etc. are too much time consuming to be of value in the routine assay of enzyme activities.

h) Inhibitors

COMT is a relatively new enzyme and an understanding of its mode of action would be facilitated if specific inhibitors could be found. More specifically, inhibitors active at physiological concentrations *in vivo* would be highly desirable. Many years ago, Bacq demonstrated that pyrogallol and other catechols markedly increased the duration of response to adrenaline on sympathetic nerve stimulation *in vivo*[61]. However, he erred in the interpretation of this early phenomena by attributing the potentiation to the antioxidant properties of the polyphenols. Axelrod and Laroche[83] were the first to report on the inhibitory properties of pyrogallol towards COMT. The formation of metanephrine and normetanephrine *in vitro* was competitively inhibited approximately 50% at 1×10^{-5} M concentration of pyrogallol. Experiments with H^3-adrenaline in mice which had received pyrogallol revealed that the metabolism of adrenaline and the formation of metanephrine were blocked. Iproniazid had no such effect. Thus began an intensive search for better inhibitors, the ultimate being to discover a non-competitive antagonist of COMT that would be active at physiological concentrations.

Bacq[84] also reported that catechol at equimolar concentrations inhibits competitively the inactivation of adrenaline by COMT *in vitro* by about 50%. Since COMT methylates all known catechol derivatives, it is clear that they

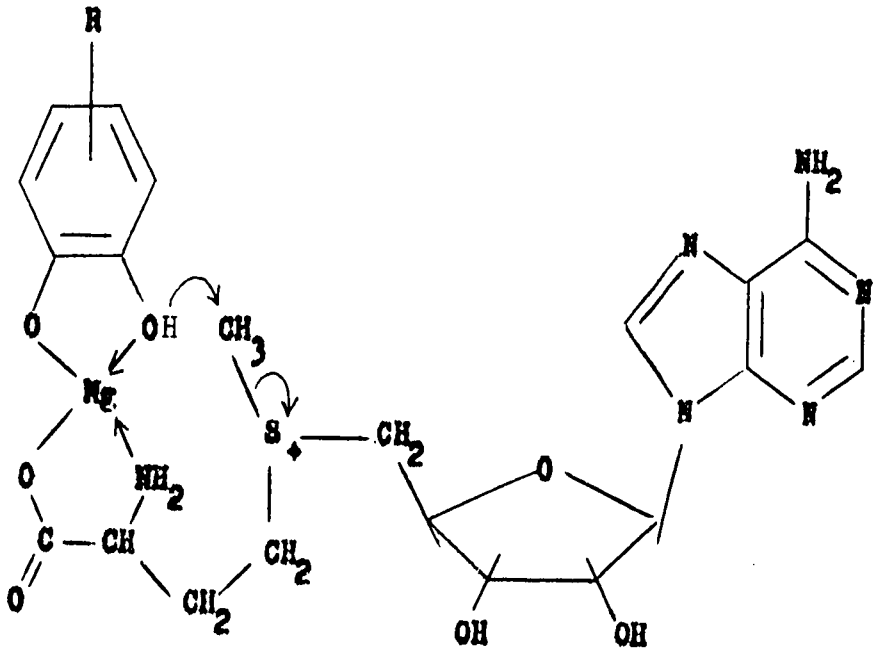
do not act as inhibitors but simply as competitors of adrenaline and noradrenaline. Their action must be regarded as resulting from a scavenging of methyl groups. In fact, catechol itself is a better substrate for COMT than the catecholamine hormones. Hence, they cannot be considered as competitive inhibitors of COMT. Many plant phenols with a catechol nucleus in their structure sensitize smooth muscle to adrenaline. This strongly indicates competition for COMT at that level.

In vivo studies with several substances known to interfere with noradrenaline metabolism[85] and which act as substrates for either the N- or O-methylating enzymes were made at about the same time. The compounds tested were pyrogallol, glycocyamine, 3,4,5-trihydroxyphenethylamine, adnamine, (5-methylaminomethyl-2,3,7,8-tetrahydroxydibenzo-[a,e,]-cycloheptatriene), catechol and nicotinamide. It was found that glycocyamine, pyrogallol and catechol were the most effective competitors leading to a doubling of the half-life of administered noradrenaline. Although the formation of normetanephrine could be blocked almost completely, the effect on the half-life of noradrenaline was not commensurate with the degree of inhibition. Alternate routes of metabolism may be assumed to operate in the absence of COMT. The fact that pyrogallol is O-methylated efficiently has been shown unambiguously[7]. It is reasonable to assume that other

polyphenols suffer the same fate both *in vivo* and *in vitro*. A good pharmacological evaluation of pyrogallol, catechol, adrenalone, arterenone, gallic acid and cocaine as O-methyl transferase inhibitors *in vivo* and *in vitro* has been reported by Wylie et al[62]. He observed augmentation of the pharmacological effects of catecholamines on the blood pressure, the nictitating membrane and general toxicity with all the inhibitors studied, but with the exception of cocaine. Crout[86] reported a non-competitive component in the activity of pyrogallol as a COMT inhibitor, although he maintained that it acts largely as a competitive substrate.

From the foregoing investigations it can be concluded that pyrogallol is a good competitive inhibitor of COMT, but is also a good substrate for the enzyme as are all of the other polyphenol type of inhibitors. It is extremely labile biochemically, and toxic at the doses used. It is therefore an unsatisfactory inhibitor for quantitative studies *in vivo* and *in vitro*.

COMT is a metal-ion dependent enzyme and on that basis a chelation mechanism has been proposed which accounts nicely for complex formation between catecholamines and the enzyme[74]. The mechanism of this enzymic methylation has been pictured as a displacement reaction on S-adenosylmethionine by the more nucleophilic hydroxyl group of a given catechol.



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This mechanism suggests that an ideal inhibitor would be a powerful but highly specific chelating agent that would not abstract the metal from the enzyme; it should be isosteric with the catechol nucleus and resistant to oxidation. Such a substance would form a complex with the enzyme and would most probably lead to non-competitive type inhibition: i.e. it would not be displaced by an excess of substrate or metal ion, and would truly fit the theory of enzyme kinetics. The tropolone ring (2-hydroxycyclohepta-

trienone) seemed to possess all of the above qualifications and as a prototype of this unusual class of aromatic compounds, 4-methyltropolone was selected for our studies.

1) Biological Activity of Tropolones

Since the elucidation of the structure of the tropolone ring more than ten years ago, the parent compound and many of its natural and synthetic analogues have been assayed for a variety of biological activities. It was discovered early that tropolone had bacteriostatic activity[87]. This work was extended to a wide variety of natural and synthetic derivatives of tropolone (such as 3,5,7-tribromo tropolone) and the fact that bacteria had no tendency to develop resistance[86a] is of some interest. However they were found ineffective against sarcoma in the rat[87a]. The powerful metal chelating property of the tropolone ring was used to advantage as a protective agent in beryllium poisoning in rats[88].

Logical extensions of these investigations of the biological activity of tropolone compounds led eventually to the observation that they can inhibit the virus of Japanese encephalitis *in vitro*[89]. More work is necessary to assess the true effectiveness of tropolones as inhibitors of other viral strains *in vivo* and *in vitro*.

Tropolone was found to have peculiar activity on smooth muscle, which hints that it may have some effect on adrenergic transmission. At low concentrations it stimulated but at high concentrations inhibited the tone and spontaneous movement of diestrus or estrus uteri of mice *in vitro*[90].

It is of interest to note that trees of the family Cupressaceae and cedar trees, which produce thujaplicins, hinokitiol and other tropolones are highly resistant to fungal infection. In fact, it was subsequently shown that tropolone and hinokitiol as well as some brominated analogs had anti-fungal activity *in vitro*[91]. They are approximately equipotent to the antibiotics, trichomycin and mycostatin. The mechanisms of these effects are unknown.

It was observed also that tropolone, Na hinokitiolate, 3-carboxy-4-carboxymethyltropolone, ammonium tropolone-5-sulfonate, 3-carboxymethyl tropolone, 4-methyl tropolone, 2-chlorotropolone and 3-bromo tropolone-mercury, produced hyperglycemia and diuresis after intravenous injection into rabbits at doses of 5-70 mg/kg[92]. The blood sugar curve showed mostly a phase of hyperglycemia continuing for several hours after the administration of the compounds followed by a hypoglycemic phase. Later, the blood sugar level normalized slowly. Histological examination indicated that the β-cells

of the pancreas were most severely injured after the administration of these troponoids at the suggested doses. In some cases the majority of the β-cells were necrotised and only the α-cells remained intact in the islets of Langerhans'. In the kidney, the main parts of the renal tubuli were reversibly impaired.

These investigations suggest that the parallelism between the extent of the hyperglycemic effect and the injury to the β-cells, may be related to inhibition of insulin secretion whereas the parallelism between the extent of the diuretic effect and the injury to the renal tubuli would originate in the inhibition of reabsorption by the renal tubuli. It is possible that inhibition of COMT by the troponoids <u>in vivo</u> may be partially responsible for the hyperglycemic effect. An activity at the level of the antidiuretic hormone secretion should also be considered in relation to the diuretic effect.

The antimitotic action of colchicine is well documented. Another interesting activity of tropolone is its antagonism to the metaphase arresting action of colchicine[93]. No interpretation of this interesting effect is available. The mode of action of colchicine has eluded investigators for many years and there is much confusion even to date. Since the fifth century, it has been used in the treatment of gout, and its antimitotic action is widely known[94].

During the past few years a wide variety of seemingly disconnected activities of colchicine have been reported[94,95,96,97,98]. Among the more prominent ones are its ACTH like action and its potentiating effect on the action of adrenaline (i.e. a sympathomimetic effect, hypertensive activity and a depressive effect on the respiratory centre).

When the methyl ether on ring C of colchicine is cleaved there results colchiceine which has its tropolone ring unblocked. Hence, the possibility arises that many of the above observed effects of colchicine might be explained on the basis of COMT inhibition if colchiceine is produced in vivo or is present as a contaminant.

Axelrod[99] has described an enzyme localised in the microsomes of liver and which has the ability to cleave a wide variety of aromatic ethers, among which is colchicine. Bacteria have also been shown to possess the same ability[100]. However, in the case of colchicine it has not been shown which methoxy group is removed from the molecule. It may be recalled that colchicine has four methoxy groups of which only one is on the tropolone ring.

To determine whether the tropolone ring can be unblocked in vivo, a biochemical assay procedure was devised, wherein colchicine was incubated with a rat liver homogenate following Axelrod's procedure[99]. The resulting solution was then assayed for COMT inhibition.

Another interpretation of these strange effects of colchicine, may be that the investigators have used a colchicine preparation containing a small percentage of minor alkaloids which have the tropolone ring intact. There is evidence that older preparations may have been so contaminated[101].

j) Metal-Complexing Agents

The use of enzyme inhibitors which bind metal ions, is another important means of investigating the mode of action of an enzyme[102]. Such inhibitors have been postulated to diminish catalytic activity by binding to the metal in situ, i.e. on the apoenzyme, forming a mixed complex as shown in the equation below:

$$[EMe] + nI \rightleftharpoons [EMe]\,In \qquad (7)$$

The inactivity of the complex [EMe]In is related to the filling of the coordination sphere of the metal. Alternatively the inhibitor I might inactivate the enzyme by removing the metal from the enzyme. The reversibility of this reaction might then be governed by the ability of the protein to reassociate with the metal ion once dissociation has occurred as per the equation below:

$$[EMe] + nI \rightleftharpoons E + MeIn \qquad (8)$$

both equilibria depicted in equations (7) and (8) are functions of temperature, time, inhibitor concentrations etc. These equations are based on established principles governing the action of such inhibitors and although they may apply to many metal-binding agents, they may not necessarily apply to all.

If a chelating agent interacts specifically with a metal ion bound to an enzyme, it should be possible to prevent inhibition by occupying the site of chelation in the ligand with a metal atom prior to exposing the enzyme's metal to the same ligand. Although the metal-chelate complex is in equilibrium with metal ions and chelating agent, this dissociation should be of negligible magnitude and can be disregarded, because the equilibrium;

$$Me + In \rightleftharpoons MeIn \qquad (9)$$

is far to the right due to an excess of metal ions usually used. Consequently, no change in activity should be observed and this is generally the case.

Once a metal-enzyme inhibitor complex has formed as described in equation (7), a critical excess of metal ion should reverse the inhibition by competition with the metalloenzyme for the bound inhibitor:

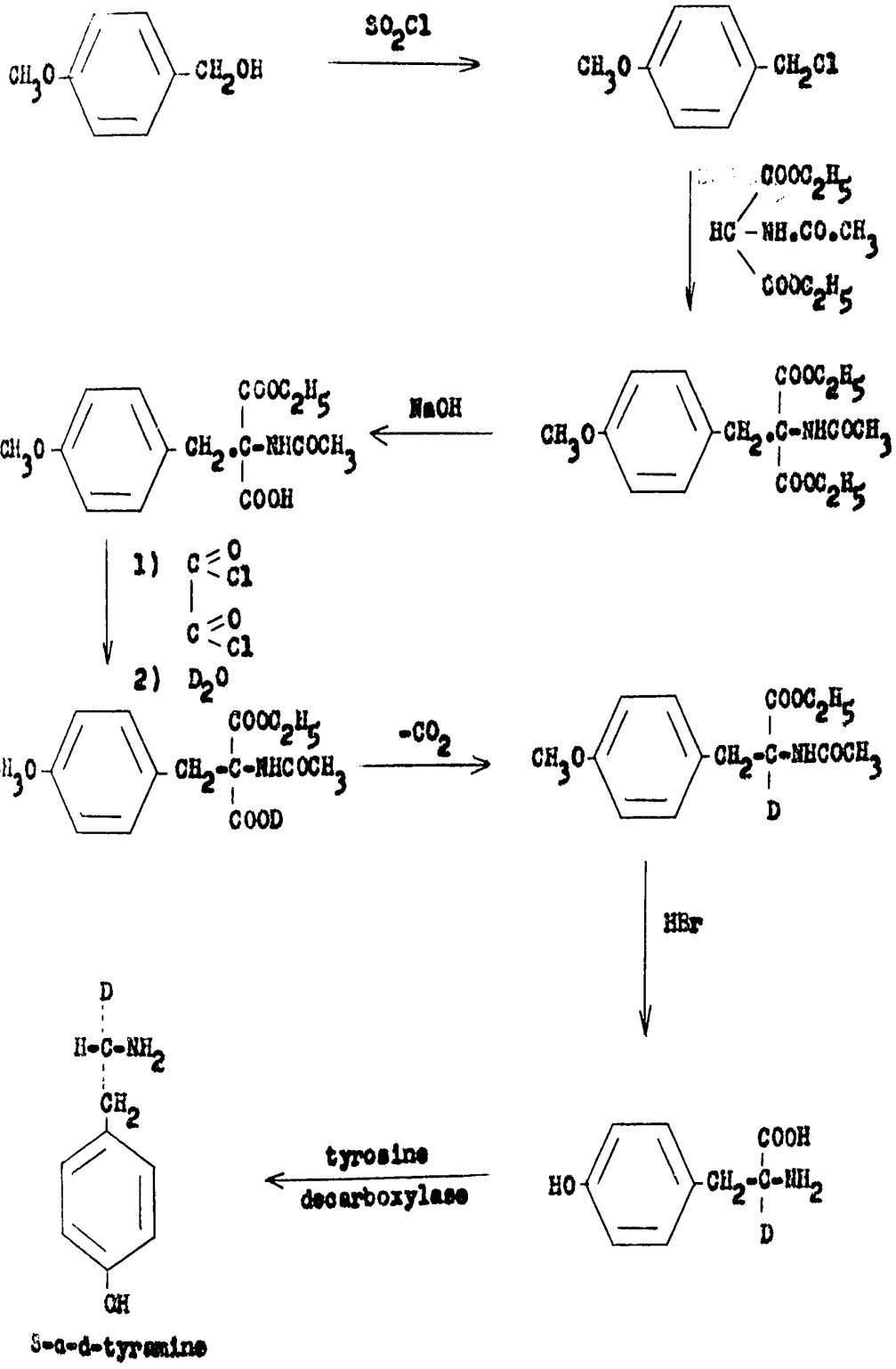
$$[EMe]In + Me \rightleftharpoons [EMe] + [MeIn] \qquad (10)$$

Thus activity should be restored by removal of the inhibitor from the metalloenzyme according to the mass action law. Thus, reversibility results from a characteristic of a dissociable metal-enzyme chelate complex. Since many of the metal ions may themselves inhibit such enzymes possibly by interaction with accessory sites on the apoenzyme, the stoichiometry of all reactants must be known before drawing conclusions.

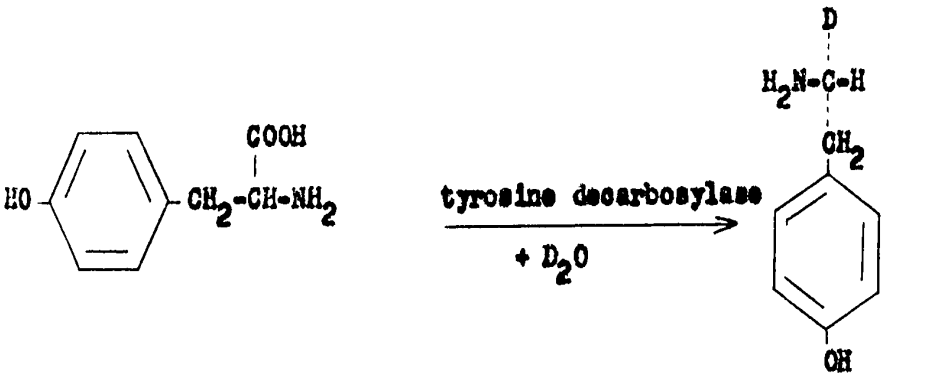
Hence, it is now apparent that one must differentiate between mechanisms (7) and (8) with regard to COMT inhibition by tropolones, and this is difficult to do on the basis of inhibition kinetics alone. Also, when a metal enzyme is exposed to concentrations of ligand which should lead to occupancy of available coordination bonds of the ion, the stoichiometry of the metal chelate that is formed will be an excellent guide to the mechanism of action of the ligand. Measurements of any of the physical or chemical changes that many chelating agents undergo as a result of complex formation with metals may serve as gauges of complex formation. Our only gauge is the percentage of inhibition index because the enzyme has not as yet been sufficiently purified. Job's Method of Continuous Variation was however used to advantage[103]. This method which suffices for our purposes was applied as follows: two components forming a complex are mixed, while their molar sum is kept constant. A metal is

varied from 100 to 0 mole %; simultaneously, in order to keep the molar sum constant, the complexing agent concentration is varied from 0 to 100 mole %. The complex will exhibit a maximal change in a physical or chemical property (such as spectrophotometric absorption) when the relative amounts of metal and ligand correspond to the stoichiometry of the complex. When the change in extinction coefficient at one wavelength is plotted against concentration of metal ions (0 to 100 mole %), the maximal change for a 1:1 [ML_1] complex will occur at 50 mole % of metal; for a 1:2 [ML_2] complex, at 33 mole % of metal; and for a 1:3 [ML_3] complex at 25 mole % of the metal. Unfortunately spectrophotometric absorption cannot be applied to crude enzyme preparations, so that percentage of inhibition changes were used instead. A sufficiently pure enzyme preparation was nevertheless used as otherwise there might be too many interfering factors in the crudor preparations used in the kinetic studies.

