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NORSTRAND, M.D., Iris Fletcher, 1915-
PURIFICATION AND SOME PROPERTIES OF HUMAN
LIVER MONOAMINE OXIDASE.

The City University of New York, Ph.D., 1972
Chemistry, biological

PURIFICATION AND SOME PROPERTIES
OF
HUMAN LIVER MONOAMINE OXIDASE

by

IRIS FLETCHER NORSTRAND, M. D.

A dissertation submitted to the Graduate
Faculty in Biochemistry in partial fulfillment
of the requirements for the degree of Doctor of
Philosophy, The City University of New York.

1972

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosphy.

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ACKNOWLEDGMENTS

1) To my husband, who has shared all of my problems and frequent despair over this project, and who has been a constant source of strength and encouragement.

2) To Dr. Morton D. Glantz, my thesis adviser, without whose support, empathy and excellent guidance I would never have been able to complete this study.

3) To Dr. Sanford Spraragen, Chief of Radioisotope Service, Dr. Philip Persons, Director of the Dental Research Laboratory, and Mr. Martin Sass, Director of the Medical Research Laboratory at Brooklyn VA Hospital, in whose respective laboratories I worked, for their graciousness and hospitality.

4) To Dr. Herbert Zipper, Mr. Carmen Caruso, Dr. Benjamin Sahagian, Mr. Ludwig Biermann, and Mrs. Helen Hamlin for their many kindnesses and suggestions.

5) To the members of the Office of the Medical Examiner at Kings County Hospital, in particular, to Dr. Dominick De Maio and Dr. Frank Melomo, who frequently went out of their way to help me obtain specimens.

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ABSTRACT OF THESIS

Human liver monoamine oxidase [monoamine:O_2 oxidoreductase (deaminating), E.C.1.4.3.4] was purified by two different methods. By Method #I, an original and somewhat shorter method, which does not depend on the isolation of mitochondria, and in which vacuum dialysis, (during which the enzyme separates out as a yellow precipitate), is an important step in purification, a final specific activity of 550 and fold purification of 42 was obtained. By Method #II, using the procedure described by Yasunobu et al[67] for the purification of beef liver MAO, a final specific activity of 1010 and a fold purification of 12 was obtained. By both methods the same end result was secured, namely, a highly purified preparation showing three active bands on polyacrylamide disc electrophoresis. By Method #I a single peak was obtained in the analytical ultracentrifuge, and a sedimentation constant of 6.78 was noted. The enzyme has a pH optimum at 8.65, with no activity below pH 5.45 or above 11.80. Using benzylamine hydrobromide as the substrate, the Michaelis constant at pH 7.4 and 27° was found to be 1.7×10^{-3} M. Human liver MAO is not inhibited by cyanide or by carbonyl reagents such as semicarbazide, hydroxylamine or aminoguanidine, but is sensitive to thiol-characterizing reagents, as evidenced by the fact that the enzyme is partially inhibited by iodoacetic acid, suggesting that it has one or more essential SH-groups. The enzyme activity was determined over a 10 minute period at various temperatures ranging from $0-80^{\circ}$. There was no activity at 0°. Maximal activity occurred at 60°, after which there

was a progressive decline, with loss of all activity above 80°. After pre-incubating the enzyme for 15 minutes at various temperatures ranging from 23°-70°, rapidly cooling to 23°, and determining the activity, human liver MAO was found to be completely inactivated at 70°, 70% denatured at 50°, and 47% at 37°. The enzyme, which is quite stable, does not contain pyridoxal phosphate as a coenzyme, and is a flavo-protein, as shown by absorption and fluorescence spectra. It contains approximately 9 moles of FAD per 100,000 grams of protein. The absorbance index ($E^{1\%}_{1cm}$) is 12.4. The amino acid composition of the enzyme was determined, with leucine and glycine present in greatest amount. The C-terminal group was found to be glycine. Repeated attempts to determine the N-terminal group were unsuccessful, possibly due to the fact that the N-terminal group may be bound by a phospholipid moiety.[196b] (On at least two occasions, a very faint but unmistakable fluorescent spot corresponding to that of the PTH-derivative of leucine was identified on TLC, but this result could not be confirmed by the amino acid analysis of the hydrolyzed PTH-derivatives.)

CHAPTER I

ISOLATION AND SOME PROPERTIES OF HUMAN LIVER MONOAMINE OXIDASE

INTRODUCTION

While monoamine oxidase has been crystallized from extracellular sources such as beef[192] and hog plasma[27] and from a species of fungus (aspergillus niger)[195], and obtained in a highly pure form from rabbit[121] serum, human plasma[120], rat[197], rabbit[63] and beef liver[67], bovine kidney[54] cortex, and human placenta[199], a human liver preparation showing three active bands on polyacrylamide electrophoresis and a single peak in the analytic ultracentrifuge has never before been obtained or characterized.

This thesis describes the purification of human liver monoamine oxidase [monoamine: O_2 oxidoreductase (deaminating), E. C. 1.4.3.4] by two different methods. By Method #I, an original and simple method, which does not depend on the isolation of mitochondria, the enzyme separates out as a yellow powder on vacuum dialysis, an important step in purification. By Method #II, the enzyme is purified, using the procedure described by Yasunobu[67] et al for the purification of beef liver MAO.