Enzymic Synthesis of S-α-d-Tyramine



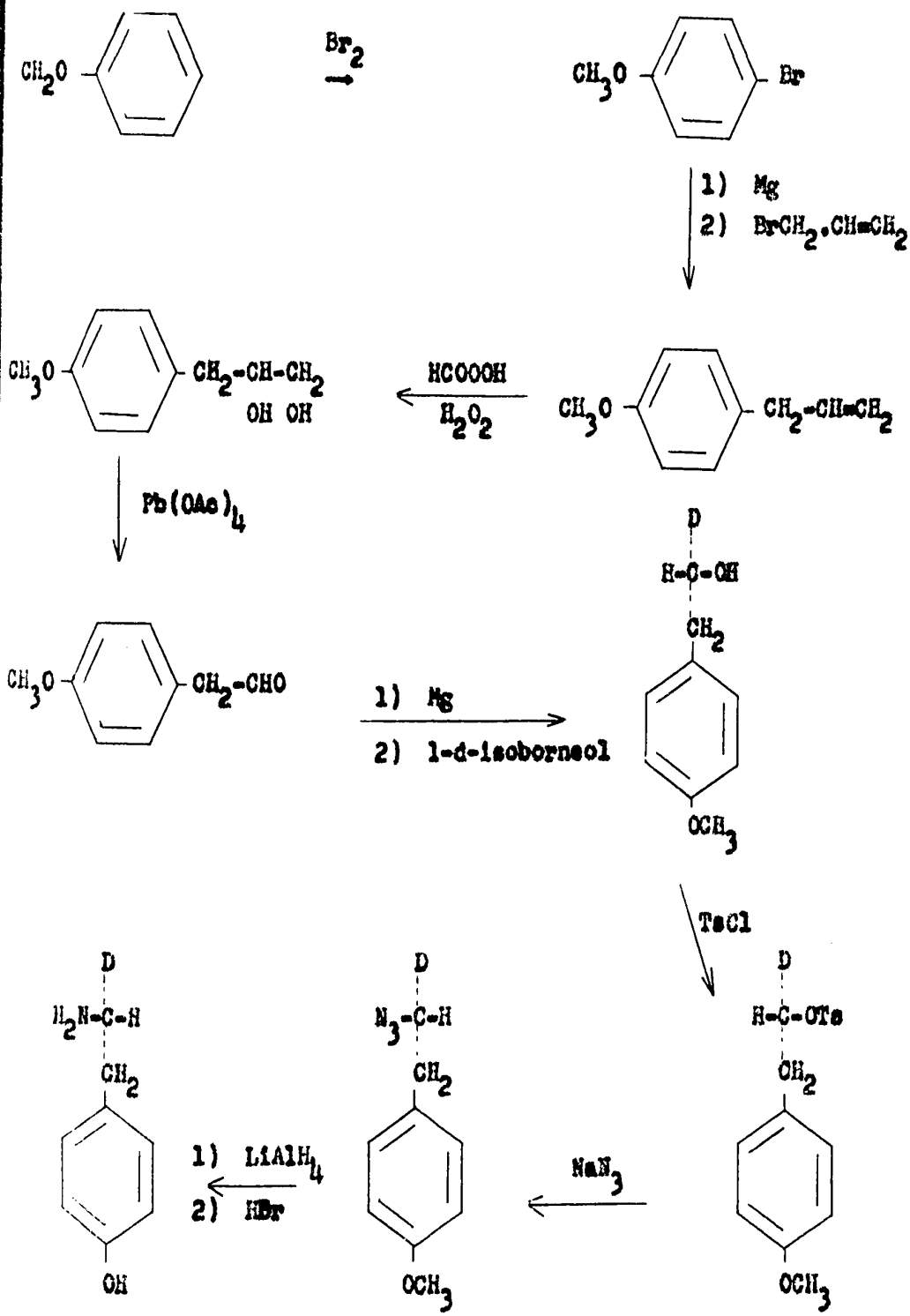
Enzymic Synthesis of R-α-d-Tyramine



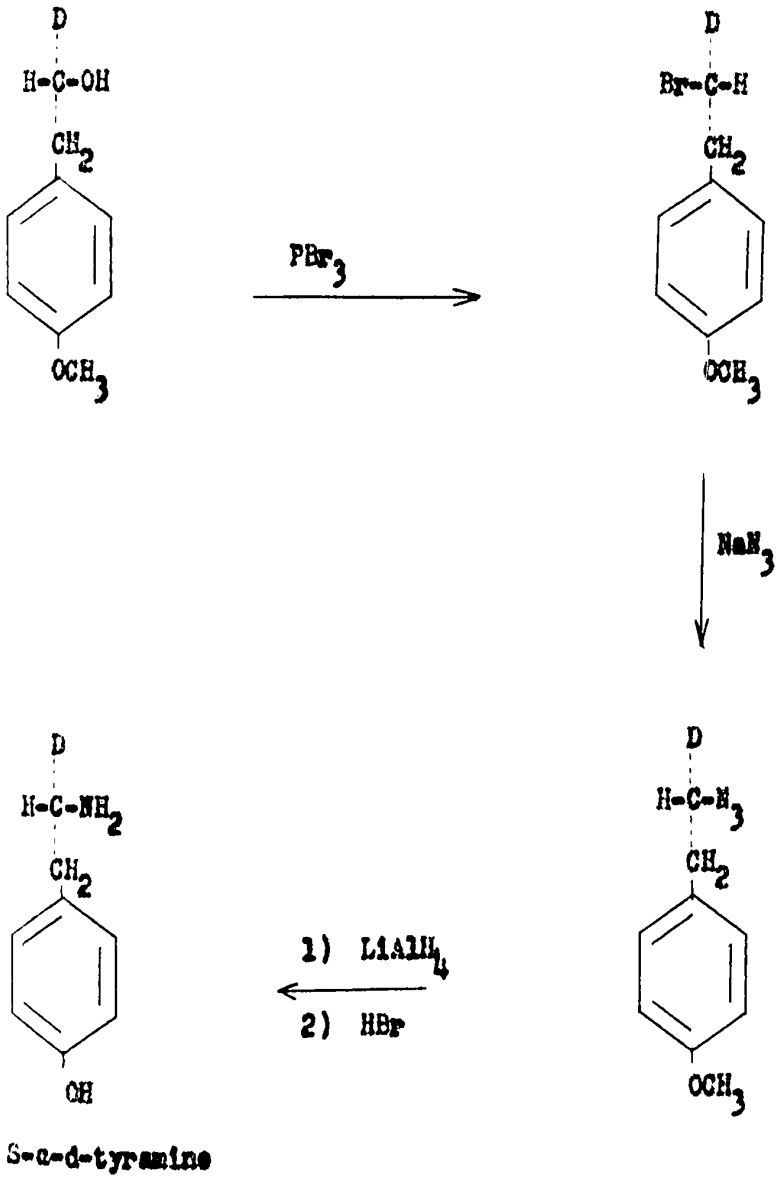
DL-tyrosine

R-α-d-tyramine

Chemical Synthesis of R-α-d-Tyramine

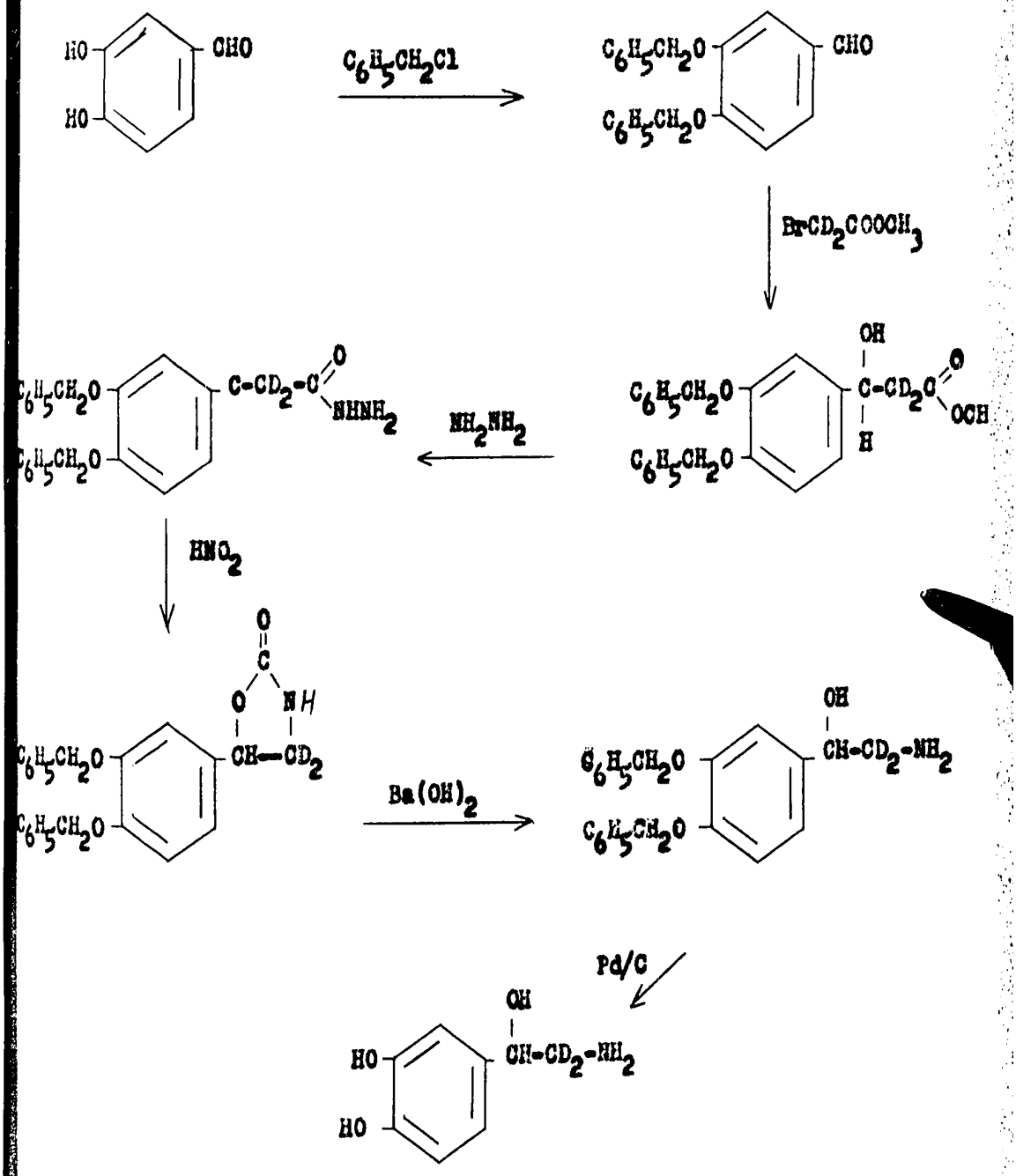


Chemical Synthesis of S-α-d-Tyramine



S-α-d-tyramine

Synthesis of l-Bis-α-Deuterionoradrenaline



dl-Bis-α-deuterionoradrenaline

EXPERIMENTAL

A. SYNTHETIC PROCEDURES

1. 3-α-DEUTEROTYRAMINE* (ENZYMATIC)

a) 4-Methoxy Benzyl Chloride (Anisyl Chloride)

Several reported procedures were attempted, to prepare this chloride from anisyl alcohol with little success. The following procedure proved the most fruitful: 35 grams of anisyl alcohol in 60 mls. of chloroform and 24 mls. of pyridine was treated for approximately 45 minutes with 37 grams of thionyl chloride at 0-5° C. The reaction mixture turned black. Stirring was continued at 5° C for 30 more minutes, and the resultant mixture was poured into water and extracted with ether. The ether was washed with dilute NaOH solution (3 times), dried with anhydrous sodium sulfate and evaporated. The residue was distilled under reduced pressure (122-125° C/25 mm.) to give 10.15 grams of colorless distillate.

b) 2-Acetamido-2-carbethoxy-3-(p-methoxyphenyl) Propionic Acid

The next two steps which begin with the addition of ethyl acetamidomalonate and anisyl chloride to give ethyl

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* Specification of asymmetric configuration according to R.S. Cahn, C.K. Ingold and V. Prelog, Experientia 12, 81 (1956).

2-acetamido-2-carbethoxy-3-(p-methoxyphenyl) propionate, and subsequently 2-acetamido-2-carbethoxy-3-(p-methoxyphenyl) propionic acid are described by Berlinguet[104].

c) Deuterated 2-acetamido-2-carbethoxy-3-(p-methoxyphenyl) Propionic Acid

To 6 grams of 2-acetamido-2-carbethoxy-3-(p-methoxyphenyl) propionic acid was added 10 mls. of triethylamine, and 150 mls. of dry benzene. The acid dissolved on shaking. Then 3 mls. of oxalyl chloride was added dropwise over a period of 10 minutes, under ice cooling and magnetic stirring. Stirring was continued at 0° C for another three hours. The triethylamine hydrochloride was then filtered off and the clear solution was evaporated under vacuo at 35° C. The residue was a heavy yellow oil, which on addition of twice the theoretical amount of heavy water, crystallized out upon triturating and cooling. The crude product was used in the subsequent reaction.

d) 2-Deuteriomethyl-3-(p-methoxyphenyl)-2-acetamido propionate

In a dried flask fitted with a thermometer and connected to a vacuum, was placed about 6 grams of deuterated 2-acetamido-2-carbethoxy-3-(p-methoxyphenyl) propionic acid. The flask was then immersed in an air bath at 150° C for 25 minutes, and decarboxylation took place vigorously. The

resultant oil was taken up in ethyl ether, and petroleum ether was added till crystallization began. The flask was placed in the cold overnight and a solid mass of crystals was collected. The product was recrystallized from ether, which removed most of the color.
Yield 2.9 grams; m.p. 70-71° C. lit. (104) 72-73° C.

e) α-Deuterio-DL-tyrosine

2.5 grams of 2-deuterioethyl-3-(p-methoxyphenyl)-2-acetamidopropionate was placed in a flask with 50 mls. of 40% HBr and refluxed overnight. The excess HBr was evaporated in vacuo and the residue taken up in water. Dilute NaOH was then added until a pH of 5.7 was reached, (iso-electric point of tyrosine). The crude α-d-DL-tyrosine was recrystallized from water; (1 gram to 200 mls. of boiling water).
yield 1 gm; m.p. 270-272° C (decomp.).

f) Enzymatic β-α-d-tyramine Hydrochloride

In a three-neck flask was placed 60 mls. of pH 5.6 acetate buffer (0.873 mole fraction of sodium acetate plus 0.127 mole fraction of acetic acid); 200 mg. of tyrosine decarboxylase (Nutritional Biochemicals Ltd.); 720 mg. α-d-DL-tyrosine and 10 mg. of pyridoxal phosphate. The flask was kept at 38° C overnight with mild stirring; also a continuous stream of nitrogen was passed over the reaction mixture (one bubble/sec.). The pH of the reaction mixture was checked

at hourly intervals for the first three hours, then about every 3-4 hours. Addition of dilute acetic acid was necessary to maintain the pH at 5.6.

After the incubation period, the contents of the flask were boiled gently for 3-4 minutes, and then centrifuged. The clear supernatant was decanted and the residue was washed with about 75 mls. of water and centrifuged again. The pH of the clear supernatant and washings was adjusted to 7.5 by addition of dilute NaOH.

The method for isolation of pure d-d-tyramine was in essence that of Davis and Awapara[105]. 100 Grams of amberlite CG -50 H^+ 200-400 mesh was placed in a one litre flask and the above supernatant and washings were added. The flask was shaken for an hour and the contents were poured into a chromatography column which contained a plug of glass wool, a thin bed of celite and 10 grams of dry resin. The flask was rinsed with 2 x 25 mls. portions of distilled water and the washings were transferred to the column. The column was then washed with about 7 litres of distilled water under mild pressure. S-d-d-Tyramine was then eluted with 2 litres of 4N acetic acid. The residue, upon evaporating the acetic acid in vacuo, was taken up in boiling ethanol. The insoluble fraction was filtered off, and the ethanol was concentrated down to about 10-15 mls., and dry HCl was bubbled into the ethanol solution for about 3-4 minutes. The insoluble inor-

ganic precipitate was filtered off, and to the clear ethanol solution of S-α-d-tyramine hydrochloride was added 5 mls. of ethyl ether. On scratching and cooling, fine crystals of S-α-d-tyramine hydrochloride crystallized out. The product was recrystallized from a minimum quantity of boiling ethanol.

Yield 100 mg.; m.p. 250-255° C. (agrees with commercial sample).

2. R-α-DEUTERIOTYRAMINE (ENZYMATIC)

The procedure used here is identical to the one employed for the preparation of S-α-d-tyramine from α-d-DL-tyrosine; except that 360 mg. of L-tyrosine was employed and the incubation was carried out in heavy water (D_2O). Also, a calcium chloride tube was fitted to the incubation flask.

3. R-α-DEUTERIOTYRAMINE (SYNTHETIC)

a) p-Allylanisole (Estragol)

200 Grams of anisole and 750 grams of acetic acid were mixed in the cold. Bromine (310 grams) was then added in an air stream until the solution was yellow. The reaction mixture was then poured into 4 litres of water. The water was evaporated and the residue distilled at 93-94° C/13 mm. Yield of p-bromoanisole was 187 grams.

Then 24 grams of magnesium power was activated with 5 grams of iodine in 420 mls. of ethyl ether. p-bromo-anisole (187 grams) was then added dropwise. Then, 121 grams of allyl bromide was added over 4 hours. After evaporation of the ether, the residue distilled at 86° C/13 mm. Yield of p-allylanisole was 122 grams.

b) <u>1,2-Dihydroxy-3-(p-methoxyphenyl)-propane</u>[106]

500 mls. of performic acid (90%) and 115 mls. of hydrogen peroxide (30%) were stirred vigorously, and to the stirring mixture was added approximately 20 grams of estragol. Stirring was continued till the temperature rose to 40° C (some heating was necessary). Then with alternate cooling in ice, was added dropwise 102 grams of estragol, and the temperature was maintained at 40-45° C. The reaction mixture was stirred overnight at room temperature; then evaporated in vacuo. To the residue was added a solution of 66 grams of NaOH in 140 mls. of water, the temperature being maintained at 40-50° C. The mixture solidified. Then 200 mls. of water was added and the solution was heated at 50° C for 1 hr., cooled, and 100 mls. of concentrated HCl was added. The resultant mixture was extracted with chloroform, which was subsequently evaporated and the residue distilled at 158° C/2.5 mm.

Yield of 1,2-dihydroxy-3-(p-methoxyphenyl)-propane was 100 grams.

c) p-Methoxyphenylacetaldehyde*

18.2 Grams (0.1 mole) of the glycol 1,2-dihydroxy-3-(p-methoxyphenyl)-propane was dissolved in 1 litre of benzene. Then, 100 mls. of the benzene was distilled off, the solution was cooled, and 44.3 grams of lead tetraacetate in small portions was added with stirring to the solution. The solution was then stirred for one hour, and filtered. The filtrate was washed with water, followed by a saturated solution of sodium bicarbonate. It was then dried with anhydrous sodium sulfate and evaporated in vacuo. The residue distilled at 78-79° C/0.1 mm.

Yield of p-methoxyphenylacetaldehyde was 10 grams, (65% overall yield).

d) d-1-d-p-methoxyphenethyl alcohol

1.6 Grams of magnesium was added to 8.5 grams of n-propyl bromide in 75 ml. of ether. Then with the temperature maintained at 0° C, was added 10.3 grams of 1-d-isoborneol dissolved in 75 ml. of ether[107]. The 1-d-isoborneol was prepared by the reduction of d-camphor with lithium aluminum deuteride in ether at -70° C [35]. It was at least 97% labeled

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* Plieninger, H., and Kiefer, B., Chem. Ber. 90, 617 (1957) reported the preparation of this aldehyde using different methods which proved unsatisfactory in our hands.

on the carbinol carbon as determined by n.m.r. analysis*. Then 150 mls. of dry benzene was added, and the mixture was distilled until the boiling point of the vapors was 75° C. The reaction mixture was cooled to room temperature, and 10 grams of p-methoxyphenylacetaldehyde in 60 mls. of benzene was added[36]. It was stirred for 4 hours and then 5.5 mls. of water was added to stop the reaction. 10% HCl was then added, and the benzene layer was separated, washed twice with dilute $NaHSO_3$ solution, dried with anhydrous Na_2SO_4 and evaporated in vacuo. The residue plus 300 mls. of water was heated under reflux, and the undesireable terpenes were removed as a sublimate. The removal is complete after 6-7 hours of reflux. The mixture was cooled, and extracted with ether. The ether was dried with anhydrous Na_2SO_4, and evaporated in vacuo. The residue distilled at 94-96° C/1 mm.[108]. Yield of S-1-d-p-methoxyphenethyl alcohol 4.1 grams (40%). $[\alpha]_D^{24}$ -1.44° (neat).

e) Tosylate of S-1-d-p-methoxyphenethyl alcohol[109,110,111]

1.52 Grams of the above alcohol was dissolved in 15 mls. of dry pyridine and cooled to -10° C. Then 2.1 grams. of p-toluenesulfonyl chloride was added; the temperature was

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* We are grateful to Dr. R.R. Fraser for the interpretation of the n.m.r. spectra.

kept at 0° C for 2 hours. The reaction mixture was diluted with 25 mls. of water at 0° C. The crystals formed were recrystallized from ethyl ether-petroleum ether.
Yield 2 grams. m.p. 50° C.

f) R-l-d-p-Methoxyphenethyl Azide[38]

2 Grams of the above tosylate was dissolved in 12 mls. of methyl alcohol and 2.5 mls. of water to which 1 gram of sodium azide was added. The reaction mixture was boiled overnight, and evaporated to dryness. The residue was taken up in water and extracted with ethyl ether. The ethereal solution was dried with anhydrous Na_2SO_4 and evaporated in vacuo. The residue was used as such in the subsequent reaction.

g) R-l-d-tyramine Hydrochloride

The residue from the ethereal solution of the previous reaction was dissolved in 25 ml. of ether, and the solution was added to a solution of 1 gram of $LiAlH_4$ in 100 mls. of ethyl ether. The mixture was boiled for one hour, cooled, and the excess $LiAlH_4$ was decomposed with 1 ml. of water. Then 1 ml. of 20% NaOH and 4 mls. of water was added, and the resultant mixture was filtered. The ethereal solution was washed with 10% NaOH, dried with anhydrous Na_2SO_4 and evaporated in vacuo. The residue was taken up in a minimum quantity of ethyl ether, and dry HCl was

passed into the solution until crystals formed. 810 mg. of colorless crystals of R-l-d-p-methoxyphenethyl amine hydrochloride were collected, placed in 20 ml. of 40% HBr, and boiled overnight. The resultant solution was evaporated to dryness. The crystalline residue was taken up in methyl alcohol containing a few mg. of Ag_2CO_3. The solution was filtered and evaporated to dryness. The residue was then taken up in minimum quantity of ethyl alcohol and dry HCl was passed into the solution until it was saturated, and then filtered hot. The clear filtrate was concentrated to a small volume, and ether was added till crystallisation began. The crystals of R-l-d-tyramine hydrochloride were recrystallized twice from ethanol. m.p. 269°C.

4. S-a-DEUTERIOTYRAMINE (SYNTHETIC)

a) R-l-d-p-methoxyphenethyl Bromide

1.5 Grams of S-l-d-p-methoxyphenethyl alcohol was dissolved in 10 mls. of dry chloroform and 1.2 grams of collidine; then 0.4 mls. of PBr_3 was added, and the reaction mixture was stirred at 50° C for 5 minutes. The mixture was poured onto ice. The chloroform layer was washed with dilute HCl, dried with anhydrous sodium sulfate and evaporated in vacuo. The residue was distilled at 138-142° C/13 mm. Yield of R-l-d-p-methoxyphenethyl bromide was 0.570 grams, (35%).

b) R-l-d-p-methoxyphenethyl Azide

The R-l-d-p-methoxyphenethyl bromide (0.570 grams) plus 1 gram of sodium azide in 2.5 mls. of water, and 15 mls. of methyl alcohol were refluxed overnight, and the product was isolated as in the procedure for R-l-d-p-methoxyphenethyl azide. The residual oil gave a strong peak in the R-N_3 region of the I.R. (2150 $cm.^{-1}$).

c) S-a-d-tyramine Hydrochloride

The residual oil from the above reaction was reduced as per the procedure for R-a-d-tyramine, using 0.5 grams of $LiAlH_4$. The yield of 155 mg. of S-l-d-p-methoxyphenethyl amine hydrochloride was boiled overnight in 10 mls. of 40% HBr, and the resultant S-a-d-tyramine hydrochloride was isolated as in the previous procedure for R-a-d-tyramine hydrochloride. m.p. 269°C.

5. l-BIS-a-DEUTERIONORADRENALINE

The most obvious procedure appeared to be the preparation of the cyanohydrin from 3,4-dihydroxybenzaldehyde, and then reduction with deuterium gas. However, this procedure only produced starting material after repeated attempts of cyanohydrin formation. Success was achieved with a modification of the method of Crewe and Herberg[112].

a) Protocatechualdehyde (3,4-Dihydroxybenzaldehyde)

The procedure employed here was essentially that of Buck and Zimmerman[113]. Piperonal was used as starting material.

b) 3,4-Dibenzyloxybenzaldehyde

10 Grams of sodium was dissolved in 200 mls. of methyl alcohol. Nitrogen was bubbled in for approximately twenty minutes, and then 23 grams of protocatechualdehyde was added under reflux and with a continuous stream of nitrogen being passed over the reaction mixture: Then 51 mls. (56 grams) of benzyl chloride was added, and the resultant solution was refluxed for approximately 15 hours, under a continuous stream of nitrogen. The sodium chloride was then filtered off, and the methanolic solution was evaporated in vacuo. Crystals separated out, which were recrystallised five times from ethanol.

Yield of 3,4-dibenzyloxybenzaldehyde was 21.2 grams, m.p. 90-91°C.

c) Bis-C-deuteriomethylbromoacetate

The procedure used here in a modification of the one used by Auwers and Bernhardi[114].

To a mixture of 5 grams of deuterated acetic acid (CD_3COOD) and 0.875 grams (0.85 grams theory) of dry red

phosphorous was added gradually 57.5 grams (20 mls.) of dry bromine, with suitable cooling if necessary. The mixture was heated under reflux for 10 hours on a water bath rather than the recommended 5, since the reaction takes longer to reach completion due to an isotope effect.

A considerable amount of bromine was recovered. Then the reaction mixture was treated slowly with 2-3 times the calculated amount of methanol (10 mls.). The mixture was diluted with water and the product was extracted into ether, which was then washed with 20% aqueous sodium bisulfite solution, dried with anhydrous Na_2SO_4 evaporated in vacuo and the residue was fractionally distilled. b.p. 65° C/ 60 mm.

Yield of bis-α-deuteriomethylbromoacetate was 5.43 grams.

d) <u>β-Hydroxy-β-(3,4-dibenzyloxyphenyl)-bis-α-deuteriomethylpropionate</u>

About 15 grams of 20 mesh zinc was washed with 10% HCl, then acetone then anhydrous ether, and dried thoroughly. In a 3-neck flask equipped with stirrer, 3 grams of 3,4-dibenzyloxybenzaldehyde was dissolved in a mixture of 50 mls. of anhydrous ether and 50 mls. of dry benzene. Then 1.6 grams of bis-α-deuteriomethylbromoacetate (5% excess) was added, and finally 3 grams of washed zinc. A few crystals of iodine were added to activate the zinc, and heat

was applied from a water bath. The mixture was refluxed for 3 hours. When the source of heat was removed, the reaction ceased. At the end of the first hour of reflux, an additional 3 grams of zinc was added, and still an additional 3 grams was added after the second hour of reflux. The mixture was cooled, and shaken with 10% HCl, then with water, then with dilute NaOH, and finally with water again. The ether-benzene solution was dried with anhydrous Na_2SO_4 and evaporated in vacuo. A thick light yellow oil was obtained which failed to crystallise. This crude ester product of the Reformatsky reaction was used as such in the subsequent reaction.

e) <u>β-Hydroxy-β-(3,4-dibenzyloxyphenyl)-bis-α-deuteriohydrazide of Propionic Acid</u>

To approximately 3 grams of the crude ester from the previous reaction was added 2 mls. of ethanol and 2.5 grams of 95% hydrazine hydrate. After heating at 50° C for a few minutes, a white crystalline mass separated out, which was filtered and dried.

Yield, 1.5 grams m.p. 190-191° C.

f) <u>4-Bisdeuterio-5-(3,4-dibenzyloxyphenyl) Oxazole-2-one</u>

To a suspension of 1.5 grams of the previously prepared hydrazide in 10 mls. of water, 10 grams of ice, and 2 mls. of glacial acetic acid, was added drop by drop

under intensive stirring a solution of 1.3 grams of $NaNO_2$ in 2.8 mls. of water. Stirring was continued for an additional 4 hours under ice cooling. Then the temperature of the reaction mixture was allowed to rise to 40° C during a two hour time interval. Finally the solution was heated to the boiling point, and an oil separated out which became very viscuous on cooling. The oil was extracted with chloroform, and the chloroform extract was washed with 5% HCl, water and dilute NaOH. The chloroform layer was then dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residual viscuous mass was taken up in a minimum quantity of ethyl acetate and crystals formed on scratching the side of the container. The crystals were redissolved in a minimum quantity of ethyl acetate and a small insoluble portion was filtered off. Then ethyl ether was added to the clear solution until crystallisation began.

Yield of 4-bisdeuterio-5-(3,4-dibenzyloxyphenyl) oxazole-2-one was 0.992 grams. m.p. 146-148° C.

g) <u>Bis-α-deuterio-β-hydroxy-β-(3,4-dibenzyloxyphenyl) Ethylamine Hydrochloride</u>

900 mg. of the oxazole obtained from the previous reaction, was placed in a solution of 2.2 grams of $Ba(OH)_2.8H_2O$ in 60 mls. of methanol plus 40 mls. of water. The resultant solution was refluxed for 3 hours, after which

CO_2 was bubbled in under reflux for 15 minutes to remove excess barium hydroxide. The solution was boiled an additional 15 minutes. The $BaCO_3$ precipitate was centrifuged and the supernatant was filtered through fine filter paper. The $BaCO_3$ precipitate was washed twice with methanol and centrifuged again. The washings and clear filtrate were combined and evaporated in vacuo. The residue was a heavy light yellow oil which was taken up in iso-propyl alcohol. The alcoholic solution was then evaporated to a few mls. and anhydrous ether was added until slight cloudiness was observed. Iso-propyl alcohol was added drop by drop until the cloudiness just disappeared. Dry HCl was then bubbled in carefully until the solution was just on the acid side. Crystallization began immediately. The extremely hygroscopic crystals were recrystallized from iso-propyl alcohol and ether. Yield of bis-α-deuterio-β-hydroxy-β-(3,4-dibenzyloxyphenyl) ethylamine hydrochloride was 635 mg.

h) <u>dl-Bis-α-deuterionoradrenaline</u>

595 mg. of bis-α-deuterio-β-hydroxy-β-(3,4-dibenzyloxyphenyl) ethylamine hydrochloride was dissolved in 25 mls. of methanol containing 1% water*. Then, 595 mg. of 10% Pd/C

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* The water was necessary to prevent the formation of the methyl ether at the β-hydroxy position.

was added to the hydrogenation flask, and hydrogenation was carried out at slight positive pressure. The hydrogen uptake stopped abruptly when the theoretical amount was reached. The solution was filtered through celite and evaporated in vacuo. The residue was taken up in 3 mls. of water and 6 drops of conc. NH_4OH was added. After scratching the container, crystals of dl-bis-α-deuterionoradrenaline separated out, and were let stand in the cold for several hours.

Yield of dl-bis-α-deuterionoradrenaline was 149 mg. m.p. 185° C.

i) l-Bis-α-deuterionoradrenaline-d-bitartarate

The resolution of dl-bis-α-deuterionoradrenaline was carried out according to the procedure of B.F. Tullar[115].

6. 4-ETHANAMOLAMINOTROPOLONE

a) 4-Styryltropolone

Using 3-carboxy-4-styryltropolone as starting material*, 4-styryltropolone was prepared according to the procedure of Tarbell et al[117].

b) 4-Formyltropolone by Periodate-osmate Oxidation of 4-Styryltropolone

The procedure of Tarbell et al[117] was also employed here.

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* 3-Carboxy-4-styryltropolone was prepared by Mrs. M. Triggle using Haworth's procedure[116].

c) 4-(β-Hydroxy Nitroethane) Tropolone

2 Grams of 4-formyltropolone was dissolved in 50 mls. of methanol, and 4 mls. of pure triethylamine was added. The reaction mixture was cooled to 5° C and 10 mls., of nitromethane was added. The mixture was let stand for 48 hours at 5° C. Then the solution was acidified in the cold with 20% aqueous HCl. Almost complete discoloration occurred. The resultant solution was evaporated to dryness in vacuo at 20-25° C. The residue was triturated with ice cold water, and light yellow crystals were formed. m.p. 138-139° C. The crystals were recrystallized from acetone-heptane to give cream colored plates with a m.p. of 140.5-141° C.

d) 4-Ethanolaminotropolone

Hydrogenation of the nitroalcohol obtained from the previous reaction, with PtO_2 produced a product with no discernible melting point and whose carbon, hydrogen analysis was far from theoretical. Residues of 6-9% were produced also after combustion during C, H, analysis. The use of 5% rhodium on alumina gave an insignificant yield of an unknown compound. The use of 10% Pd/C as catalyst and mole for mole of glacial acetic acid proved successful.

In a hydrogenation flask was placed 340 mg. of the nitroalcohol, 150 mg. of 10% Pd/C, 1 ml. of a solution of 0.92 mls. of 99.8% acetic acid in 10 mls. of water, and

30 mls. of ethanol. Hydrogenation was carried out for 24 hours under slight positive pressure. The theoretical uptake of hydrogen was 129 mls., while the actual uptake was 110 mls. The solution was then filtered, and evaporated in vacuo. To the residual viscuous oil was added a minimum quantity of methanol, then anhydrous ethyl ether was added until crystallisation occurred. A large crop of fine light yellow crystals were obtained, which were extremely hygroscopic and had to be filtered under dry nitrogen. Yield was approximately 250 mg. m.p. 145-155° C (decomp.). The N.M.R. data fits theory. C, H and N analysis gave C - 57.64%; H - 6.62% and N - 7.4%, with no residue. The theory is C - 59.6%; H - 6.1%; N - 7.7%. Also, infrared and U.V. spectra agree with expectations. A paper chromatogram developed with ninhydrin showed the product to be contaminated with traces of an unknown compound which may account for the discrepancy in elemental analysis. The contaminant could be a partially reduced tropolone ring analogue, since the tropolone ring system is more prone to reduction than the benzene ring.

7. 4-ACETAMIDOMETHYLTROPOLONE AND ITS METHYL ETHERS

The procedure applied here is essentially that of T. Nozoe et al[118] which is described in Chemical Abstracts. The original journal was not available.

a) 3-Carboxy-4-methoxycarbonylmethyltropolone

11.7 Grams of 3-carboxy-4-carboxymethyltropolone, which was previously synthetized in our laboratory, was dissolved in 100 mls. of 10% methanolic HCl. The solution was let stand for 6 hours and was selectively methylated to 3-carboxy-4-methoxycarbonylmethyltropolone. The oily residue on evaporation of solvent in vacuo was dissolved in ethyl acetate. Ethyl ether was added till crystallisation began. It was recrystallised from a minimum quantity of hot isopropyl alcohol and n-heptane.

Yield 4.7 grams; m.p. 140-145° C. lit.[118] 177° C.

b) 4-Methoxycarbonylmethyltropolone

To 2.0 grams. of 3-carboxy-4-methoxycarbonylmethyltropolone was added 50 ml. of pyridine, and the solution was heated at 100° C for 3 hours. The pyridine was then evaporated in vacuo and the dark brown oily residue was submitted to sublimation overnight (170° C at 0.025 mm.). Light yellow needles were formed.

Yield 1.3 grams; m.p. 50-55° C. lit.[118] 68°C.

c) 4-Carboxymethyltropolone

To 1.3 grams of 4-methoxycarbonylmethyltropolone was added 50 mls. of 10% aqueous NaOH and the solution was let stand overnight at room temperature. About 10 mls. of the solution was then evaporated in vacuo, and the residual

portion was acidified with dilute H_2SO_4. A crop of cream colored crystals was obtained.

Yield 0.9 grams; m.p. 140-143° C. lit.[118] 154-155° C.

d) 4-Aminomethyltropolone

The Schmidt reaction was applied here. To 0.9 grams of 4-carboxymethyltropolone was added 3 mls. of concentrated H_2SO_4. Hydrazoic acid, (evolved from 4 mole equivalents of NaN_3 into 25 mls. of $CHCl_3$) was then added with stirring. The reaction was allowed to proceed for 20 hours at 40° C. The $CHCl_3$ was then evaporated in vacuo and the residual H_2SO_4 solution was diluted with one volume of water. Concentrated NH_4OH was added until the pH was 7.5. Yellow leaflets were obtained.

Yield 0.3 grams m.p. 200° C (decomp.). lit.[118] 233-234° C.

e) 4-Acetamidomethyltropolone

300 mg. of 4-aminomethyltropolone was dissolved in 5 ml. of 1 N NaOH (twice the equivalent amount), then 0.5 ml. of acetic anhydride was added (1 1/2 times the equivalent amount), and the mixture was shaken for 30 minutes. The amine was found to be insoluble in all the available organic solvents. The resultant solution was then acidified slowly with concentrated HCl till pH 2 was reached. The solution was evaporated to dryness in vacuo, and the residue was digested with two 10 ml. portions of ethanol. The

ethanol solution was then filtered hot, to remove most of the inorganic salts, and evaporated in vacuo. The oily residue was then sublimed at 180° C and 0.05 mm. for four hours. White crystals were obtained.
Yield 194 mg. m.p. 146-147° C. lit.[118] 146° C.

f) Methyl Ethers of 4-Acetamidomethyltropolone

An excess of an ethereal solution of diazomethane was reacted with 150 mg. of 4-acetamidomethyltropolone. This afforded two isomeric ethers; 6-acetamidomethyl-2-methoxytropone and 4-acetamidomethyl-2-methoxytropone. These ethers could not be crystallized, although they have been reported as crystalline[118] with two widely different melting points. A solution of the ethers was used for biological studies.

B. PREPARATION OF ENZYMES

1. RAT LIVER MONOAMINE OXIDASE

The procedure followed is a modification of that of Hawkins[119]. All operations were carried out at 0° C, with previously cooled reagents and apparatus.

Freshly excised livers from decapitated rats were freed of connective tissue and minced in a Latapie mincer with 0.25 M sucrose as the perfusing medium. The resulting suspension was brought up to a volume 10 times (w/v) that of the original tissue wet weight. The suspension was then

homogenized in a glass-teflon homogeniser (No. 42888 size C, Arthur H. Thomas Co.) for 40 seconds at 600 R.P.M. allowing for intermittant periods of cooling. The homogenate was then centrifuged in an International Refrigerated Centrifuge (0° C, Head No. 860) for 5 minutes at 1000 R.P.M. after which the supernatant was removed and the sedimented cell debris discarded. The supernatant was then centrifuged in a Spinco Ultracentrifuge (Rotor No. 40) for 45 minutes at 23,000 R.P.M. (33,000 x g.) and the supernatant was discarded. The precipitated pellet was resuspended in 0.25 M sucrose so that the final volume was 5 times the original wet weight of the tissue, and stored at 0° C.

2. RAT BRAIN MONOAMINE OXIDASE

The method used to prepare rat liver MAO applied to rat brain MAO gave completely unreliable results. The following preparation gave good reproducible results.

Four rats were decapitated and the brains were removed immediately. Each brain was homogenised immediately on removal at 0° C in 0.25 M sucrose using the glass-teflon homogeniser. The total volume of sucrose used for the four brains was 25 ml. The cell debris was removed by centrifuging twice at 600 g 0° C (900 R.P.M.) on the refrigerated International centrifuge as in the liver MAO preparation. The enzyme was used as such, in its crude form.

3. CRUDE COMT

The procedure was essentially that of Axelrod et al[74], except that an equal volume of isotonic KCl per weight of wet tissue was employed. The excised livers were homogenized on a Lourdes homogeniser at 70% max. voltage, for 1 1/2 minutes with the container being kept at 0° C. Intermittant cooling periods were allowed. The crude enzyme was divided in 5 ml. aliquots and stored at -30° C.

4. PURIFIED COMT

The procedure employed here was that of Axelrod and Tomchick[6], except that the purification procedure was stopped after dialysis for 6 hours against 0.001 M phosphate buffer pH 7.0. The enzyme was used as such, and stored at -30° C. Calcium phosphate gel elution produced an enzyme with little activity.

5. CRUDE AROMATIC ETHER CLEAVING ENZYME (DEMETHYLASE)

Five grams of fresh rat liver obtained from decapitated rats was homogenized with w/v of isotonic KCl. The preparation was used as such.

C. METHODS

1. ASSAY FOR TYRAMINE

The procedure employed was a modification of that of Udenfriend and Cooper[78].

a) Reagents

Borate Buffer: 165 mls. of 10 N NaOH is added to 9h.2 grams of boric acid in 3 litres of water. The solution is then saturated with n-butanol and sodium chloride. The final pH is approximately 10.

n-Butanol and n-Heptane: The reagent grade solvents were washed with equal volumes of 0.1 N NaOH, water, 0.1 N HCl and finally twice with distilled water.

1-Nitroso-2-naphthol Reagent: A 0.1% solution of 1-nitroso-2-naphthol in 95% ethanol was made fresh every month.

Nitrous Acid Reagent: To a solution of 4 mls. of concentrated HNO_3 and 20 mls. of water is added 12 mg. of $NaNO_2$ just prior to use.

b) Incubation with Rat Liver MAO

Enzyme, equivalent to 600 mg. liver wet weight, (3 mls. of MAO preparation per previous procedure), 0.5 ml. phosphate buffer pH 7.4, 0.5 M, and approximately 30 μ moles of tyramine were brought to a total volume of 5 ml. with distilled water in 30 ml. beakers. The mixture was incubated in a Dubnoff metabolic shaker at 37° C in air. The enzyme reaction was quenched by transferring 0.4 ml. aliquots of the incubation mixture into 2 ml. of 20% aqueous Na_2CO_3 at various intervals.

i) Extraction of tyramine:

To each of the 50 ml. flasks containing the incubation mixture and Na_2CO_3 solution was added 1 ml. of borate buffer, 3 grams of NaCl and 20 ml. of butanol. The flasks were shaken for 30 minutes and a 10 ml. aliquot of the organic phase was transferred to another 50 ml. flask containing 15 mls. of n-heptane and 3 mls. of 0.2 N HCl. These flasks were shaken for another 30 minutes, and the acid layer was removed.

ii) Colorimetric assay:

To 2 ml. of the acid extract in a 15 ml. centrifuge tube was added 1 ml. each of 1-nitroso-2-naphthol, and nitrous acid reagents. The tube was stoppered, shaken and placed in a water bath at 55° C for 30 minutes. Then, 10 mls. of $CHCl_3$ was added and the tube was shaken vigorously to extract the unchanged nitroso-naphthol. The tubes were centrifuged at low speed and the acid layer was transferred to a cuvette. The optical density was read on a Bausch and Lomb Spectronic 20 at 450 mμ. It was found that the optical density is proportional to concentration up to 0.8 μ moles of tyramine. Enzyme blanks and standards were carried through the same procedure.

c) Incubation with Rat Brain MAO

The incubation and assay was essentially the same as with rat liver MAO except that 6 ml. of the crude enzyme preparation was used; 1 ml. of phosphate buffer pH 7.4, 0.5 M and 15 μ moles of tyramine. The incubation mixture was brought to a total volume of 9 ml.

2. ASSAY FOR NORMETANEPHRINE

a) Reagents

Borate Buffer: The pH 10; 0.5 M borate buffer was prepared by standard methods.

Ethylene Dichloride Containing 2% Iso-amyl Alcohol: This reagent was prepared according to the method of Axelrod and Tomchick[6].

Reagents for Colorimetric Assay: Identical with the ones used for MAO assay.

b) Incubation with Crude COMT

5 ml. of the crude COMT preparation, 2 ml. of phosphate buffer pH 7.9; 0.5 M, 5 mg. of S-adenosylmethionine iodide, (California Corporation for Biochemical Research) 400 μM of $MgCl_2.6H_2O$; 3 μM of 4-methyl tropolone was brought to a total volume of 11 ml. in 30 ml. beakers and preincubated for 10 minutes before addition of the substrate, l-noradrenaline-d-bitartarate, (20-100 μM). The mixture was

incubated in a Dubnoff metabolic shaker at 37° C. Then, 2 ml. aliquots of the reaction mixture were withdrawn at various intervals and placed in 50 ml. flasks containing 1 ml. of pH 10; 0.5 M borate buffer. Standard curves were obtained by omitting the 4-methyl tropolone. Also, the 4-methyl tropolone was substituted by other tropolones and pyrogallol.

i) Extraction of normetanephrine:

To each of the 50 ml. flasks containing the borate buffer and the quenched enzyme reaction mixture was added 25 ml. of an ethylene dichloride, 2% iso-amyl alcohol mixture. The flasks were shaken for 30 minutes, and a clear 20 ml. aliquot of the organic phase was placed in another 50 ml. flask, containing 3 ml. of 0.1 N HCl. The flasks were shaken for another 30 minutes.

ii) Colorimetric assay:

2 ml. of the acid layer from the previous extraction procedure was carried through the same colorimetric assay procedure as described for tyramine, except that the color was read at 400 mμ rather than at 450 mμ.

a) Incubation with Purified COMT

The method of continuous variation was applied here. Let 100 μM of $MgCl_2.6H_2O$ equal 100 mole %, and 1 μM of 4-methyltropolone was let equal 100 mole %. The concen-

trations of $MgCl_2.6H_2O$ and 4-methyltropolone were varied as follows:

	MOLE %					
	1	2	3	4	5	6
$MgCl_2.6H_2O$	100	90	75	50	25	500
4-methyltropolone	0	10	25	50	75	100

The incubation and assay procedures with purified COMT were essentially the same as that with crude COMT except for the following modifications in the concentration of the reactants in the incubation mixture: 1.5 ml. of purified COMT (19.4 mg. protein/ml.), 10 μM l-noradrenaline-d-bitartarate, $MgCl_2.6H_2O$ (0-500 μM), 4-methyltropolone (0-1 μM), 6.0 ml. of phosphate buffer pH 7.8, 0.1 M.

3. ASSAY FOR DEMETHYLASE ACTIVITY WITH COLCHICINE AS SUBSTRATE

a) Incubation with Crude Demethylase

A 5 gm. homogenate (wet weight) of rat liver, 200 μM of $MgCl_2.6H_2O$, 200 μM of nicotinamide, 5 μM of TPN^+ *, 3 ml. of phosphate buffer 0.2 M, pH 7.4, and 100 μM of col-

* Triphosphopyridine Nucleotide.

chicine were brought to a total volume of 13 mls. in a 30 ml. beaker and incubated in a Dubnoff Metabolic Shaker at 37° C for 2 1/2 hours. At the end of the incubation period the proteins were denatured by placing the incubation mixture on the steam cone for 3 minutes. The proteins were centrifuged out and the clear supernatant was assessed for COMT inhibitory activity. A blank which contained no colchicine was carried through the same procedure. From previous data[99] it was assumed that the minimum quantity of colchicine that would be cleaved to produce colchiceine or a phenol would be 10% of the original concentration of colchicine.

b) Bioassay for Demethylase Activity

1 ml. of the clear supernatant from the demethylase incubation mixture above, was used to replace 4-methyltropolone in the assay for normetanephrine with crude rat liver COMT. This aliquot was assumed to contain 1 μM of colchiceine, and was assayed for COMT inhibitory activity as in the procedure for crude rat liver COMT. Blanks were also carried through the same procedure.

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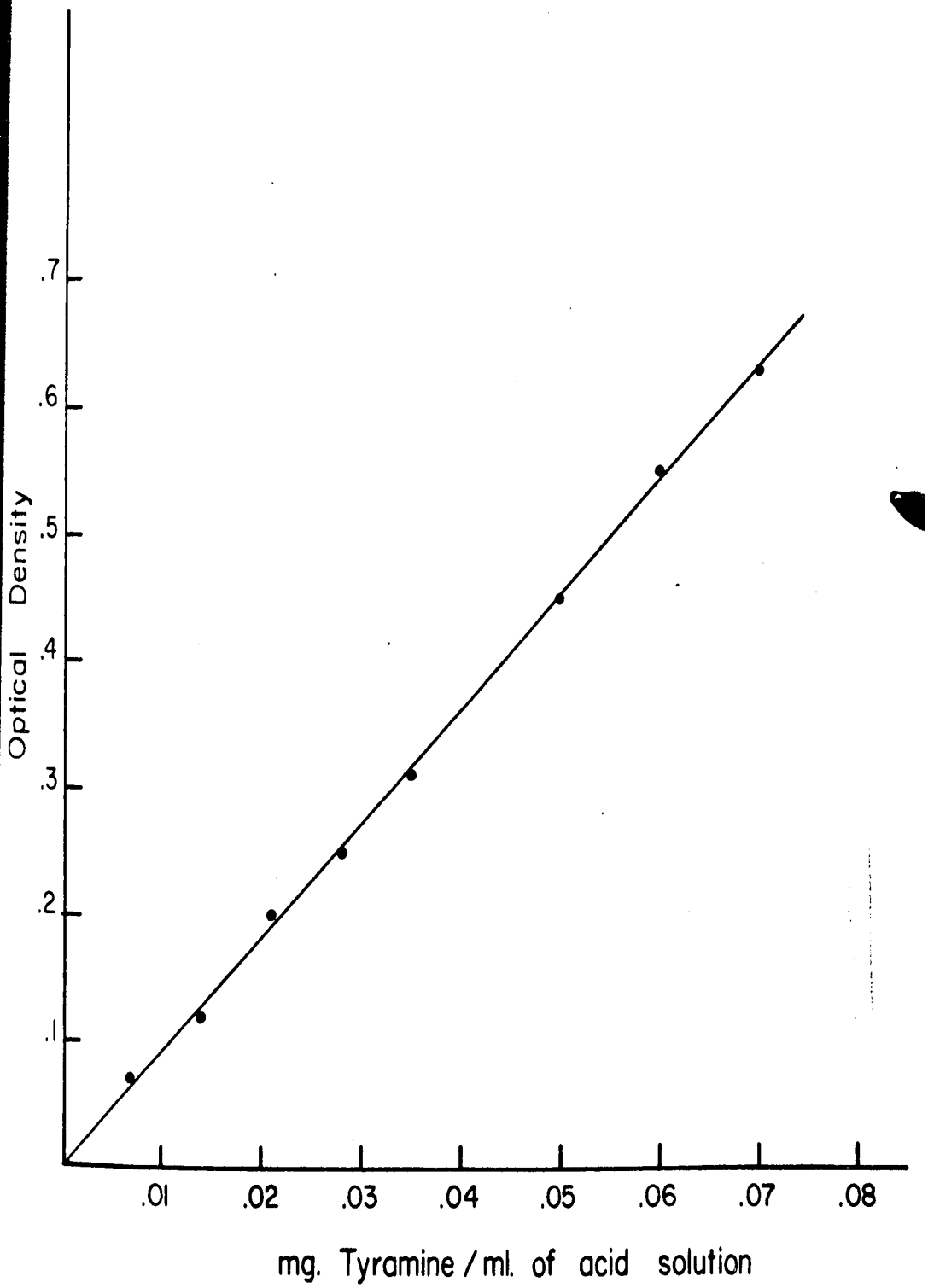
RESULTS

Since the results of MAO and COMT assay procedures rely heavily on the 1-nitroso-2-naphthol colorimetric method it was thought advisable to first plot a standard curve and observe to what extent Beer's Law is followed with the procedures. Also the standard curve will be used to determine the extent of substrate degradation by the enzymes. Fig. 1 shows that a linear relationship exists between the concentrations of tyramine, that are extracted in acid solution and the optical density.

Figure 1. Standard curve for mg. of tyramine extracted into acid solution vs. optical density, using the 1-nitroso-2-naphthol assay technique.


Optical Density
.7
.6
.5
.4
.3
.2
.1
.01
.02
.03
.04
.05
.06
.07
.08
mg. Tyramine / ml. of acid solution

A. OXIDATION OF DEUTERATED AND NON-DEUTERATED SUBSTRATES BY MAO

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*, (Fig. 4). When synthetically prepared R- and S-α-d-tyramine were incubated with MAO, the results shown in Fig. 2 were obtained. It can be seen that tyramine and S-α-d-tyramine were oxidised at approximately the same rate, whereas R-α-d-tyramine was degraded at half the rate of tyramine. The enzymically prepared enantiomers of α-d-tyramine (Fig. 3) show that the one obtained by decarboxylation of L-tyrosine in D_2O (R-α-d-tyramine) is oxidised 2.3 times slower than the one obtained from decarboxylation of DL-α-d-tyrosine in water (S-α-d-tyramine). The results with rat brain MAO (Fig. 5) show that the brain enzyme displays the same absolute optical specificity as the liver enzyme.

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* α-d_2-tyramine was prepared by J. Moran.

Figure 2. Relative rates of oxidation by rat liver MAO for tyramine, R- and S-α-d-tyramine (synthetic), using the 1-nitroso-2-naphthol technique. The substrate concentration in all cases was 5×10^{-3} moles/litre.

$$\frac{K_H}{K_D} = \frac{\text{tyramine}}{\text{R-α-d-tyramine}} = 2.00$$

$$\frac{K_H}{K_D} = \frac{\text{tyramine}}{\text{S-α-d-tyramine}} = 1.25$$

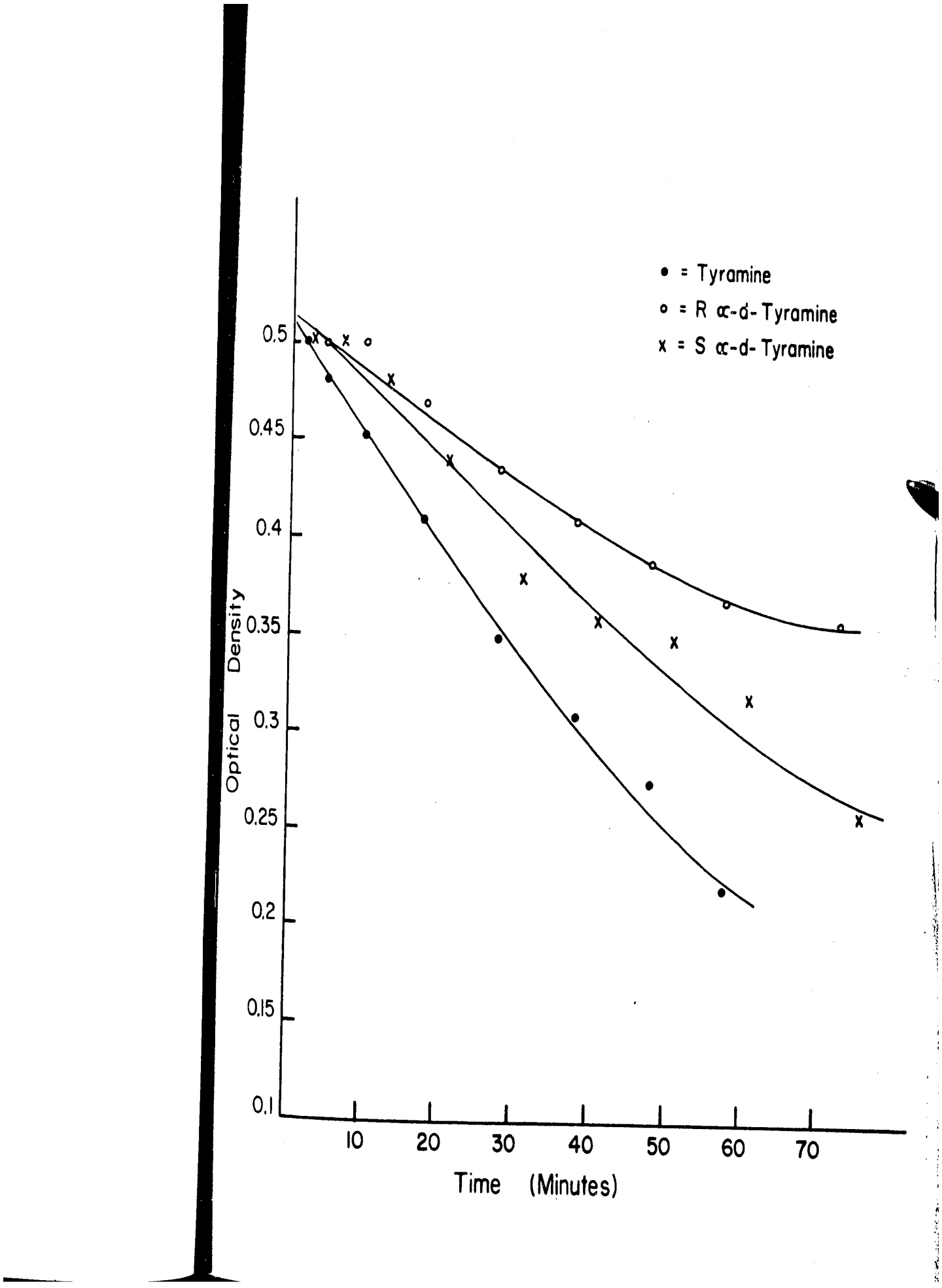

• = Tyramine
o = R α-d-Tyramine
x = S α-d-Tyramine
Optical Density
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
10
20
30
40
50
60
70
Time (Minutes)

Figure 3. Relative rates of oxidation by rat liver MAO for R- and S-α-d-tyramine (enzymically prepared), using the 1-nitroso-2-naphthol technique. The substrate concentration in both cases was 5×10^{-3} moles/litre.

$$\frac{K_H}{K_D} = \frac{\text{S-α-d-tyramine}}{\text{R-α-d-tyramine}} = 2.3$$

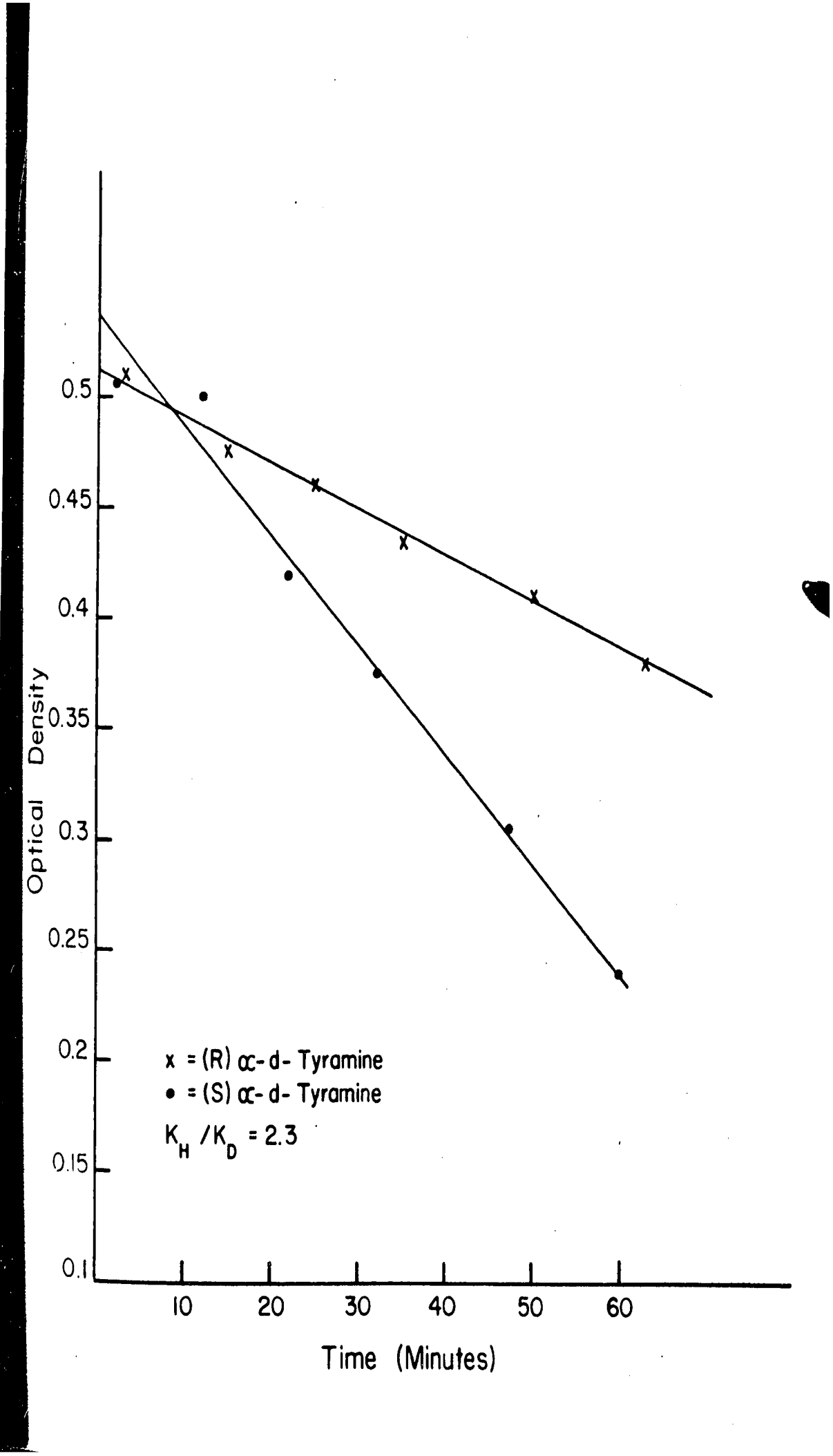

0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
Optical Density
x = (R) α- d- Tyramine
• = (S) α- d- Tyramine
K_H / K_D = 2.3
10
20
30
40
50
60
Time (Minutes)

Figure 4. Relative rates of oxidation by rat liver MAO for tyramine and α-d_2-tyramine, using the 1-nitroso-2-naphthol technique. The substrate concentration in both cases was 5×10^{-3} moles/litre.

$$\frac{K_H}{K_D} = 2.3$$

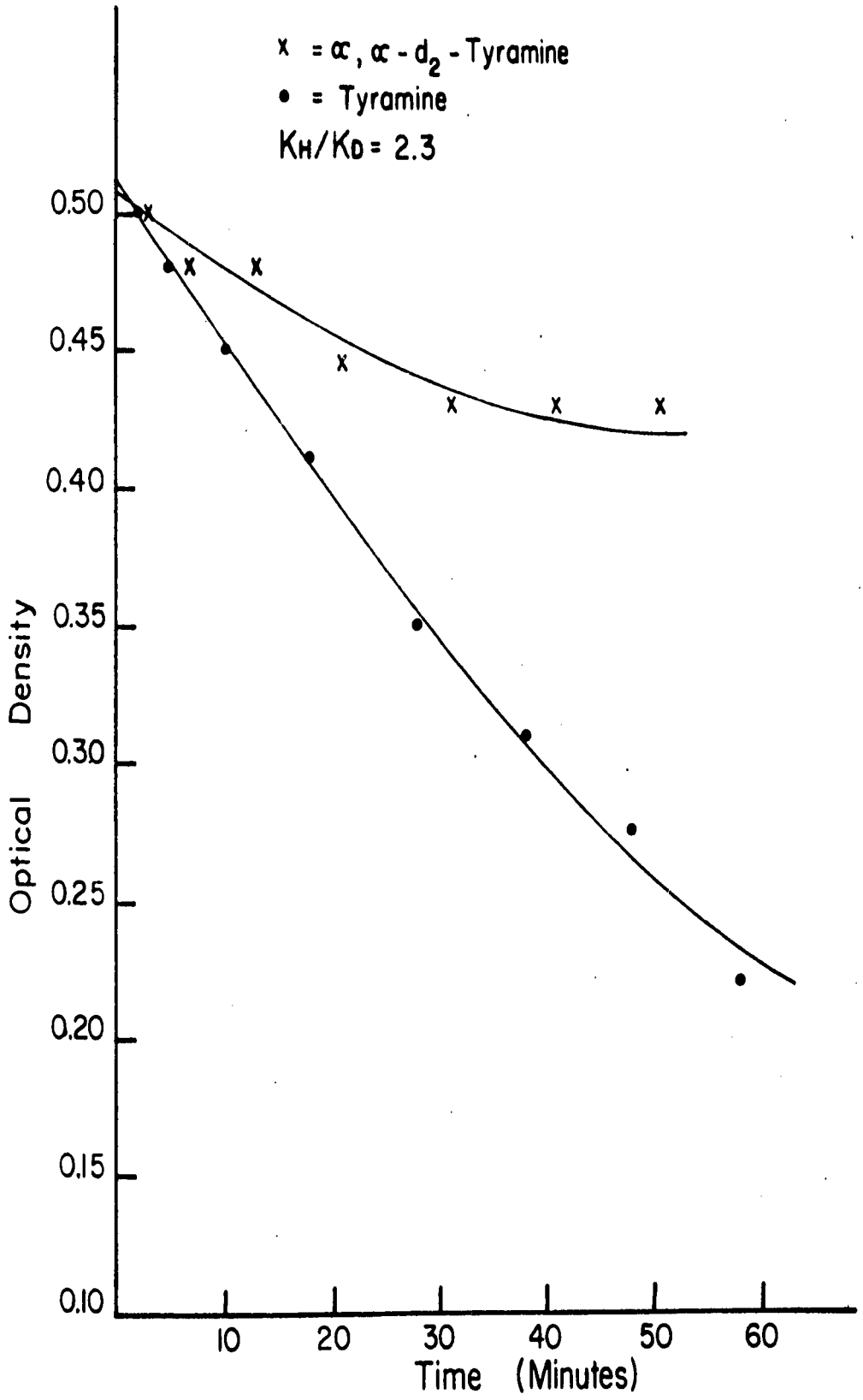
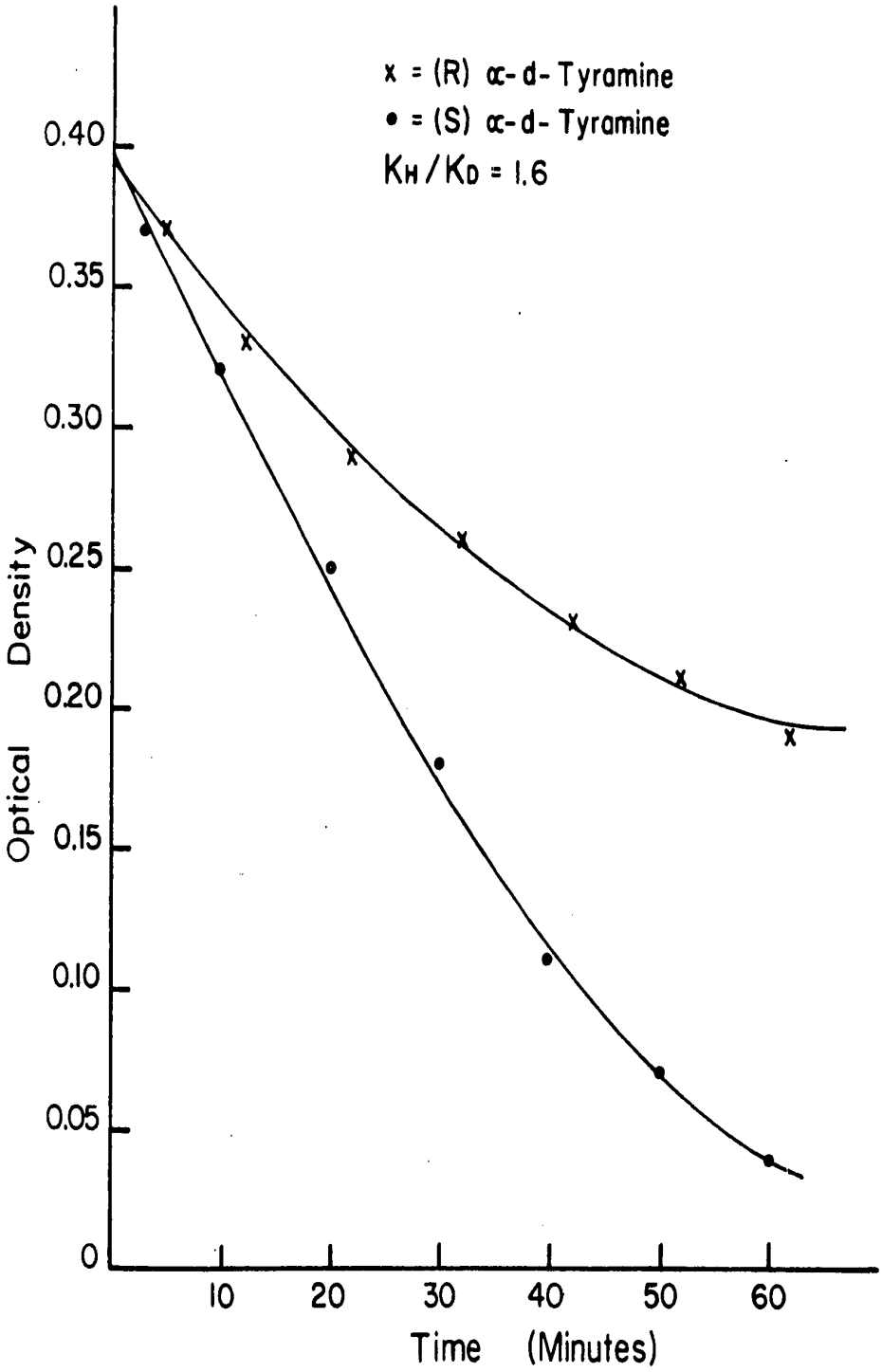

x = α, α - d2 - Tyramine
• = Tyramine
KH/KD = 2.3
Optical Density
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
10
20
30
40
50
60
Time (Minutes)

Figure 5. Relative rates of oxidation by rat <u>brain</u> MAO for R- and S-α-d-tyramine (enzymically prepared) using the 1-nitroso-2-naphthol technique. The substrate concentration in both cases was 2.5×10^{-3} moles/litre.

$$\frac{K_H}{K_D} = \frac{\text{S-α-d-tyramine}}{\text{R-α-d-tyramine}} = 1.6$$


x = (R) α-d-Tyramine
• = (S) α-d-Tyramine
KH / KD = 1.6
Optical Density
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
10
20
30
40
50
60
Time (Minutes)

B. RESULTS OF DEUTERATED SYMPATHOMIMETIC AMINES ON ADRENERGIC RESPONSES (IN VIVO)*

Pentobarbitalized cats were prepared for recording blood pressure and nictitating membrane contractions in the conventional manner. Tyramine and α-d_2-tyramine (98 percent isotopic purity) ((XLIX) p. 27, $R_1 = R_2 = D$) were administered intravenously in equivalent doses. Figure 6 illustrates the effects of the two materials on arterial pressure and membrane contraction in typical experiments. Table 1 summarizes the results of all studies carried out. The magnitude of the pressor response was essentially the same for both compounds, but the duration of the effect (reflected in pressor area) was twice as prolonged with α-d_2-tyramine (statistical analysis giving a p value < 0.001). Both the magnitude and duration of the nictitating membrane contractions were twice as large after administration of α-d_2-tyramine as with tyramine ($p < 0.001$). The greater magnitude of contraction observed in the latter instance is probably related to the lack of compensatory mechanisms such as exists for blood pressure maintenance. When α-d_2-tryptamine (synthetized previously in our laboratories) was compared with tryptamine under the

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* We are deeply indebted to Dr. M. Pindell for pharmacological data.

TABLE 1

Comparison of tyramine and α-d_2-tyramine on sympathetic receptors in the cat

	No. tests	Intravenous dose (μg/kg)	Mean maximum blood pressure increase (mm-Hg)	Mean pressor area*	Mean nictitating membrane contraction	
					Maximum	Area$_{50}$*#
Tyramine	17	800	82	142	11	54
α-d_2-tyramine	9	800	84	291	22	179
Ratio: α-d_2-tyramine/tyramine				2.1	2.0	3.3
p			0.75	< 0.001	< 0.001	< 0.001

* Areas determined with planimeter.

\# Areas under curve to 50 percent recovery.

same conditions, an identical pattern of differences was observed. However, when l-bis-α-deuteronoradrenaline (see "Experimental" for synthesis) was compared to l-noradrenaline, no difference whatsoever could be observed in their potencies [see Fig. 6 (1.-A, 1.-B)].

1. STEREOSPECIFICITY OF THE DEUTERIUM ISOTOPE EFFECTS IN VIVO

In view of the above results, it was of considerable interest to examine the possibility that the observed isotope effects may be stereospecific, that is, assuming a three-point contact between substrate and enzyme or receptor only one of the two possible optical isomers of α-d-tyramine ((XLIX) p. 27, R_1 = H; R_2 = D; and (XLIX), R_1 = D; R_2 = H) may be responsible for the isotope effect observed with the bisdeuteric amine. Both optical isomers of α-d-tyramine were prepared enzymically from tyrosine (see "Experimental") and assayed as above. The results are shown in Fig. 6 (2.-C and 4.-B)*, and clearly establish that the isotope effect is

* The unusual blood pressure effect of the enzymically prepared tyramine (Fig. 6, 2.-C) has been traced to the presence of a minute amount of a phenolic impurity which is formed when oxygen is not excluded from the incubation mixture. A purely synthetic sample of optically active α-d-tyramine produced a typical blood pressure response (Fig. 6, 3.-B).

completely stereospecific in accordance with a three-point contact between tyramine and MAO. The absolute dependence on configuration of the isotope effect on the nictitating membrane response fully agrees with our deductions based on <u>in vitro</u> studies with liver MAO (see Fig. 3).

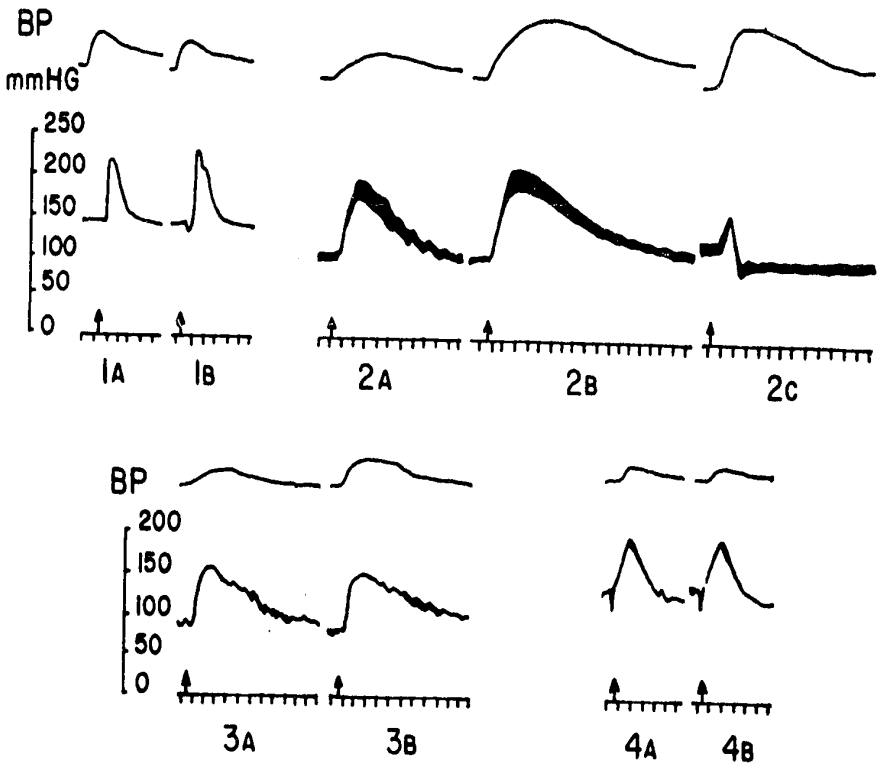


Figure 6. Effects of tyramine and bis-α,α-deuteriotyramine on arterial pressure and membrane contraction. Female cats (3.0 to 3.5 kg.), under pentobarbital anesthesia were used. From top to bottom (1, 2, 3 and 4): isotonic contraction of nictitating membrane, arterial blood pressure, time in minutes. Test compound given at arrows (intravenously): noradrenaline, 16 µg/kg., all tyramine compounds, 800 µg/kg.

1.-Cat 3.1 kg: A.- l-noradrenaline, B.- l-bis-α-deuterio-noradrenaline.
2.-Cat 3.0 kg: A.- tyramine; B.- bis-α,α-deuteriotyramine; C.- α-monodeuteriotyramine (prepared enzymically).
3.- Cat 3.5 kg: A.- tyramine; B.- α-monodeuteriotyramine (same isomer as in 2.-C but prepared synthetically).
4.-Cat 3.2 kg: A.- tyramine; B.- α-monodeuteriotyramine (opposite isomer of that shown in 2.-C and 3.-B, prepared enzymically).

C. RESULTS WITH COMT INHIBITORS

COMT inhibition studies with various analogues of tropolone at a concentration of 3×10^{-4} M show no great differences in potency (Table 2 and Figures 8, 13, 14, 15, 16, 17, 18, 19 and 20). It is noted that 1-methylamino-7-thioxo 1,3,5 cycloheptatriene is the weakest inhibitor as might be expected (see "Discussion" on COMT), while colchicine is not an inhibitor of COMT. Percentage inhibition studies with 4-methyltropolone (Figs. 9, 10 and Table 3) show that an inhibitor concentration of approximately 3×10^{-5} M gives 50% inhibition. The following equation was used to calculate the percentage inhibition:

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

The determination for non-competitive or competitive inhibition at various substrate concentrations and at the same inhibitor concentration is shown in Fig. 11 and Table 4. The Lineweaver-Burk plot (Fig. 12) shows, without a doubt, that inhibition of COMT by 4-methyltropolone is non-competitive.

The results with the rat liver demethylase system (Fig. 21) show that there is no significant demethylation of colchicine to yield colchiceine. Within experimental error, the two curves could be said to be identical.

Job's method of continuous variation[103] used to determine, in this case, whether an excess of metal ion could reverse the inhibition by tropolones gave results as shown in Figs. 22 and 23, (Table 5).

TABLE 2

Percent inhibition with tropolone analogues

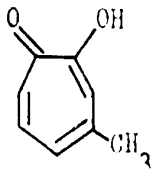
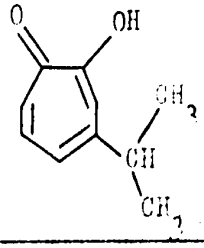
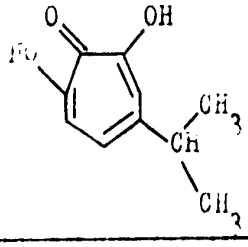
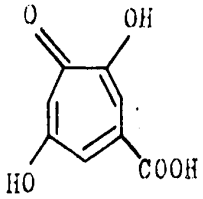
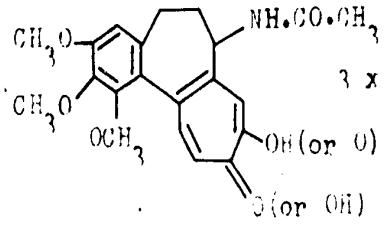
Tropolone analogue	Structure	Concentration	% inhibition
4-Methyl tropolone		3×10^{-4} M	88
β-Thujaplicin		3×10^{-4} M	68
7-Hydroxy-4-isopropyl tropolone		3×10^{-4} M	68
Stipitatic acid		3×10^{-4} M	85
Colchiceine		3×10^{-4} M	82

TABLE 2 (Cont'd.)

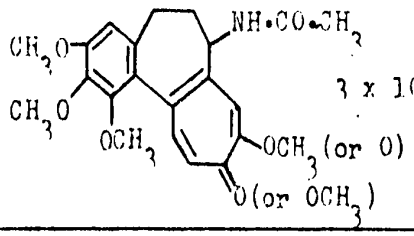
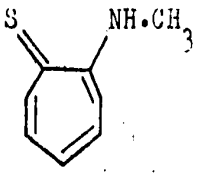
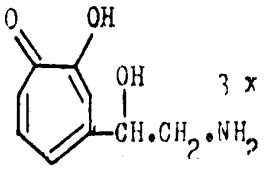
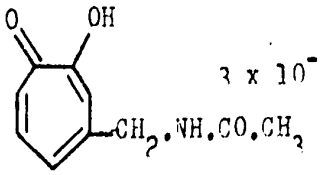
Colchicine		3×10^{-4} M	0
1-Methylamino-7-thioxo 1,3,5 cycloheptatriene		3×10^{-4} M	60
4-Ethanolamino-tropolone		3×10^{-4} M	76
4-Acetamidomethyl-tropolone		3×10^{-4} M	75

TABLE 3

Percent inhibition studies with 4-methyltropolone

Molar concentration of 4-methyltropolone	% Inhibition of COMT
3×10^{-5} M	45
8×10^{-5} M	76
3×10^{-4} M	80

TABLE 4

Data for Lineweaver-Burk plot

Substrate concentration	$\frac{1}{[S]}$ = $\frac{1}{\text{Substrate concentration}}$	$\frac{1}{V}$ = $\frac{1}{\text{Optical density (Initial rate)}}$
NO INHIBITOR		
2×10^{-3} M	500	21.5
5×10^{-3} M	200	17
1×10^{-2} M	100	16.3
4-METHYLTROPOLONE 3×10^{-4} M		
2×10^{-3} M	500	52.8
3×10^{-3} M	333	45.5
5×10^{-3} M	200	40
8×10^{-3} M	125	43.5

TABLE 5

Results of Job's method of continuous variation using a substrate concentration of 1×10^{-3} M

100 µM $MgCl_2 \cdot 6H_2O$ = 100 mole %

1 µM of 4-methyltropolone = 100 mole %

Mole % of 4-methyltropolone	Mole % of Mg^{++}	% inhibition of COMT
0	100	0
10	90	38
25	75	58
50	50	67
75	25	91
100	500	73

These results show that a large excess of Mg^{++} will not reverse inhibition of COMT by tropolones, (see "Discussion").

Figure 7. Standard curve for mg. of normetanephrine extracted into 2 ml. of 0.1 N HCl vs. optical density using the 1-nitroso-2-naphthol assay technique.

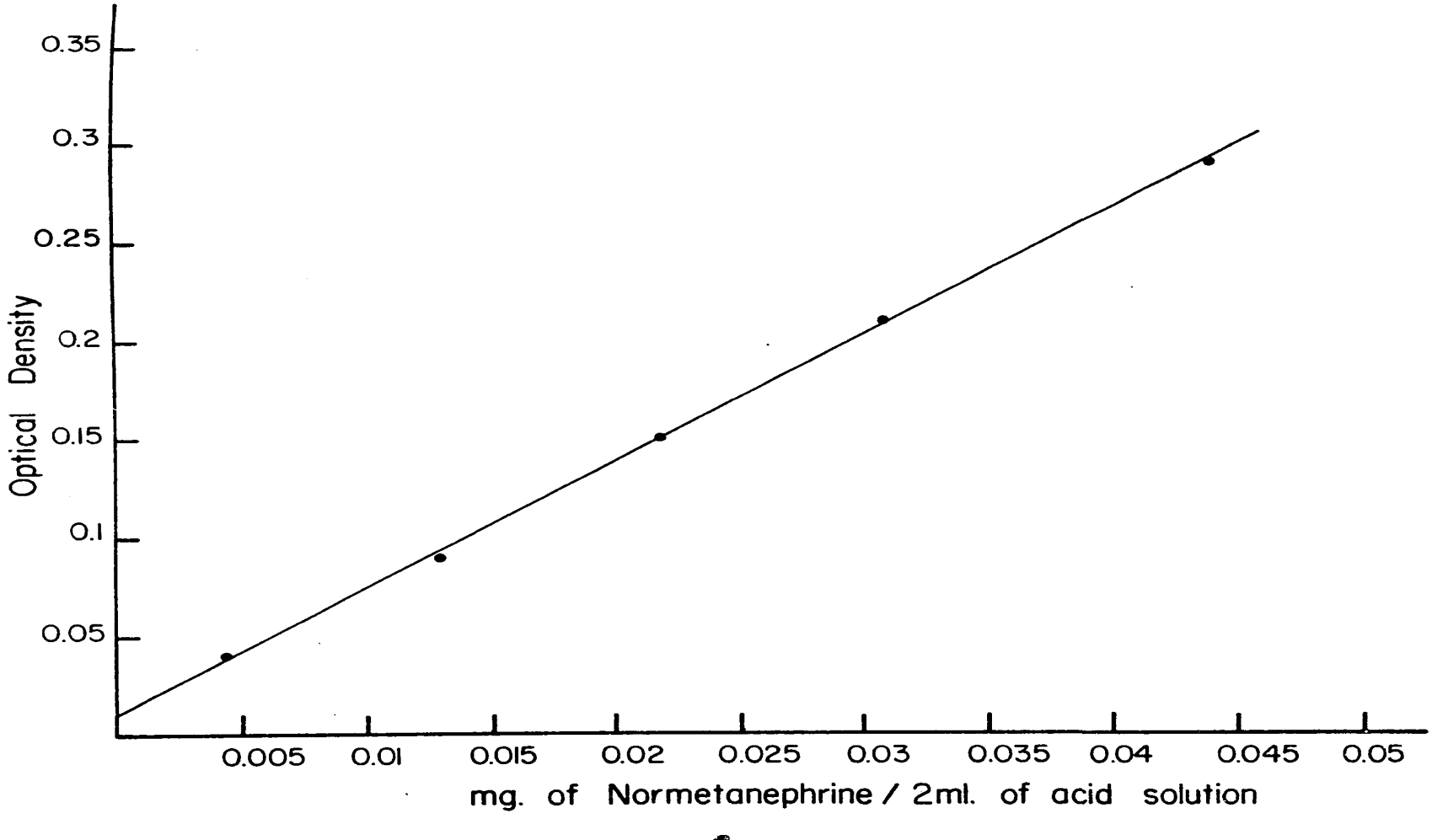

0.35
0.3
0.25
0.2
0.15
0.1
0.05
Optical Density
0.005
0.01
0.015
0.02
0.025
0.03
0.035
0.04
0.045
0.05
mg. of Normetanephrine / 2ml. of acid solution

Figure 8. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without 4-methyltropolone, and with 4-methyltropolone 3×10^{-4} M. The l-nitroso-2-naphthol assay technique is used in this and subsequent COMT studies.

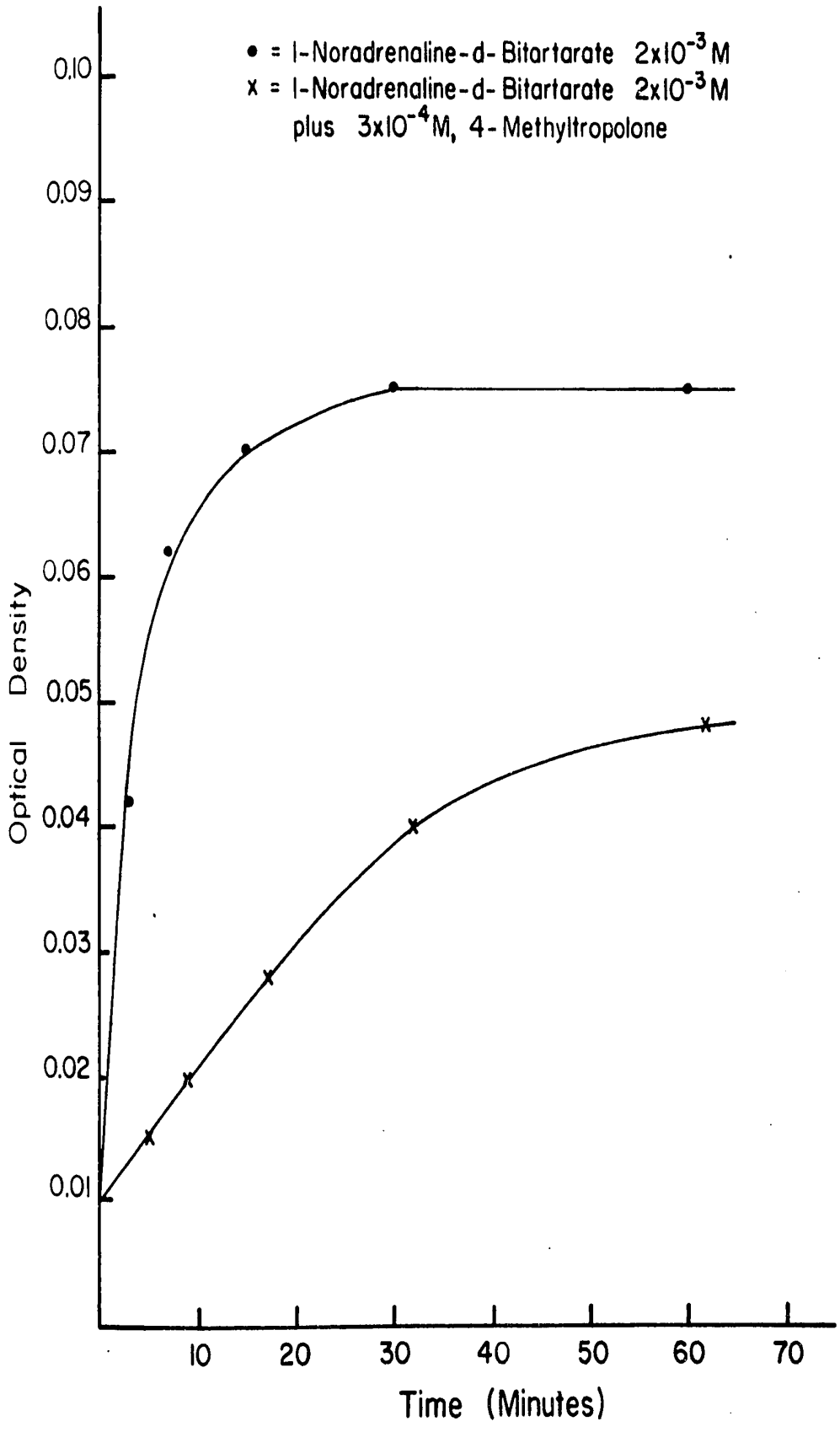

• = l-Noradrenaline-d-Bitartarate 2x10⁻³M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³M
plus 3x10⁻⁴M, 4-Methyltropolone
Optical Density
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
10
20
30
40
50
60
70
Time (Minutes)

Figure 9. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2 x 10^{-3} M without 4-methyltropolone and with varying concentrations of 4-methyltropolone.

(A) = No inhibitor

(B) = 4-Methyltropolone 3 x 10^{-5} M

(C) = 4-Methyltropolone 6 x 10^{-5} M

(D) = 4-Methyltropolone 3 x 10^{-4} M

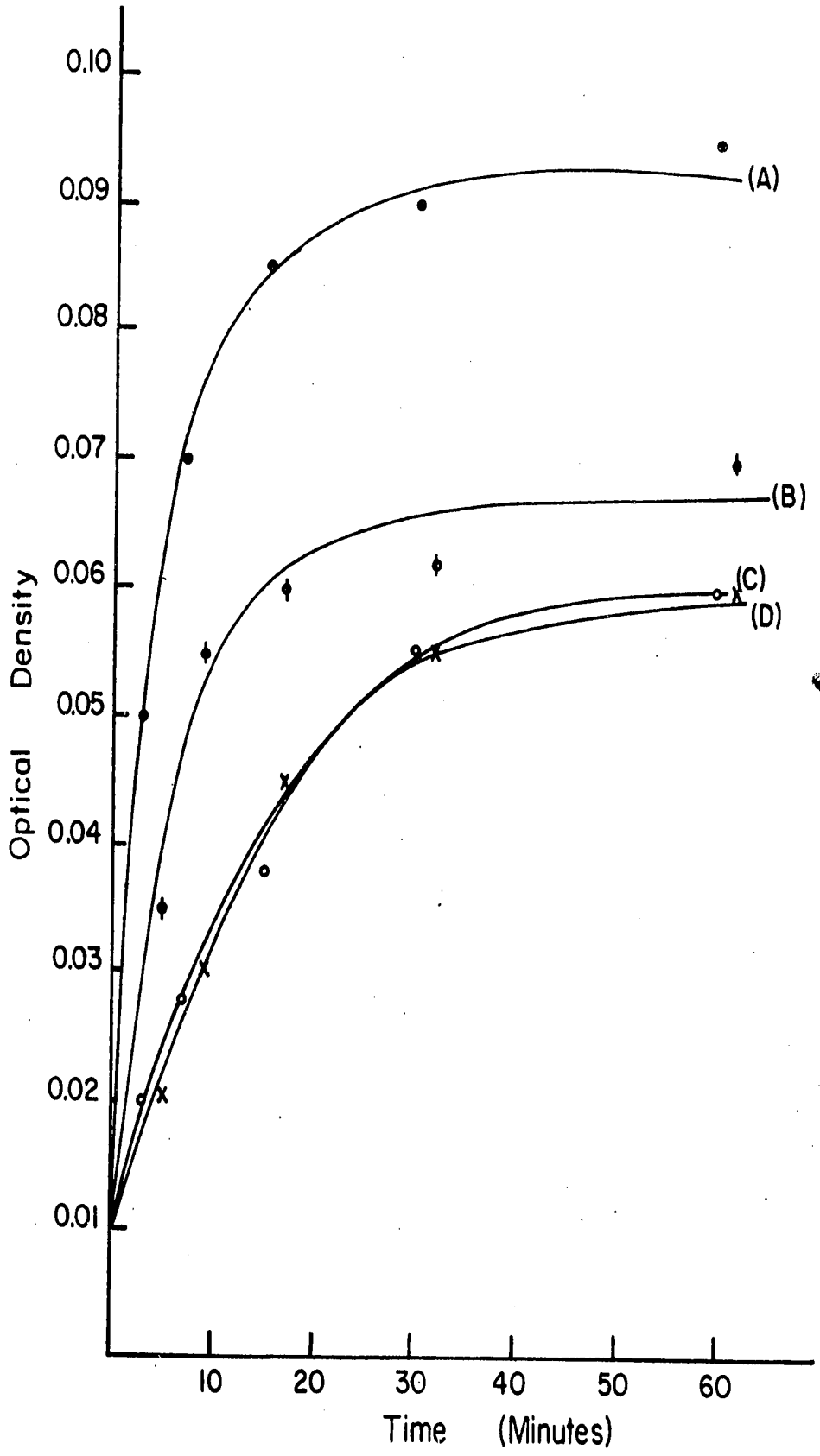

0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
(A)
(B)
(C)
(D)
10
20
30
40
50
60
Time (Minutes)

Figure 10. Percent inhibition versus log of the concentration of 4-methyltropolone. The substrate (l-noradrenaline-d-bitartarate) concentration was 2×10^{-3} M.

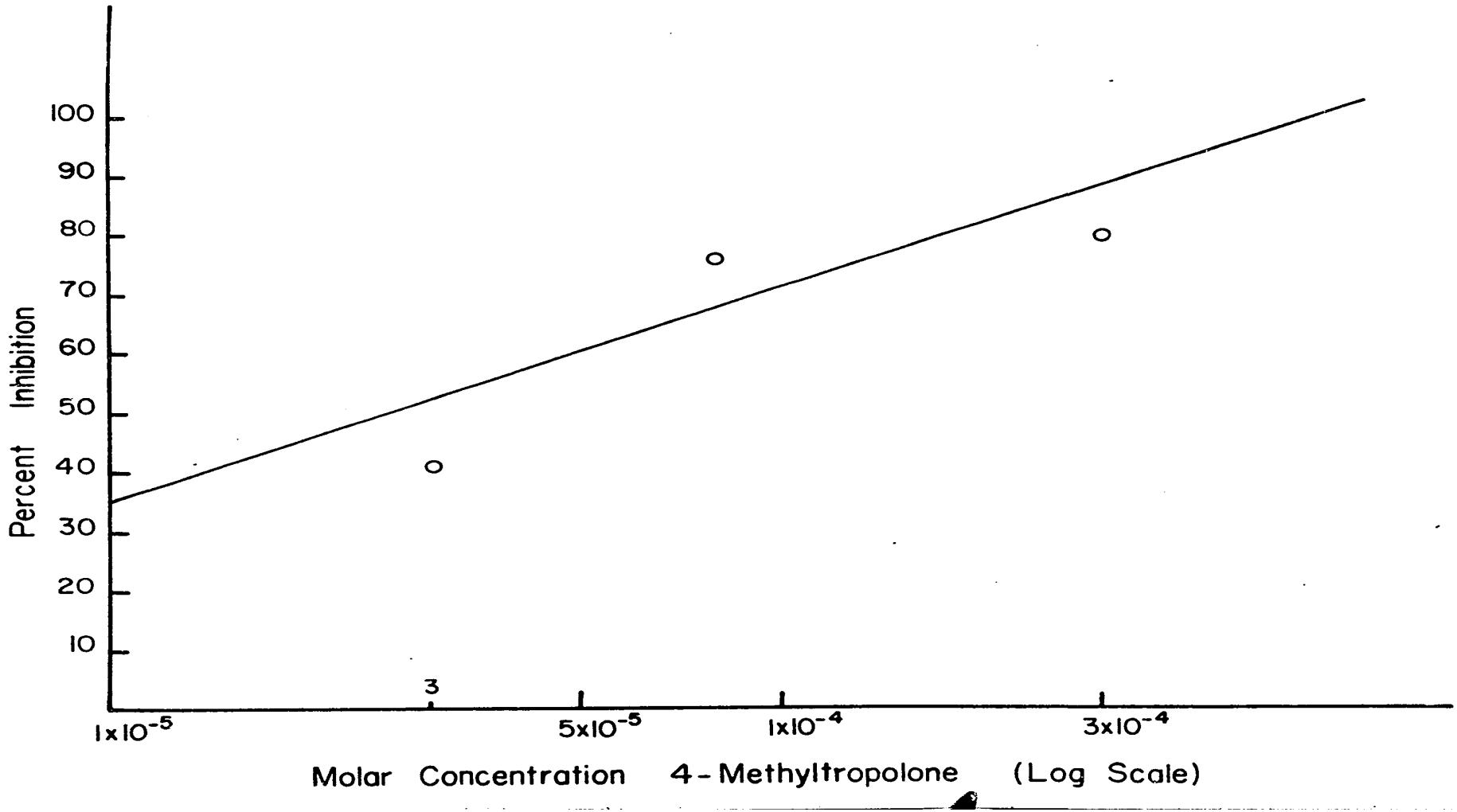

Percent Inhibition
100
90
80
70
60
50
40
30
20
10
3
1x10-5
5x10-5
1x10-4
3x10-4
Molar Concentration 4-Methyltropolone (Log Scale)

Figure 11. Relative rates of O-methylation at various substrate (l-noradrenaline-d-bitartarate) concentrations and no inhibitor (4-methyltropolone). Also relative rates of O-methylation at various substrate concentrations plus 4-methyltropolone 3×10^{-4} M.

(A) = l-noradrenaline-d-bitartarate 1×10^{-2} M

(B) = l-noradrenaline-d-bitartarate 5×10^{-3} M

(C) = l-noradrenaline-d-bitartarate 2×10^{-3} M

(D) = l-noradrenaline-d-bitartarate 8×10^{-3} M plus 4-methyltropolone 3×10^{-4} M

(E) = l-noradrenaline-d-bitartarate 5×10^{-3} M plus 4-methyltropolone 3×10^{-4} M

(F) = l-noradrenaline-d-bitartarate 3×10^{-3} M plus 4-methyltropolone 3×10^{-4} M

(G) = l-noradrenaline-d-bitartarate 2×10^{-3} M plus 4-methyltropolone 3×10^{-4} M.

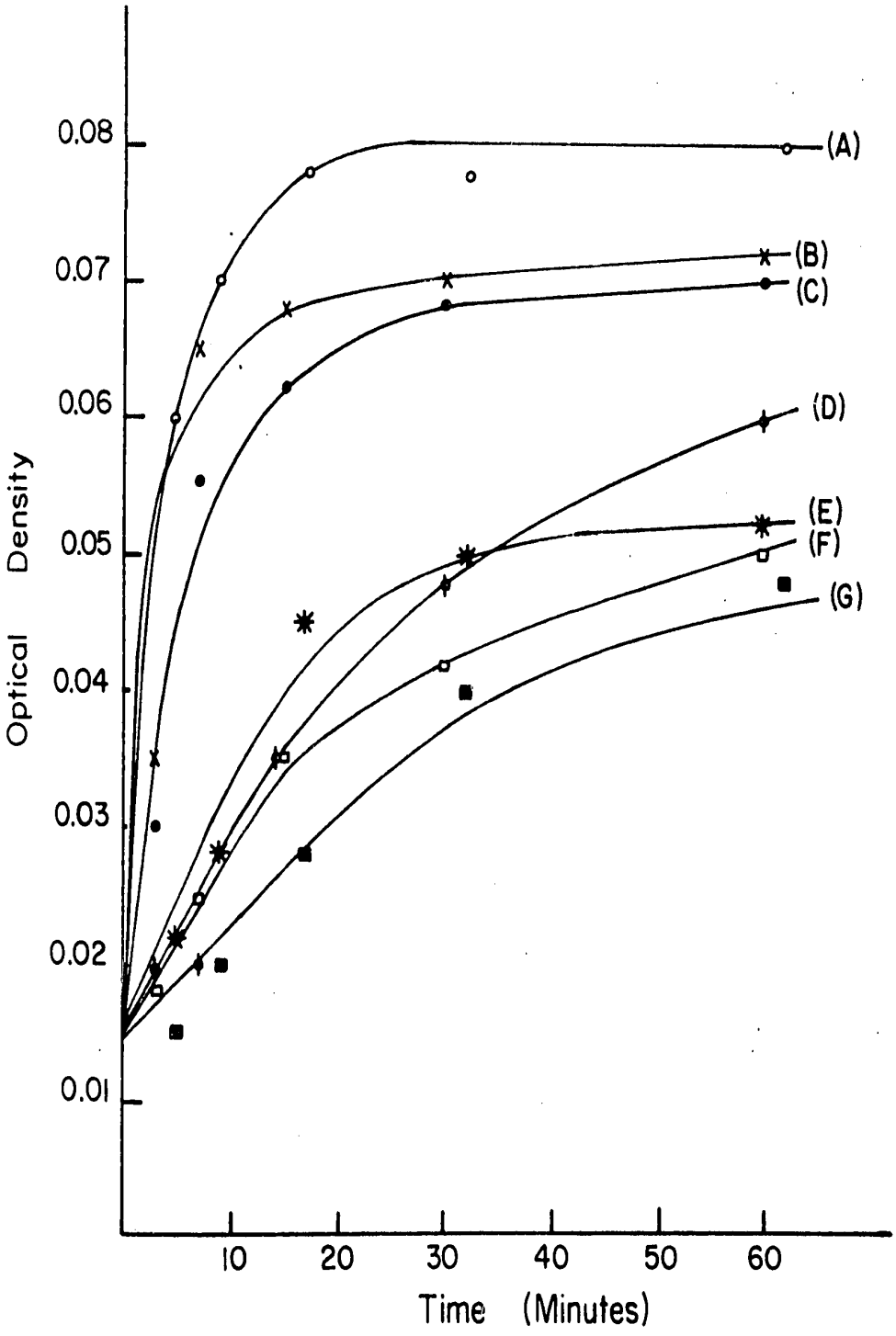

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
(A)
(B)
(C)
(D)
(E)
(F)
(G)
10
20
30
40
50
60
Time (Minutes)

Figure 12. Non-competitive inhibition of COMT by 4-methyltropolone. Data were obtained from Fig. 11. Various concentrations of the substrate were incubated as described in "Experimental". The inhibitor concentration was held constant.

V = change in optical density at 450 mμ after 5 minutes of incubation

[S] = substrate concentration.

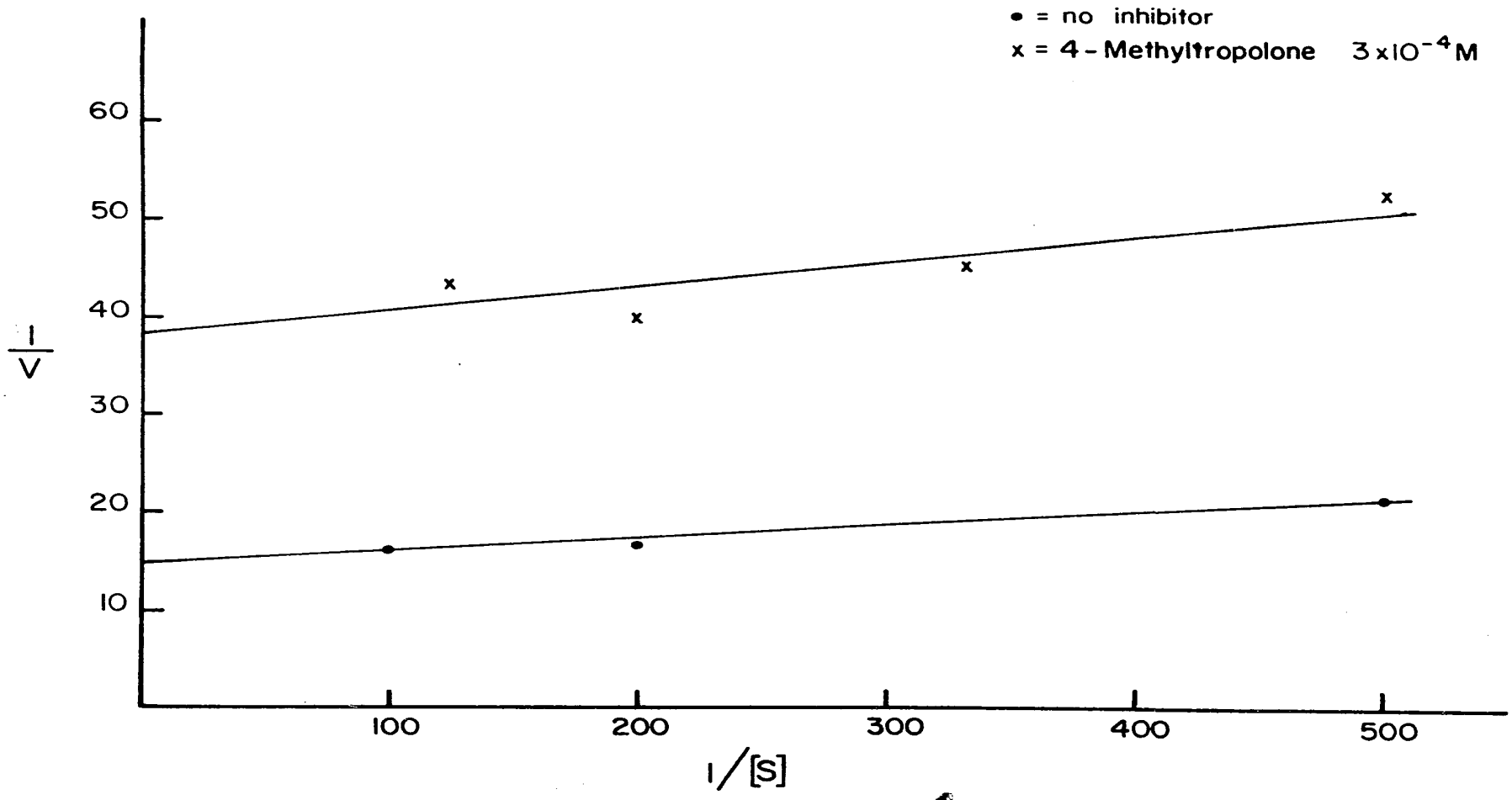

• = no inhibitor
x = 4 - Methyltropolone 3x10⁻⁴M
60
50
40
30
20
10
1/V
100
200
300
400
500
1/[S]

Figure 13. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without β-thujaplicin and with β-thujaplicin 3×10^{-4} M.

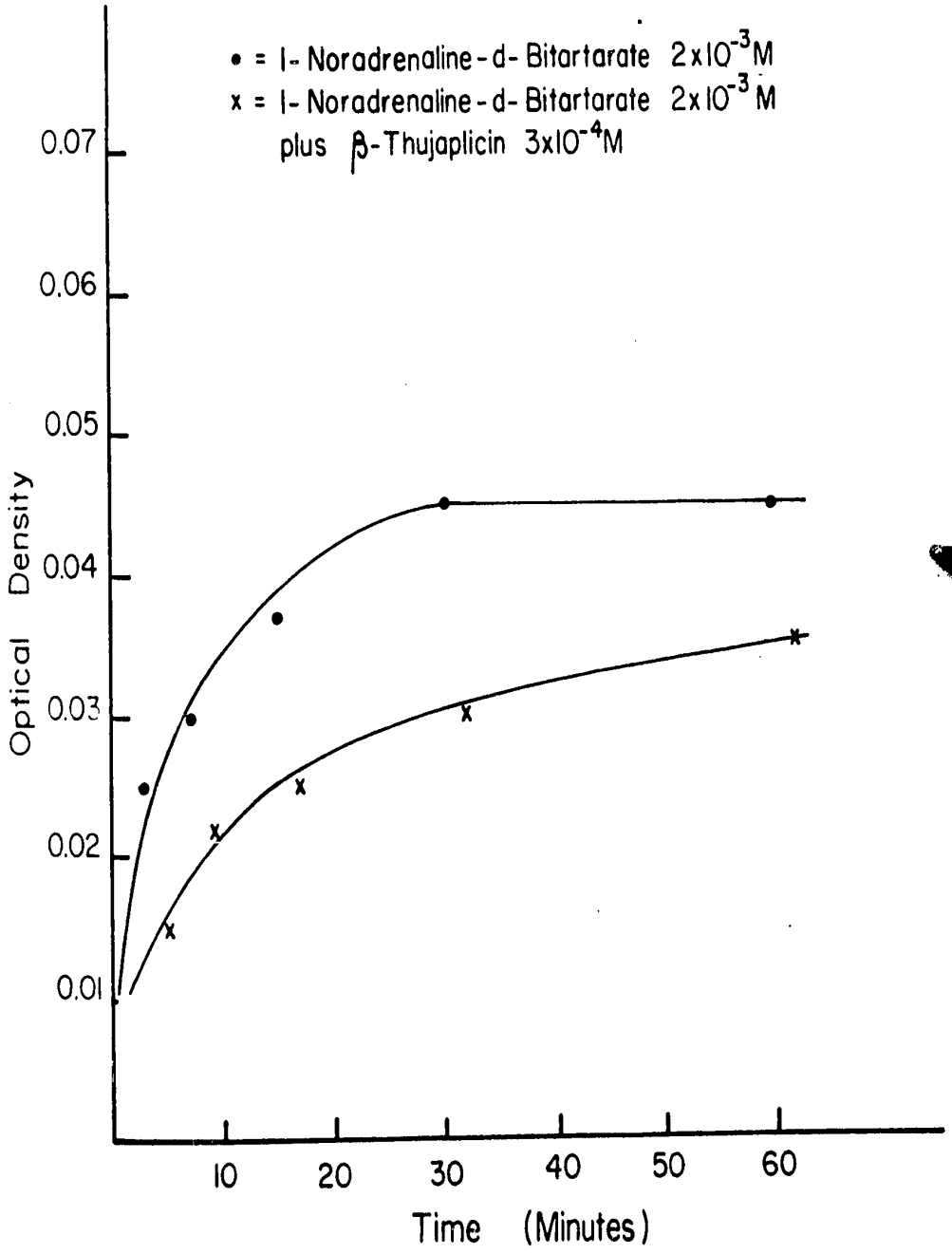

• = l- Noradrenaline - d- Bitartarate 2x10⁻³M
x = l- Noradrenaline - d- Bitartarate 2x10⁻³ M
plus β-Thujaplicin 3x10⁻⁴M
Optical Density
0.07
0.06
0.05
0.04
0.03
0.02
0.01
10
20
30
40
50
60
Time (Minutes)

Figure 14. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without 7-hydroxy-4-isopropyltropolone and with 7-hydroxy-4-isopropyltropolone 3×10^{-4} M.

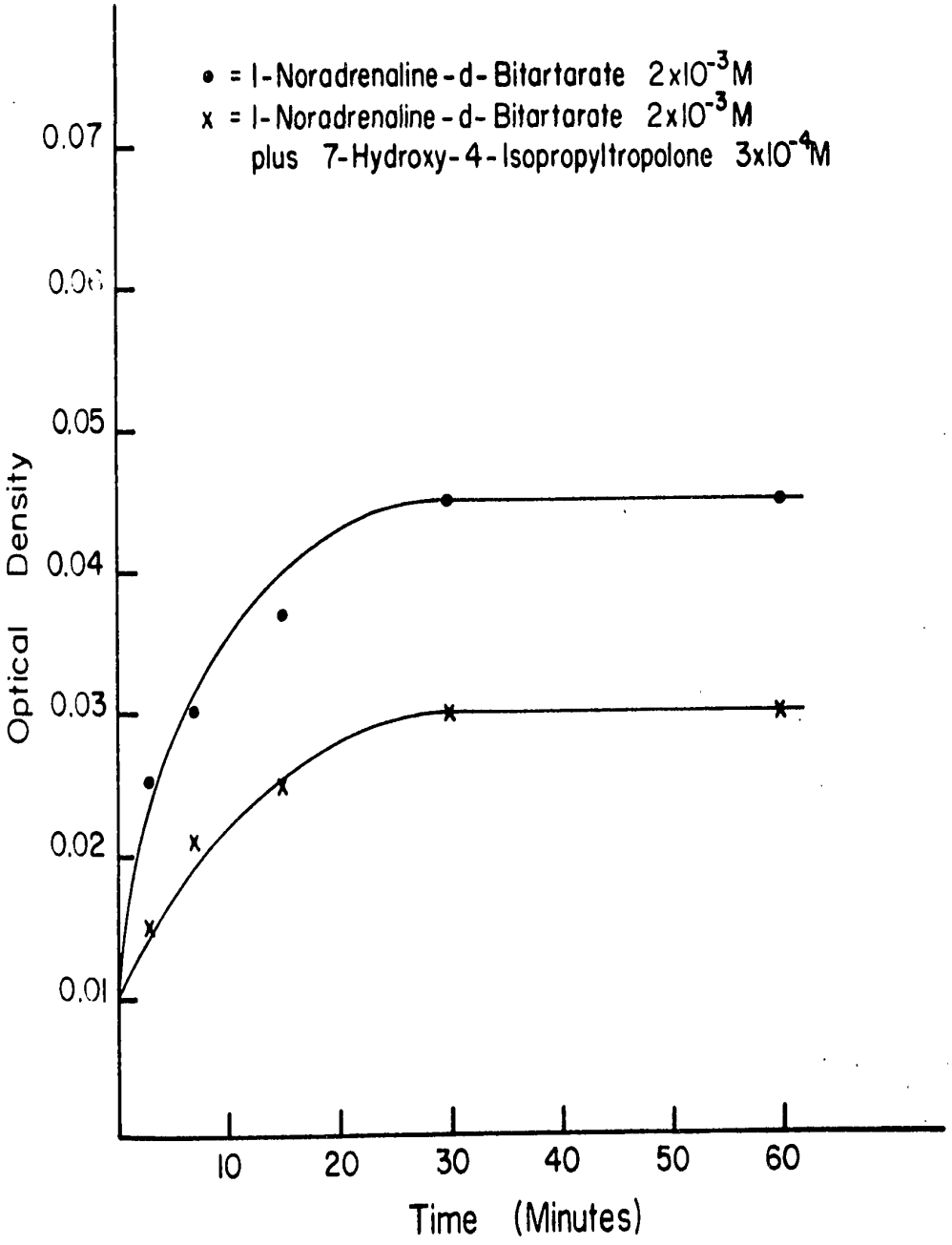

• = l-Noradrenaline-d-Bitartarate 2x10⁻³M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³M
plus 7-Hydroxy-4-Isopropyltropolone 3x10⁻⁴M
Optical Density
0.07
0.06
0.05
0.04
0.03
0.02
0.01
10
20
30
40
50
60
Time (Minutes)

Figure 15. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without stipitatic acid and with stipitatic acid 3×10^{-4} M.

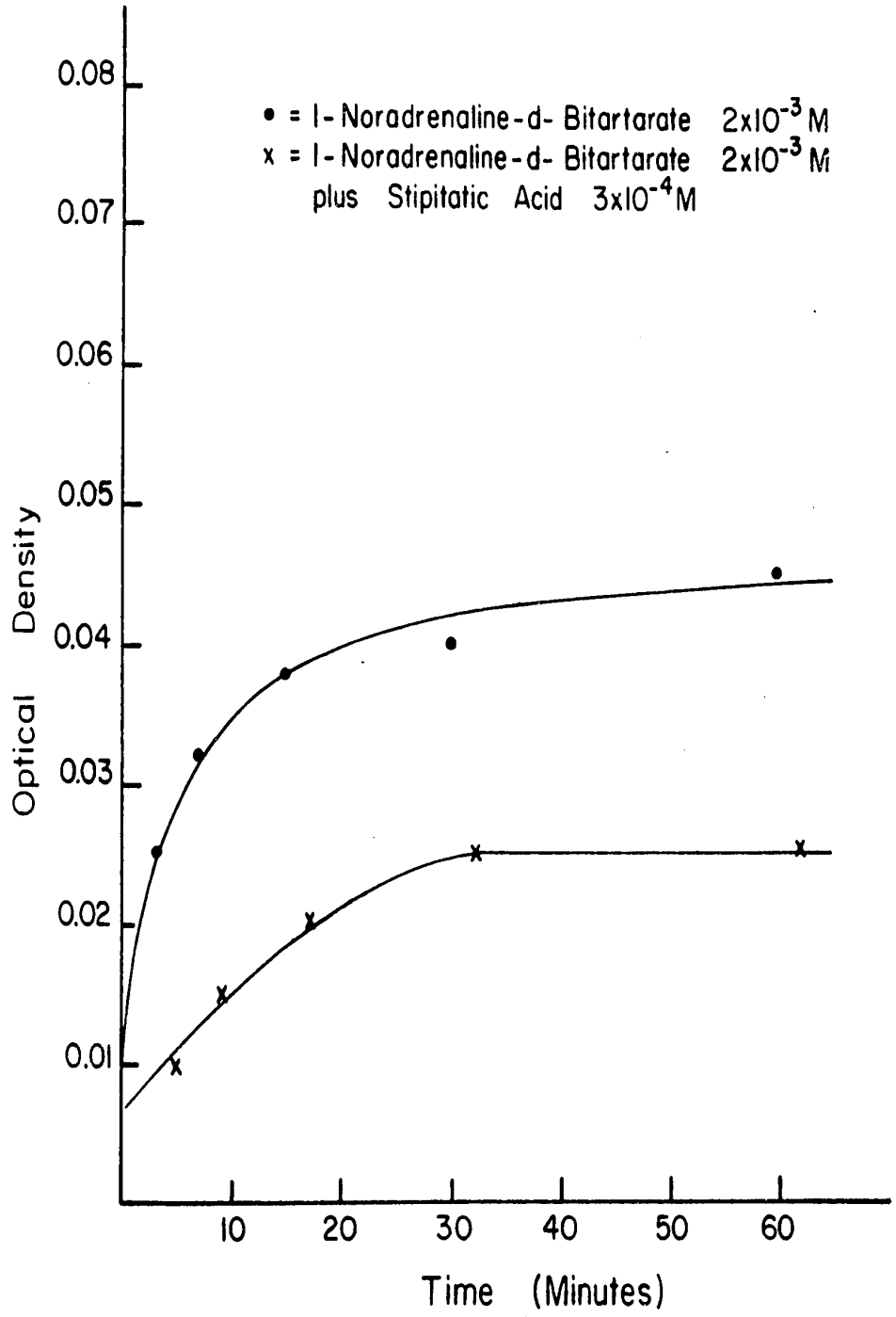

• = l-Noradrenaline-d- Bitartarate 2x10⁻³ M
x = l-Noradrenaline-d- Bitartarate 2x10⁻³ M
plus Stipitatic Acid 3x10⁻⁴ M
Optical Density
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
10
20
30
40
50
60
Time (Minutes)

Figure 16. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without colchiceine, and with colchiceine 3×10^{-4} M.

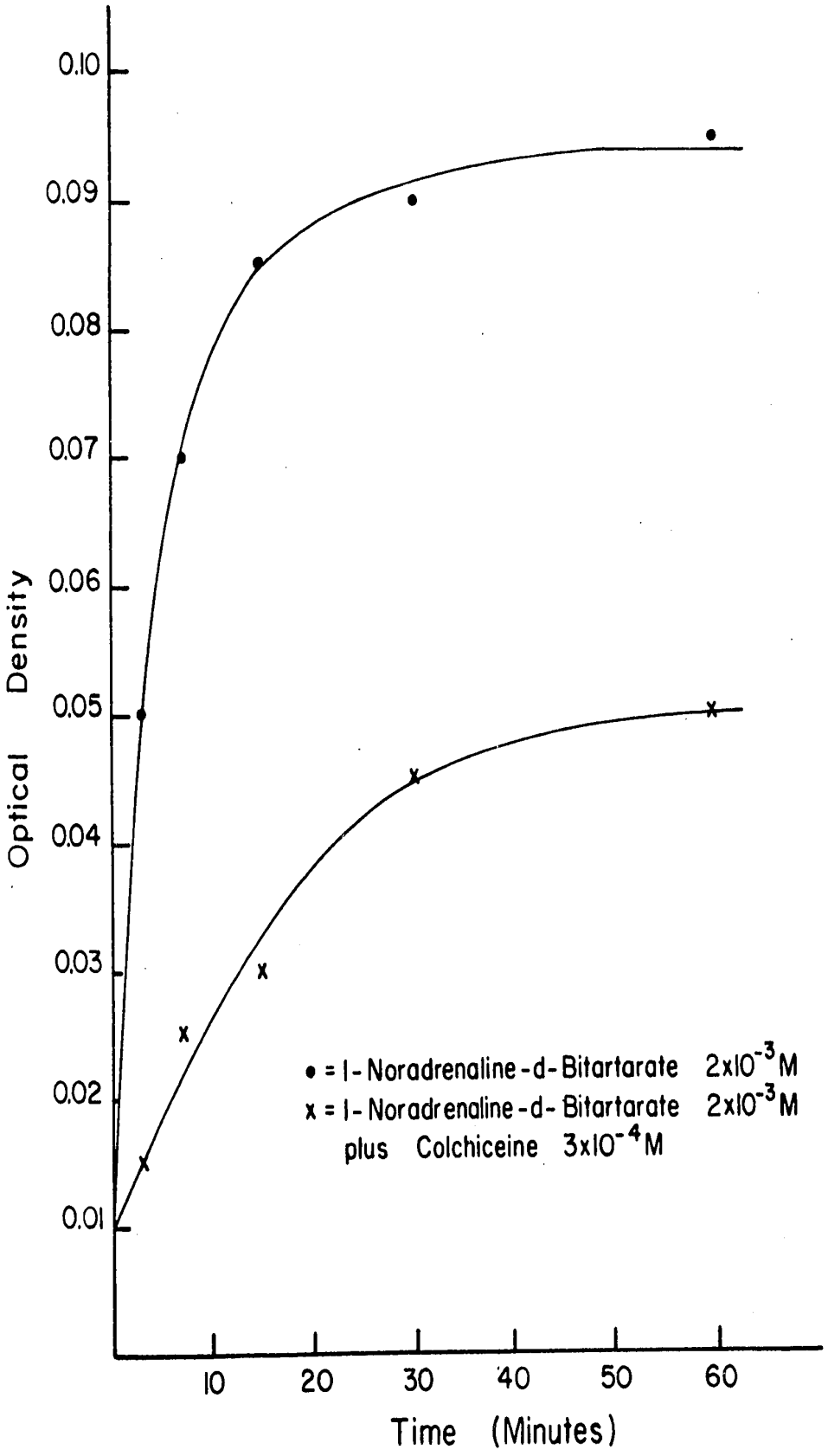

0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
10
20
30
40
50
60
Time (Minutes)
• = l-Noradrenaline-d-Bitartarate 2x10⁻³M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³M
plus Colchiceine 3x10⁻⁴M

Figure 17. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without colchicine and with colchicine 3×10^{-4} M.

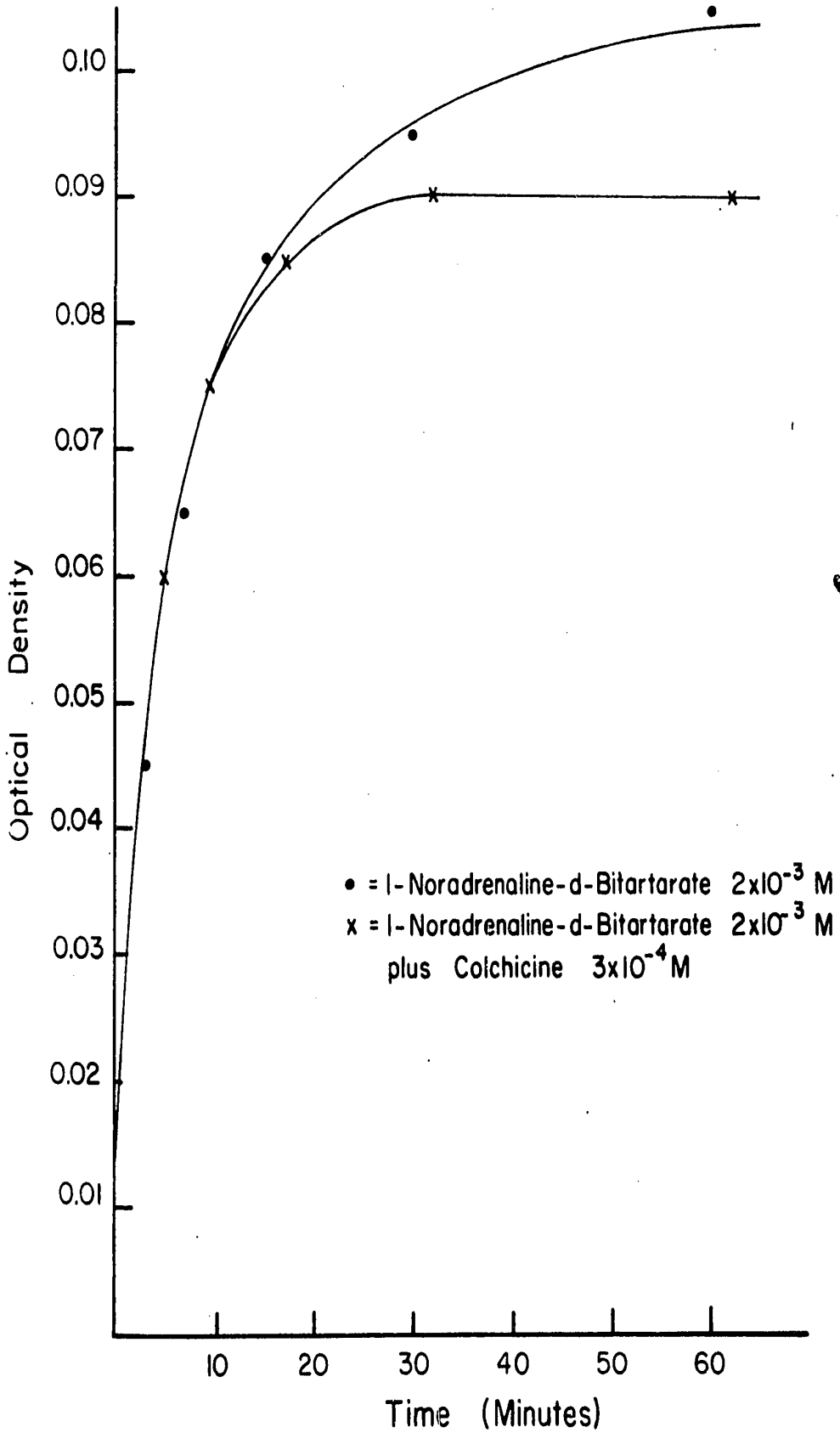

0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
10
20
30
40
50
60
Time (Minutes)
• = l-Noradrenaline-d-Bitartarate 2x10⁻³ M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³ M
plus Colchicine 3x10⁻⁴M

Figure 18. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without l-methylamino-7-thioxo 1,3,5 cycloheptatriene* and with l-methylamino-7-thioxo 1,3,5 cycloheptatriene 3×10^{-4} M.

* We are indebted to Dr. W.H. Brasen of E.I. Du Pont de Nemours and Co. for a generous supply of this compound as well as l-methylamino-7-methylimino 1,3,5 cycloheptatriene.

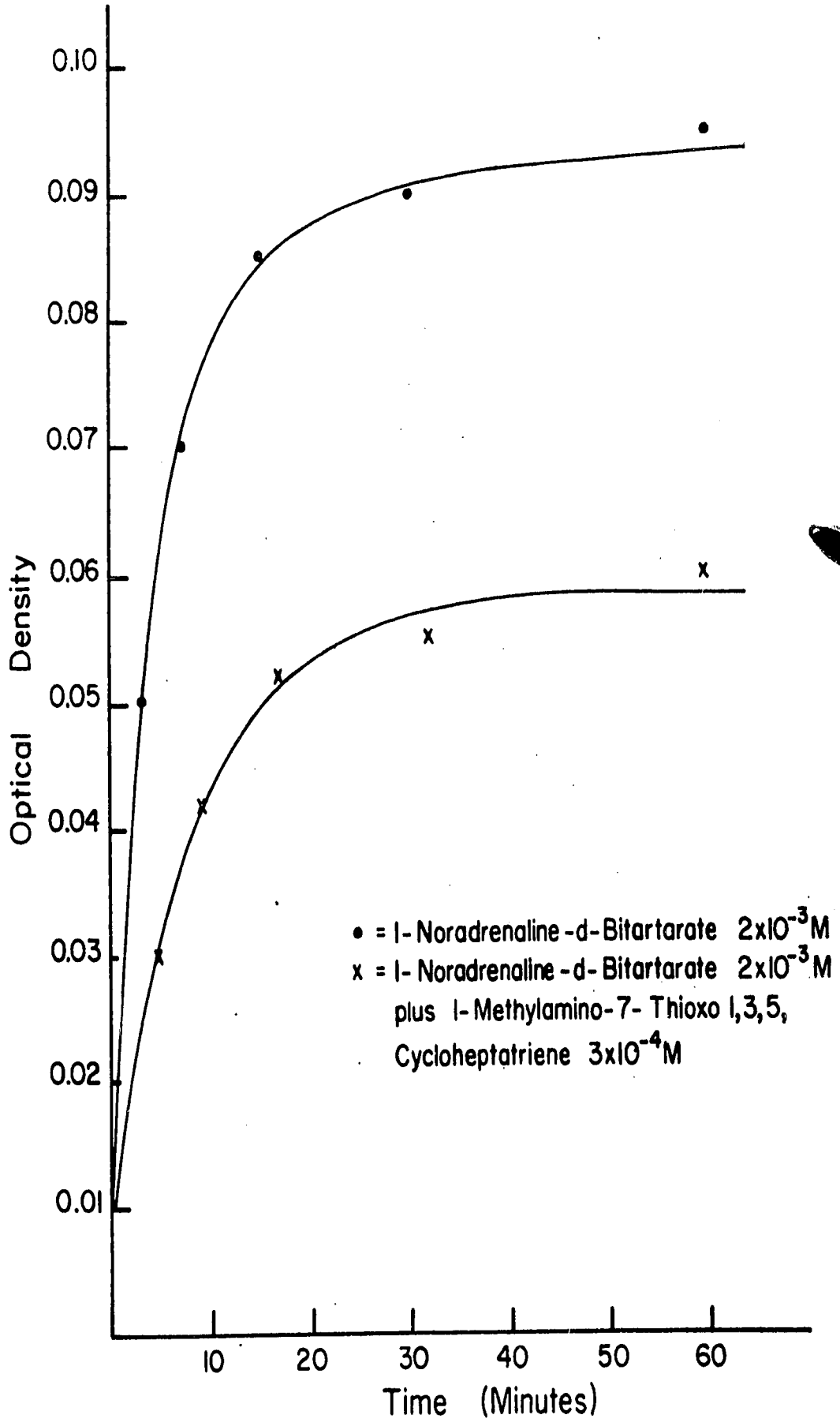

0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
10
20
30
40
50
60
Time (Minutes)
• = l-Noradrenaline-d-Bitartarate 2x10⁻³M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³M
plus l-Methylamino-7-Thioxo 1,3,5,
Cycloheptatriene 3x10⁻⁴M

Figure 19. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without 4-ethanolaminotropolone and with 4-ethanolaminotropolone 3×10^{-4} M.

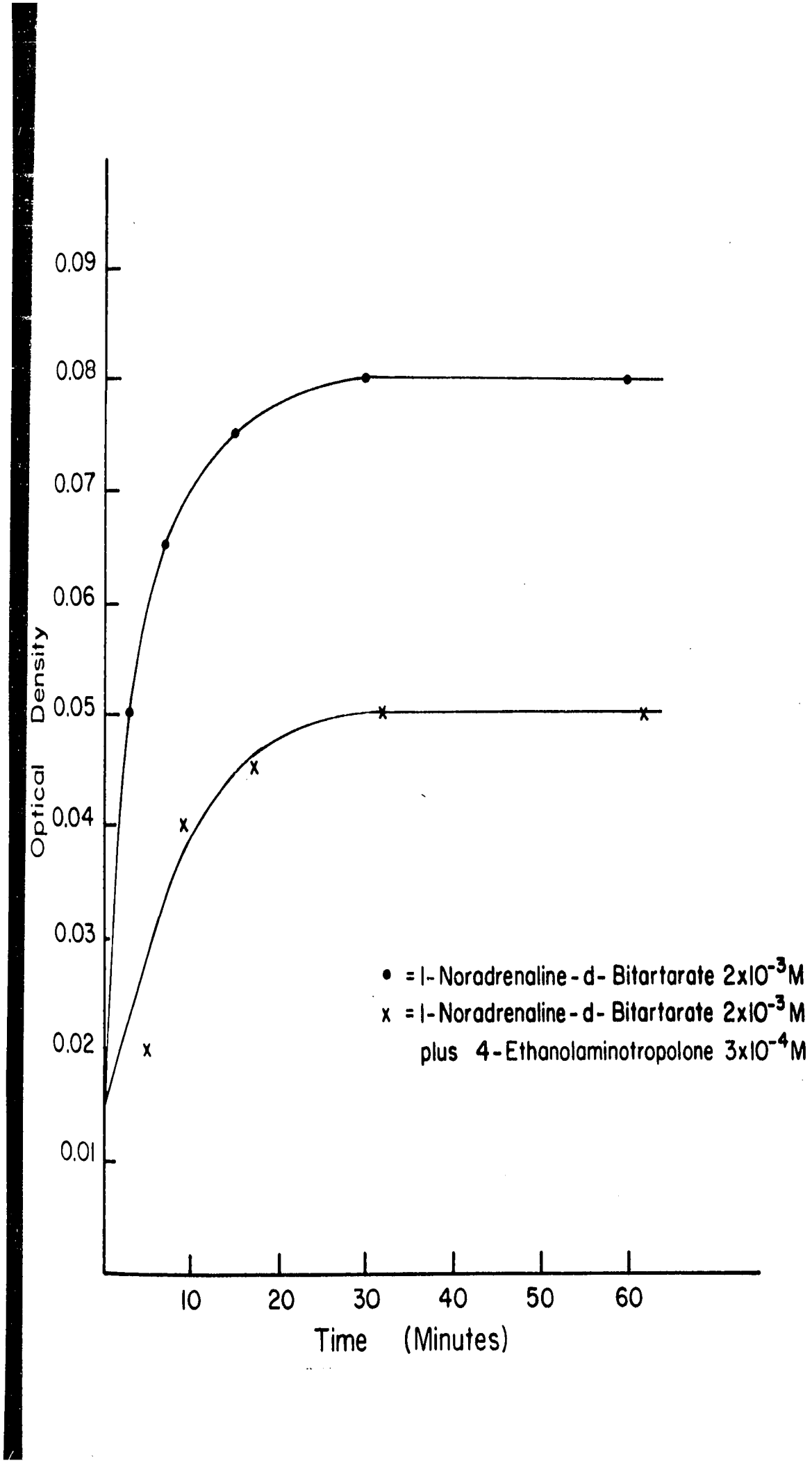

0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
10
20
30
40
50
60
Time (Minutes)
• = l-Noradrenaline-d-Bitartarate 2x10⁻³M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³M
plus 4-Ethanolaminotropolone 3x10⁻⁴M

Figure 20. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2×10^{-3} M without 4-acetamidomethyltropolone and with 4-acetamidomethyltropolone 3×10^{-4} M.

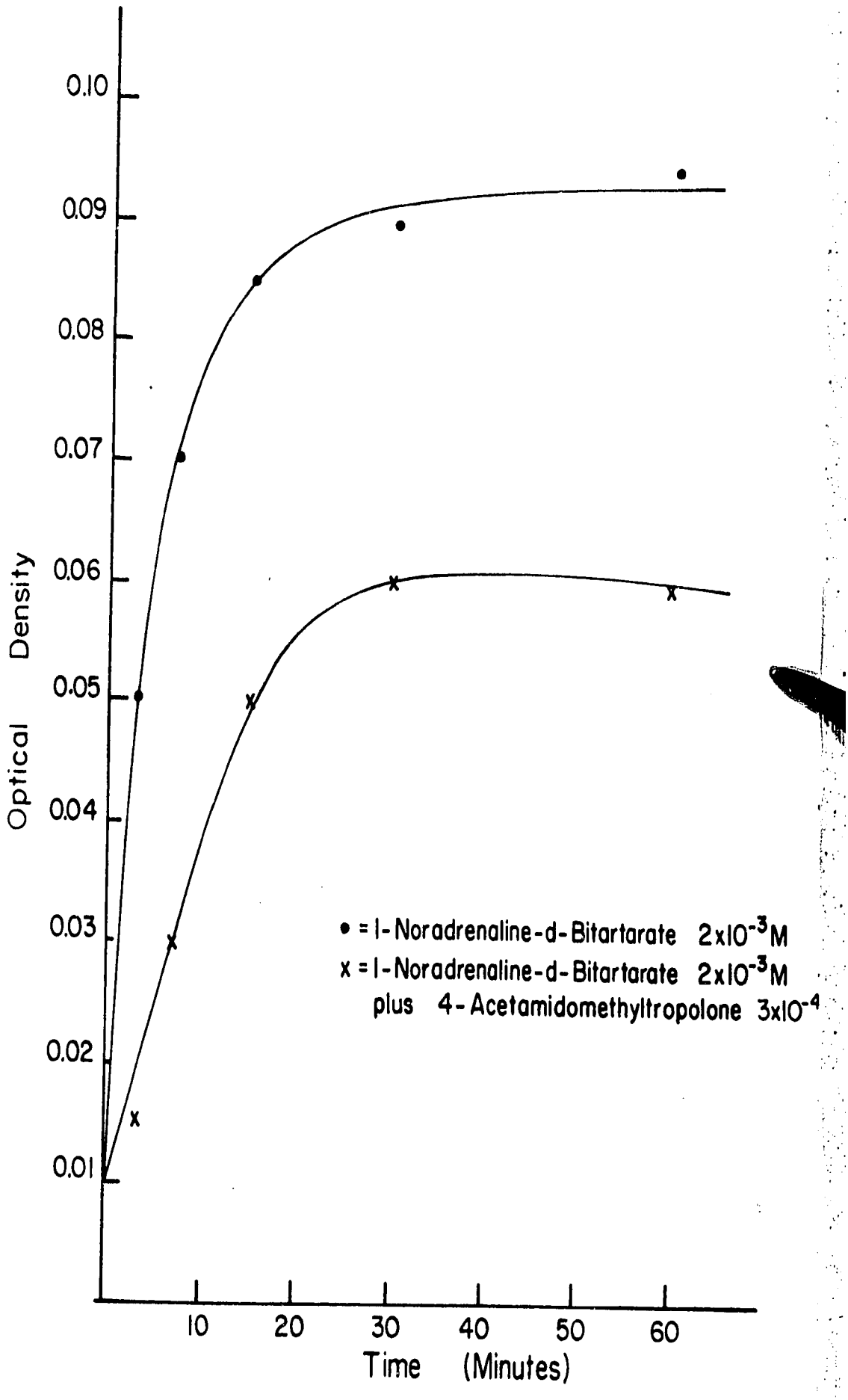

0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
10
20
30
40
50
60
Time (Minutes)
• = l-Noradrenaline-d-Bitartarate 2x10⁻³M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³M
plus 4-Acetamidomethyltropolone 3x10⁻⁴

Figure 21. Relative rates of O-methylation by rat liver COMT of l-noradrenaline-d-bitartarate 2 x 10^{-3} M without added inhibitor, and with 1 ml. of the clear incubation mixture from the demethylase system (see "Experimental").

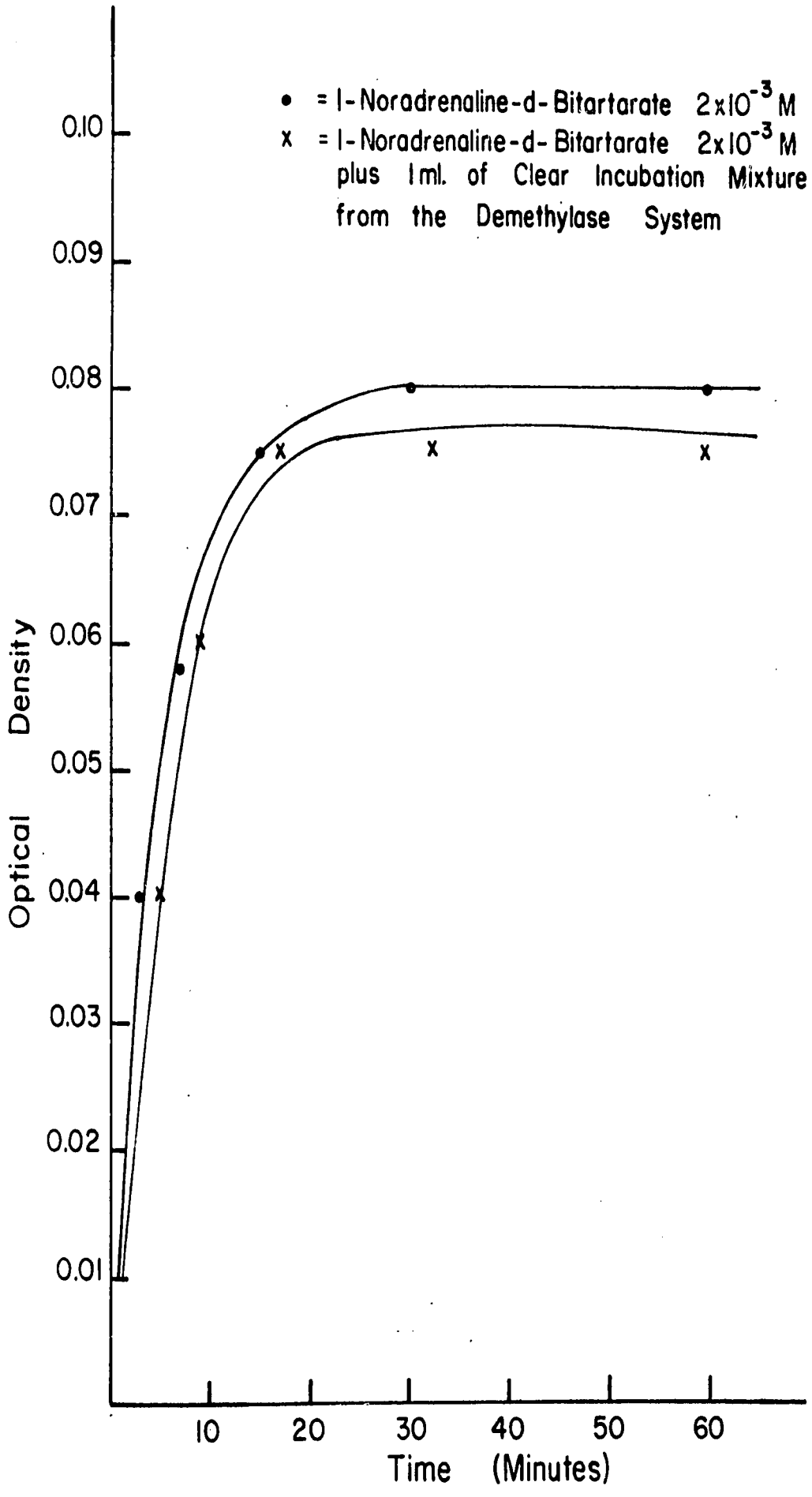

• = l-Noradrenaline-d-Bitartarate 2x10⁻³ M
x = l-Noradrenaline-d-Bitartarate 2x10⁻³ M plus 1 ml. of Clear Incubation Mixture from the Demethylase System
Optical Density
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
10
20
30
40
50
60
Time (Minutes)

Figure 22. Relative rates of O-methylation by purified rat liver COMT of l-noradrenaline-d-bitartarate 1×10^{-3} M while varying the mole % of Mg^{++} and 4-methyltropolone in opposite directions. (Job's method of continuous variation) let

100 µM $MgCl_2 \cdot 6H_2O$ = 100 mole %

1 µM of 4-methyltropolone = 100 mole %

(A) = 100 mole % Mg^{++} and 0 mole % 4-methyltropolone

(B) = 90 mole % Mg^{++} and 10 mole % 4-methyltropolone

(C) = 75 mole % Mg^{++} and 25 mole % 4-methyltropolone

(D) = 50 mole % Mg^{++} and 50 mole % 4-methyltropolone

(E) = 25 mole % Mg^{++} and 75 mole % 4-methyltropolone.

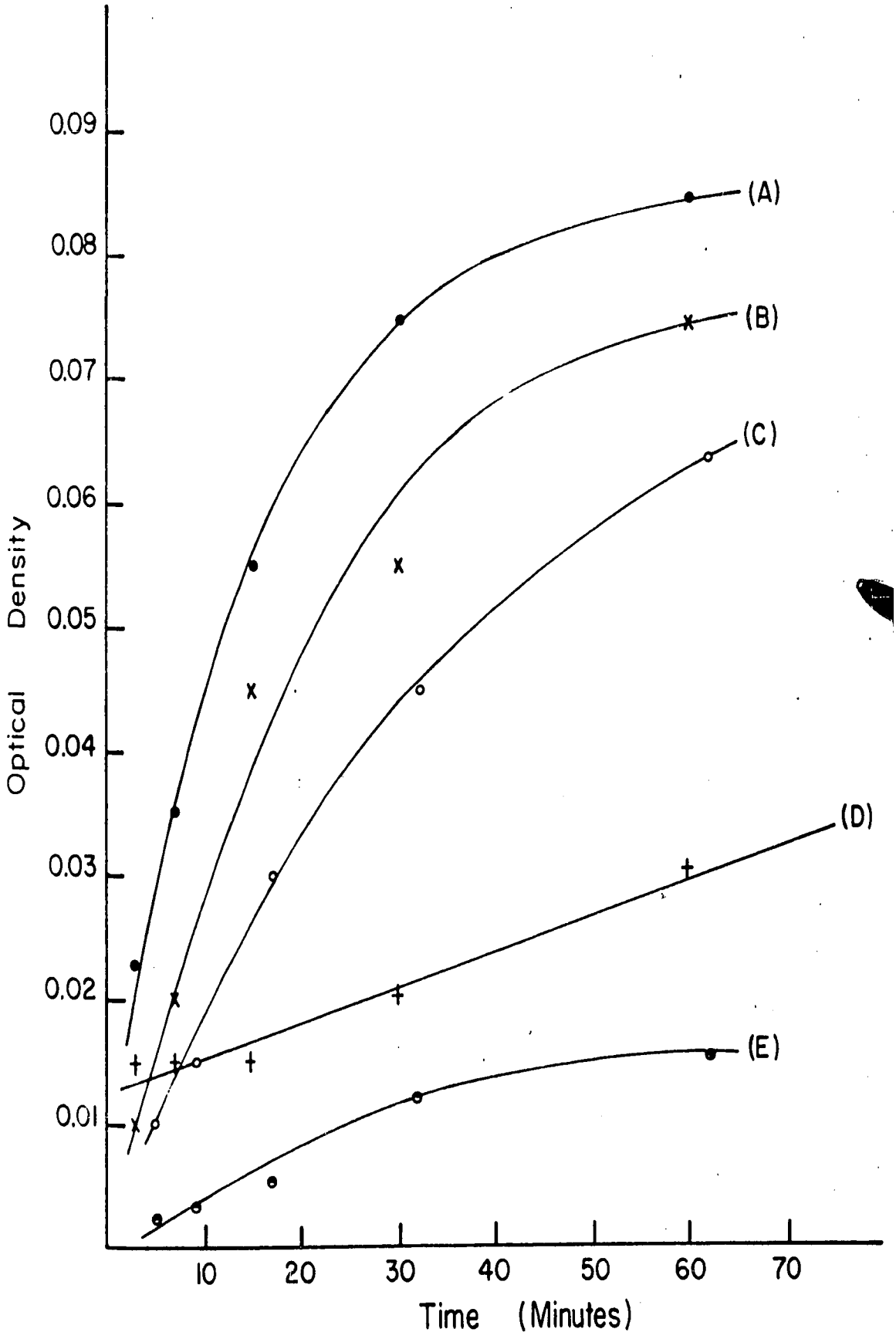
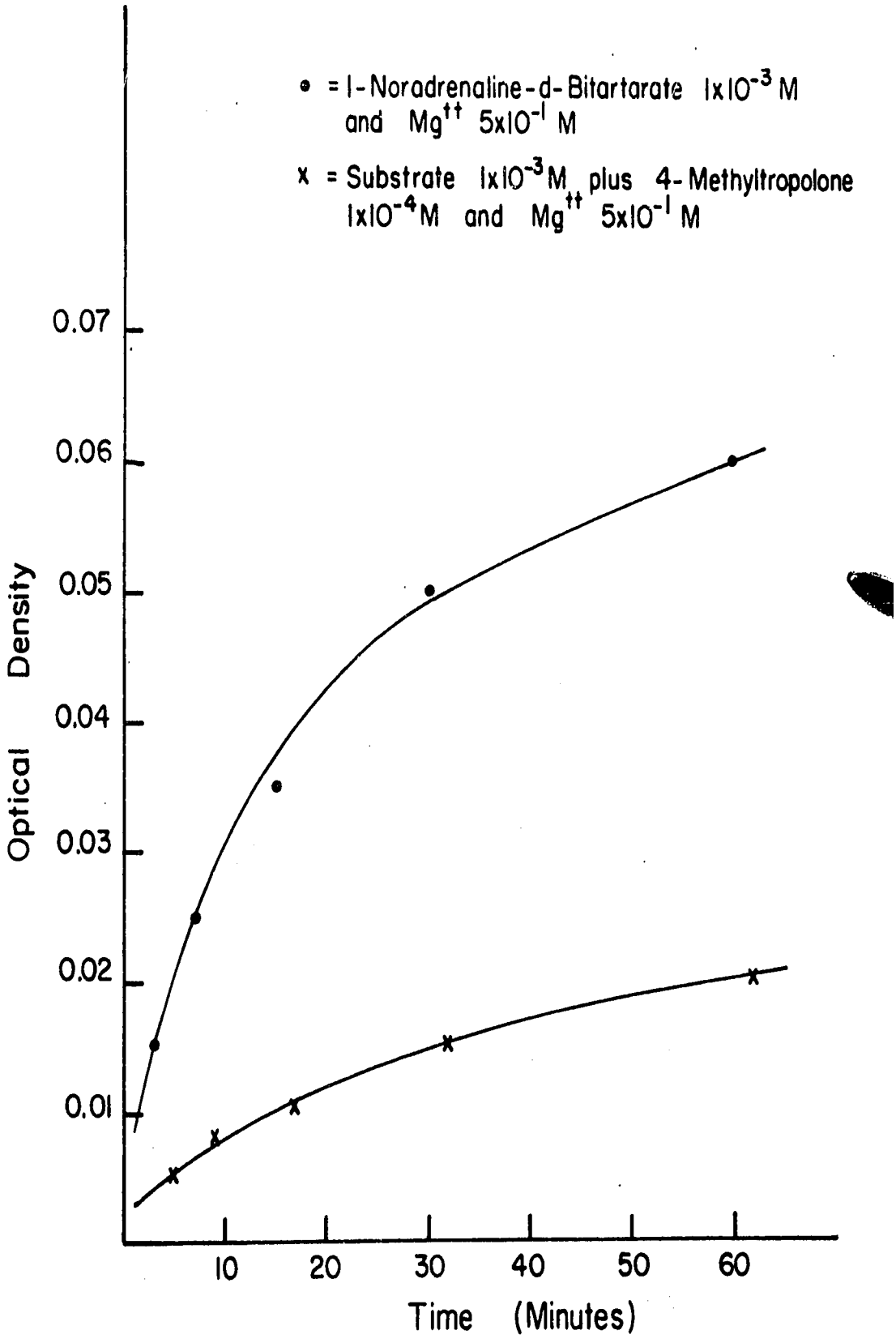

0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
Optical Density
(A)
(B)
(C)
(D)
(E)
10
20
30
40
50
60
70
Time (Minutes)

Figure 23. Relative rates of O-methylation by purified rat liver COMT of l-noradrenaline-d-bitartarate 1×10^{-3} M plus Mg^{++} 5×10^{-1} M (500 mole %) without 4-methyltropolone and with 4-methyltropolone 1×10^{-4} M (100 mole %).

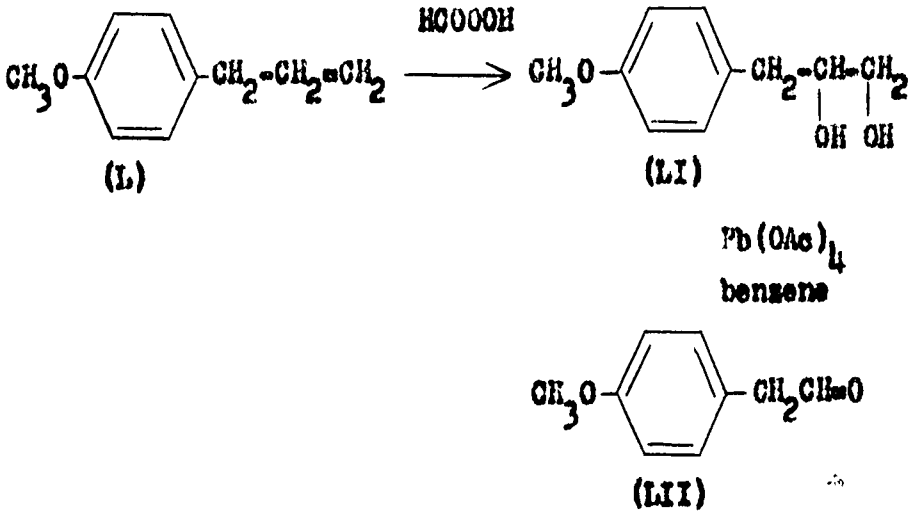

• = l-Noradrenaline-d-Bitartarate 1x10⁻³ M and Mg⁺⁺ 5x10⁻¹ M
x = Substrate 1x10⁻³M plus 4-Methyltropolone 1x10⁻⁴M and Mg⁺⁺ 5x10⁻¹ M
Optical Density
0.07
0.06
0.05
0.04
0.03
0.02
0.01
10
20
30
40
50
60
Time (Minutes)

DISCUSSION

It has been shown experimentally[30] that the decarboxylation of amino acids proceeds stereospecifically. However, with the discovery that asymmetrically deuterated biogenic amines can lead to configuration dependent isotope effects on certain pharmacological responses[120], it became imperative to establish the absolute stereochemistry of the enzymic decarboxylation of amino acids. Since retention of configuration is a characteristic of electrophilic displacement reactions, it may be expected that over-all retention of configuration occurs in the enzymic decarboxylation of amino acids. The chemical synthesis of both isomers of α-d-tyramine, (that is, R- and S-α-d-tyramine) from asymmetric intermediates of known absolute configuration was the first objective to be reached.

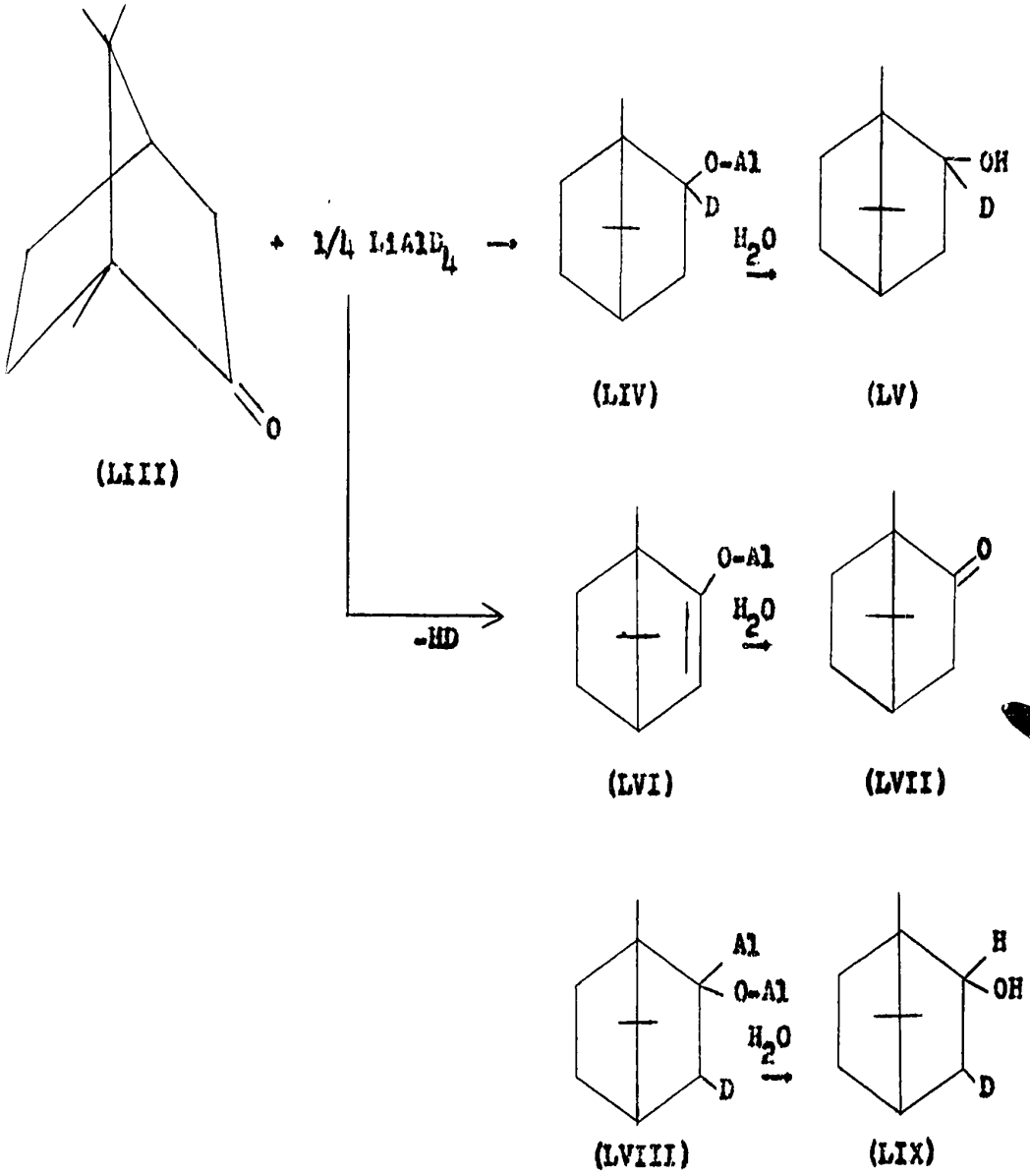
A. SYNTHETIC METHODS

Hydroxylation of p-allylanisole (estragol) with performic acid[106] gave 1,2-dihydroxy-3-(p-methoxyphenyl)-propane, which was cleaved with lead tetraacetate in benzene to p-methoxyphenylacetaldehyde. (See "Experimental" page 64).



(L) (LI) (LII)

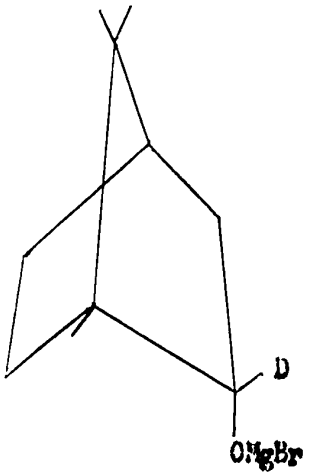
Reduction of d-camphor with lithium aluminum deuteride in ether at -70° C gave l-d-isoborneol[35] at least 97% labeled on the carbinol carbon (see "Experimental" page 64). This reduction reaction would be expected to proceed as follows[35]:



Streitwieser[35] found that the reaction of camphor with an equivalent amount of lithium aluminum deuteride at low temperature leads to incorporation of deuterium only on the carbinol carbon without the intervention of significant amounts

of enolate salts. A small amount of borneol is formed however but this is immaterial as was shown by Streitwieser.

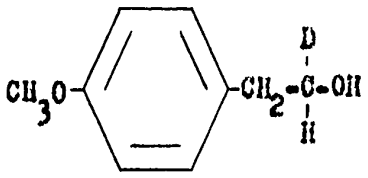
The deuterated isoborneol was converted to the bromomagnesium salt[107] and treated with p-methoxyphenylacetaldehyde according to Streitwieser's procedure[36]. There resulted a 40% yield of 1-d-p-methoxyphenethyl alcohol which exhibited a significant rotation of -1.44°.*



(LX)

+ CH_3O—$CH_2CH{=}O$ →

(LII)



(LXI)

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* In a recent paper, Streitwieser[37] has noted that with the possible exception of enzymically prepared 1-d-ethanol, all optically active deuterio alcohols of absolute configuration (LXII) are dextrorotatory. In view of the existence of π-hydrogen bonding in our alcohol (see Goldman, I.M., and Crisler, R.O., J. Org. Chem. 23, 751 (1958)), the sign of the rotation may lose significance.

This represents a specific application of the Meerwein-Ponndorf reduction. The reaction with optically active isobornyloxymagnesium bromide and benzaldehyde-d has been shown by Streitwieser et al[36] to produce an alcohol (benzyl-a-d-alcohol) of relatively high optical rotation. (See footnote on previous page). They also have demonstrated that the observed optical activity is due to the deuteric alcohol and not to an impurity.*

The use of the isobornyl system is especially convenient since it can be prepared readily from camphor which is available as the optically pure dextrorotatory enantiomer. Vavon and Antonini[107], have shown that isobornylmagnesium bromide will reduce phenyloxalic acid to yield mandelic acid of 15 per cent optical purity; borneol was inert to these conditions. Since borneol reacts so much more slowly than isoborneol, its presence in the reducing agent is not objectionable.

Doering and Young[109] proposed the involvement of a quasi six membered ring in the mechanism of Meerwein-Ponndorf

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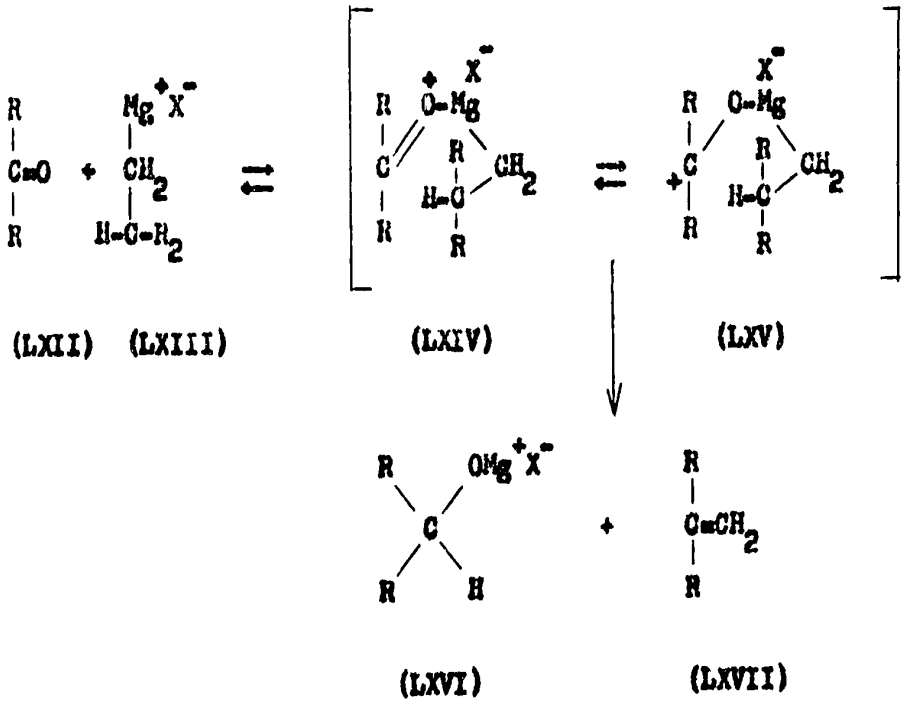
* Streitwieser[37] has also suggested that the rotation of the a-d alcohol prepared by reduction with isoborneol is rather close to the rotation of optically pure alcohol. He found that 1-butanol-1-d produced by the reduction of butyraldehyde-d with 2-octanol had only about one-fourth the rotation of the optically pure alcohol. However, that obtained from the isoborneol reduction had 0.93 ± 0.09 times the rotation of the optically pure alcohol.

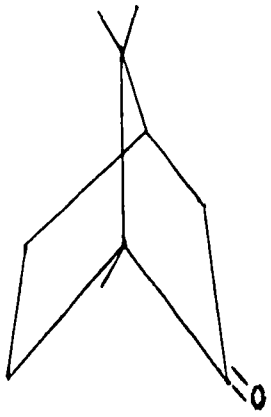
reduction of ketones with aluminum alkoxides. Mosher and La Combe[110] have also proposed a similar cyclic transition state in the mechanism of reduction by halomagnesium alkoxides:



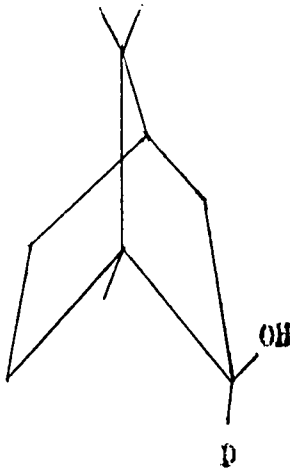
(LXII) (LXIII) (LXIV) (LXV)

(LXVI) (LXVII)

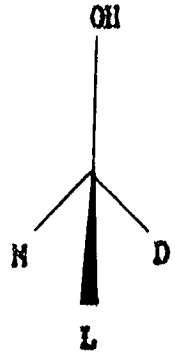
The absolute configuration of (+) camphor has been established to be as shown in (LXVIII)[121]. Hence, (-) isoborneol which is formed on reduction with lithium aluminum deuteride is (LXIX). This configuration can be represented as (LXX) in which the medium methylene group, M, is at the rear and the large quaternary carbon, L, is in front; the small deuterium atom is denoted by D



(LXVIII)

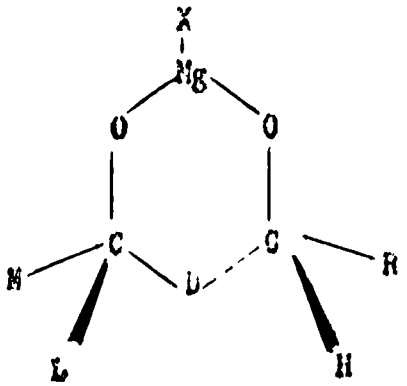


(LXIX)

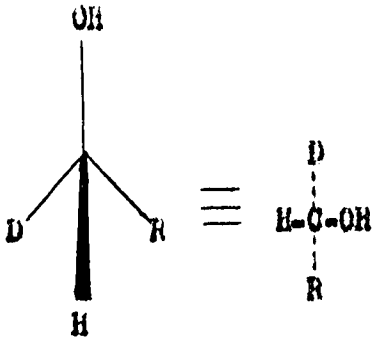


(LXX)

In the reduction of the p-methoxyphenylacetaldehyde by a halomagnesium alkoxide from (LXX), the cyclic mechanism, can theoretically proceed by way of two transition states. The more stable, depicted in (LXXI), involves steric interactions between the medium group, M, and the large group, R, and between the large group, L, and the small hydrogen. The alternative transition state involves steric oppositions between the large L group and the large R group and is consequently of higher energy. The deuterated alcohol which results from (LXXI) must have the configuration shown in (LXXII)



(LXXI)

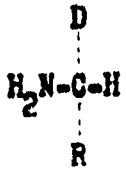


(LXXII)

According to Streitwieser[37], who has done rotation and configuration studies on a variety of optically active deuterium compounds, the above alcohol should be near optical purity.

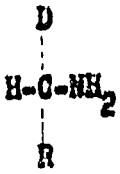
Because the mechanism of the Meerwein-Ponndorf reduction[109] is now well established, the absolute configuration (S) can be safely assigned to (LXXII) (R = p-methoxybenzyl). This alcohol (LXXII) was converted to the tosylate (a reaction which does not affect the asymmetric centre) and was subsequently converted to the corresponding azide by reaction with excess sodium azide. This direct displacement reaction with azide ion is closely analogous to the similar reaction with acetate ion in methanol, which has been de-

monstrated to proceed with complete inversion of configuration[38]. Reduction of the crude azide with lithium aluminum hydride in ether followed by hydrolysis with hydrobromic acid gave R-l-d-tyramine (XLVII) which was isolated as the



(XLVII)

crystalline hydrochloride. It follows that its deuterium content must be the same as that of the starting deuterioisoborneol. The preparation of the enantiomer S-l-d-tyramine (XLVIII) was accomplished by first treating the alcohol



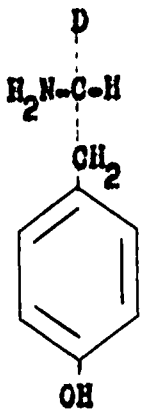
(XLVIII)

(LXXII) with phosphorous tribromide in the presence of collidine. Under these conditions (presence of collidine), virtually complete inversion of configuration should occur[39]. The bromide thus obtained was subjected to the same reaction sequence (i.e. displacement by azide ion etc.) already

applied to the above tosylate to give the desired S-l-d-tyramine (XLVIII).

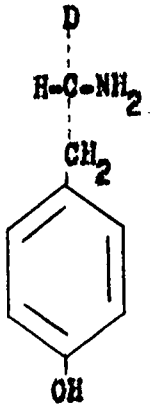
B. INTERPRETATION OF ENZYMATIC RESULTS

The rates of oxidation by rat liver MAO of these two synthetic substrates were measured using tyramine as a standard. The results are shown in Figure 2 where it can be seen that the slope ratio (at initial velocities) is 2.00 when R-α-d-tyramine is compared with tyramine and 1.25 when S-α-d-tyramine is compared.



(LXXIII)

R-α-d-tyramine



(LXXIV)

S-α-d-tyramine

The enzymatic decarboxylation of L-α-d-tyrosine in H_2O, and of L-tyrosine in D_2O, as detailed in "Experimental", had already provided us with the optically pure enantiomers of α-d-tyramine. Slope ratios of 2.3 and 1.0 have been observed with these enantiomers, (Fig. 3). It

follows that the α-d-tyramine obtained by decarboxylation of L-tyrosine in D_2O as the solvent, and which produced a slope ratio of 2.3 when compared to tyramine, (Fig. 3) has the same absolute configuration (LXXIII) as the synthetic sample, which gives rise to an isotope effect of 2.0. The smaller isotope effect obtained with (LXXIII) indicates that this preparation is optically slightly impure, although Streitwieser et al[37] claim that alcohols produced by this method should be of near optical purity. It must also be remembered that the alcohol was carried through several reactions in order to obtain the corresponding amine. However, there are many precedents suggesting that these reactions proceed with complete inversion of optical integrity. On the basis of these experiments, it is clear that the enzymic decarboxylation of tyrosine and presumably of other amino acids, (being decarboxylated via the PPal mechanism outlined in the introduction) proceeds with retention of configuration.

Now that the absolute configuration of the two enzymatic enantiomers of α-d-tyramine had been established it was a natural and necessary objective to settle the question of the absolute optical specificity of monoamine oxidase. The main factor which led us to pursue this objective was our discovery of a marked increase in the adrenergic activity of sympathomimetic amines produced by stereospecific

deuterium substitution. These in vivo results will be discussed below. MAO had already exhibited an isotope effect towards α-d_2-tyramine (Fig. 4). Subsequent work led to the discovery of a configuration dependent deuterium isotope effect in the enzymatic oxidation of asymmetrically labeled tyramine produced enzymatically. This is fully discussed in another thesis[9]. These results revealed that MAO is an enzyme that exhibits a degree of stereospecificity paralleling that of alcohol dehydrogenase. The operation of a "three point contact" between enzyme and substrate is a natural consequence and some deductions about the nature of the groups interacting with the active sites of the enzymes can be derived from these observations.

The results with rat brain MAO, (Fig. 5), show an absolute optical specificity identical to that of rat liver MAO. Hence, one must seek another explanation for the different activity of some enantiomeric drugs in the brain as compared to the periphery. The fact that the K_H/K_D ratio is 1.6 (tyramine as substrate) with rat brain MAO, while it is 2.3 with rat liver MAO might be related to differences in the purity of the respective enzyme preparations or to the lower concentration of substrate that had to be used with the brain enzyme. However, our only interest here was whether rat brain MAO showed the same absolute optical specificity as rat liver MAO. The results are nevertheless conclusive.

C. INTERPRETATION OF IN VIVO RESULTS WITH THE SYSTEM MAO-DEUTERATED SUBSTRATES

It had not yet been possible to assess quantitatively and unambiguously the role of MAO in adrenergic mechanisms at the effector cell level because previous studies relied upon the use of large doses of noncompetitive inhibitors thus creating conditions that are far from physiological. Moreover, these drugs (such as iproniazid) lack specificity in that they produce side effects such as analgesia, hypoglycemia, hypotension, etc. It is likely that in their presence, the physiology of adrenergic effector cells may be altered, thus making the interpretation of results difficult.

We have discovered a new approach, which is free of ambiguities, to the problem of the physiological role of MAO in adrenergic mechanisms. This approach is outlined in the introductory section (see page 27).

From the results obtained as described on page 95 (see also Fig. 6) the following conclusions became obvious:

i) The enzyme monoamine oxidase must be intimately associated with adrenergic effector cells and must be an important factor in the limitation of the action of tyramine and tryptamine.

ii) The MAO involved in adrenergic mechanisms displays an absolute stereospecificity which is identical to that of

liver MAO, thus making it probable that these two enzymes are very similar in properties and mechanism of action.

iii) Noradrenaline is not a substrate for the enzyme at the adrenergic effector cell level. This excludes an oxidative deamination of transmitter as part of the sequence of events leading to a response or to inactivation of the substrate.

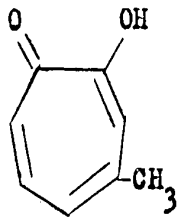
iv) The role of the enzyme in adrenergic mechanisms can best be pictured as a protective device for the rapid disposition of circulating or endogeneous nontransmitter amines.

To the best of our knowledge, the only isotope effect on a pharmacological response that had been reported was by Erlenmeyer and Lobeck[122] who claimed that acetylcholine deuterated in the methyl group was less active than acetylcholine in various tests. However these results could not be confirmed in our laboratories so that our results with deuterated adrenergic amines constitute the first report of isotope effects in the field of pharmacology. Clemo and Swan[123] have described the synthesis of completely deuterated adrenaline but could observe no difference in blood pressure response when compared to adrenaline. We feel that this method constitutes a novel approach that should prove a powerful tool in mechanism studies at the receptor level.

D. CATECHOL O-METHYL TRANSFERASE

The search for a powerful non-competitive inhibitor of COMT that is not a substrate for the enzyme seems to have

reached a climax with the discovery of the inhibitory properties of 4-methyltropolone:



(LXXV)

Figure 8 shows that 88% inhibition of the enzyme at a tropolone concentration of 3×10^{-4} M occurs. Pyrogallol was found to be approximately equipotent in our system. The tropolones are far less labile biochemically than pyrogallol, and are far less toxic.* Hence with the establishment of the inhibitory activity of 4-methyltropolone towards COMT, the question arose as to whether it acts competitively or non-competitively. Lineweaver-Burk plots (Fig. 12) clearly established that 4-methyltropolone does

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* This statement is based on the fact that the LD_{50} (i.p.) in mice of 4-methyltropolone is about 500 mg/kg. We are indebted to Dr. M. Pindell for these data.

not act competitively, in marked contrast to the polyphenol type of inhibitors. It may be recalled that Lineweaver-Burk plots provide a basis for distinguishing quantitatively between the two possible types of inhibition. See note p. 147.

It was then thought possible that inhibitory potency of tropolone inhibitors could be varied by introducing substituent groups other than methyl, provided these groups could interact with accessory sites on the enzyme surface. It was also reasoned that if a noradrenaline side chain be introduced on the tropolone ring, a better fit on the enzyme might result, noradrenaline being a natural substrate for the enzyme. As seen on page 102 (Table 2), the results of inhibition studies with the various analogues of tropolone show approximately equipotent inhibitory power regardless of the nature of the substituent on the tropolone ring.* It is noted that 1-methylamino-7-thioxo 1,3,5 cycloheptatriene is the weakest inhibitor of COMT which is not too surprising since, if the chelation mechanism (LXXVI) is operative, then one would expect this compound to have weaker chelating ability than the tropolones. Another inhibitor, 1-methylamino-7-methylimino 1,3,5 cycloheptatriene gave

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* It is interesting to note that the methyl ether of 4-acetamidomethyltropolone showed potent antimitotic activity. The compound is an analogue of colchicine, and this indicates, although more corroborating evidence is necessary, that only this part of the colchicine molecule is necessary for antimitotic activity. The experimental evidence was provided by Dept. of Agriculture laboratories in Ottawa.

identical results. Also, it is seen that colchicine shows no activity as an inhibitor (Fig. 17) at the concentrations used. This shows that the tropolone hydroxyl group must be free in order to produce inhibition.

It was seen previously that catechols like, 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylglycol, 3,4-dihydroxymandelic aldehyde, adrenaline, noradrenaline, and even catechol itself are all good substrates for COMT. Also polyphenols like pyrogallol act as excellent substrates for COMT. It is evident then that the catechol nucleus must be an integral part of any molecule in order that it can act as a substrate for COMT. The various substituents on the catechol nucleus seem to play a very minor role. It was shown (Table 2) that the tropolone nucleus was essential for inhibitory activity. Also, it was noted that the wide variety of side groups on the tropolone ring did not alter to any significant extent, the inhibitory power of tropolone. Therefore, we see a distinct parallelism between the lack of requirement for substituents on the ring of inhibitors and the nucleus of substrate molecules.

E. COMPLEX FORMATION

If the chelation mechanism (XLIX) page 49 which has been proposed for complex formation between catecholamines and COMT holds true, then one could by analogy write a similar

mechanism which would account for the inhibitory power of the tropolones *in vitro*. The mechanism of the enzymic methylation has been pictured as a displacement reaction on S-AMe by a nucleophilic hydroxyl group of the catechol ring.

The above mechanism (XLIX) can be modified to account for the non-competitive inhibitory power of tropolones, for it is obvious that the nucleophilic attack by a phenolic oxygen anion on the methyl group of S-AMe could not occur in the analogous complex (LXXVI) with tropolones.

(LXXVI)

The cycloheptatriene ring being electron deficient in its favored resonance form would most probably lead to strong interactions with electron donors on the enzyme surface. The electron density on any of the three oxygens that partake in the chelate complex should be distributed evenly in this resonating system, and the complex itself being electrically neutral cannot ionize. Therefore, no nucleophilic attack on the methyl group of S-AMe is possible. An analogy can be drawn between an alcohol and a ketone with regard to ease of methylation;

$$C{=}O \rightleftharpoons \overset{+}{C}{-}O^{-} \qquad (11)$$

$$R{-}OH \longrightarrow R{-}O^{-} + H^{+} \qquad (12)$$

Here, the alcohol on ionisation acquires a full formal negative charge and is a nucleophilic species whereas with a ketone, the resonance form (11) is electrically neutral and is not nucleophilic.

Furthermore, Witkop et al[124] studied the effects of different divalent cations on enzymatic and non-enzymatic O-methylation of 3,4-dihydroxyacetophenone and 3,4-dihydroxybenzaldehyde at different pH. They showed that the transition metal cations that withdraw electrons from the catechol hydroxyls most strongly, prevent nucleophilic displacement of the methyl group of S-AMe. This speaks strongly in favor

of the 1:1:1 enzyme-metal-substrate complex (XLIX). Experiments were performed which support mechanism (LXXVI) for the inhibition mechanism.

When a "metal enzyme" is exposed to concentrations of a ligand which in an aqueous system will occupy all the metal's available coordination sites, the stoichiometry of the metal chelate formed will be a significant guide to the mechanism of interaction. Measurements performed on any of the physical or chemical properties mentioned in the "Introduction" may serve as gauges of complex formation. Equation (7) page 54 represents a mixed complex: enzyme-metal-ligand; the metal is bound to both. The metal-ligand interaction cannot proceed fully to fill all the metal's coordination sphere, since S-AMe already occupies some of the covalent sites and indications are that it is not displaced. On the other hand if equation (8) represents the mechanism of interaction, the complexation of metal with ligand will be such that all the metal's coordination sphere is filled.

The results obtained using Job's Method of Continuous Variation (Fig. 22) show that there is a proportional decrease in inhibitory power as the mole % of 4-methyltropolone is decreased and the mole % of Mg^{++} ion is increased. Now if this system is loaded with a large excess of Mg^{++} (500 mole %, letting 100 μM = 100 mole %) while using 100 mole %

of 4-methyltropolone as inhibitor (1×10^{-4} M), there should be a large reversal of inhibition if equation (8) is operative. If mechanism (LXXVI) is correct, then the percent inhibition will approximate the percent inhibition shown by 50 mole % of Mg^{++} and 50 mole % of 4-methyltropolone. This would indicate that a 1:1:1 metal-inhibitor-coenzyme complex is formed and this further substantiates the chelation mechanism proposed by Witkop et al[124] as accounting for Michaelis complex formation. As the results show, the inhibition curve (Fig. 23) where a large excess of Mg^{++} is used, shows 73% inhibition while the curve where 50 mole % Mg^{++} and 50 mole % inhibitor are used (Fig. 22) shows 67% inhibition. Hence the results support the formulation of the complex as shown in (LXXVI) (page 142) above since the metal-inhibitor-cofactor, complex (LXXVI), is not disrupted readily by excess Mg^{++}.

CLAIMS TO ORIGINAL RESEARCH

1. Chemical synthesis of the following new compounds:
   (a) R-α-Deuteriotyramine
   (b) S-α-Deuteriotyramine
   (c) 1-α-Bisdeuterionoradrenaline
   (d) 4-Ethanolaminotropolone.
2. Enzymic preparation of the two enantiomers of α-d-tyramine.
3. The discovery that rat brain MAO displays the same absolute optical specificity as rat liver MAO, in vitro. The application of kinetic isotope effects with deuterated substrates was used for the first time as a tool for the determination of this kind of stereospecificity.
4. The discovery that MAO at the effector cell level (nictitating membrane of the cat) displays the same stereospecificity as rat liver MAO.
5. Unambiguous proof is provided that noradrenaline is not a substrate for MAO at the adrenergic effector cell level.
6. The successful application of kinetic isotope effects in the field of pharmacology.
7. The discovery of a powerful non-competitive inhibitor (4-methyltropolone) of COMT.

8. The establishment that the tropolone nucleus is all important for inhibitory activity toward COMT.
9. The development of a new assay method for COMT activity.
10. The establishment of a 1:1:1 metal-cofactor-inhibitor complex as a mechanism of COMT inhibition by tropolones.
11. A mechanism is proposed that explains the non-competitive inhibitory power of tropolones toward COMT.

NOTE

Shortly after completion of this thesis it was discovered that the Lineweaver-Burk plot (Fig. 12) was based on values of substrate concentration that approached V_{max}. Hence the apparent non-competitive nature of the Lineweaver-Burk plot becomes doubtful, and the proposed mechanism of inhibition of COMT by tropolones is no longer true. Also, Dr. D'Iorio has obtained preliminary results that indicate the tropolones to be competitive inhibitors of COMT. It is hoped to show in the near future whether the tropolones actually become methylated through the action of COMT.

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