The recognition of the central role of monoamine oxidase in the inactivation of pharmacologically important brain amines, such as the catecholamines (dopamine, epinephrine and norepinephrine), the discovery by Udenfriend of the role of amine oxidase in the metabolism of serotonin, and the possible relationship between these compounds and various disease states has stimulated a great deal of research in recent years. Among the important practical results of research on this enzyme has been the introduction into clinical medicine of the monoamine oxidase inhibitor drugs which have had a valuable place in the treatment of depression, essential hypertension, angina pectoris and rheumatoid arthritis.

Before proceeding to the actual experimental details and results, a general discussion of the enzyme would appear to be in order.

A) Classification (Historical aspects)

The first mention of the enzyme known as amine oxidase or [monoamine oxidase: O_2 oxidoreductase (deaminating) E.C. 1.4.3.4] was in 1928, when Hare[87] described an enzyme that catalyzed the oxidative deamination of tyramine. The enzyme is called amine oxidase rather than monoamine oxidase by many since monoamine oxidase acts on many diamines, and diamine oxidase acts on many monoamines. Amine oxidase catalyzes the oxidation of a primary or secondary amine to the corresponding aldehyde, presumably through a dehydrogenation reaction, with the formation of the imine compound, and subsequent hydrolysis of the compound to aldehyde plus ammonia plus hydrogen peroxide, according to the equation:

$$RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2$$

In the presence of catalase, $H_2O_2 \longrightarrow H_2O + \frac{1}{2}O_2$

In 1940 Zeller[201,207] was the first to attempt a classification of amine oxidases into two groups, the monoamine oxidases and the diamine oxidases, basing the classification on supposed substrate specificities of these enzymes. The significance of this classification became doubtful when it was found that long-chain aliphatic diamines were substrates of monoamine oxidase (MAO) and not of diamine oxidase (DAO).[20, 17] In 1947 Steensholt[166] reported the occurrence of an amine oxidase in rabbit liver that apparently oxidized not only mescaline, a typical monoamine, but also histamine and cadaverine, and was inhibited by typical DAO inhibitors. This behavior of the rabbit liver mescaline oxidase was found to be contrary to that of the mescaline-oxidizing mitochondrial enzyme of hog kidney cortex as well as of hog liver. The latter enzymes, in their reaction pattern toward inhibitors, resembled a monoamine oxidase rather than a diamine oxidase.[210, 183]

In 1953 Hirsch[92] found in beef and sheep plasma an amine oxidase showing great affinity for spermine and spermidine. This enzyme was also found to be inhibited by such typical DAO inhibitors as cyanide, semicarbazide, and hydroxylamine.

Tabor and co-workers,[169] who purified a beef plasma amine oxidase 150-200x, found the enzyme most active on spermine, spermidine, benzylamine, as well as on various aliphatic monoamines. Whereas tyramine and mescaline were rather poor substrates, tryptamine, serotonin, epinephrine and norepinephrine were not attacked at all. The enzyme did not show any activity on histamine, and of the diamines, only the decamethylenediamine was oxidized. Furthermore, the purified beef plasma enzyme differed from the particulate classical[20,87,205] liver MAO not only in substrate specificity but also in its behavior toward inhibitors. Zeller[205] et al had reported that liver and brain MAO's were both inhibited by iproniazid, but were resistant to isoniazid, whereas Tabor's plasma MAO was inhibited by both drugs.

Yamada[191] and Yasunobu succeeded in obtaining from beef plasma a crystalline MAO preparation that showed the same substrate and inhibitor specificities as Tabor's[169] plasma spermine oxidase.

Kolb[104] described another amine oxidase occurring in hog serum which strongly degraded histamine, but had only a very small effect on cadaverine. Blaschko[83] et al confirmed the observation of Kolb. In 1964 Buffoni[35] and Blaschko obtained the hog plasma amine oxidase in crystalline form, and found that its best substrate was benzylamine, with mescaline and histamine also serving as good substrates. The enzyme was inhibited by carbonyl reagents.

In[91] 1964 Hill and Mann extensively purified an amine oxidase occurring in pea seedlings which degraded aliphatic diamines as well as aliphatic monoamines, phenylalkylamines, histamine, spermidine and agmatine, and was inhibited by carbonyl reagents.

Because of all these confusing observations, in 1959 Blaschko[85] and co-workers proposed another scheme of classification of amine oxidases, based on differences in inhibitor rather than substrate specificities. Two main groups of amine oxidases were thereby distinguished, -

those resistant to carbonyl reagents and those inhibited by carbonyl reagents.

Group I: Amine oxidases resistant to carbonyl reagents. 1) Classical MAO, an intracellular, mainly mitochondrial, insoluble enzyme, present in many vertebrate and invertebrate tissues.[20,27] Amine oxidases of this group act on primary, secondary, and tertiary amines, and on long-chain aliphatic diamines. 2) Mouse liver histaminase.[101]

Group II: Amine oxidases inhibited by carbonyl reagents. 1) Classical histaminase, preponderantly an intracellular, probably mitochondrial soluble enzyme. 2) Plasma enzymes, extracellular enzymes a) Ruminant plasma amine oxidase, spermine oxidase b) Non-ruminant plasma amine oxidase, benzylamine oxidase 3) Rabbit liver amine oxidase, mescaline oxidase 4) Plant amine oxidase, pea-seedling amine oxidase 5) Bacterial amine oxidase, polyamine oxidase.

Amine oxidases belonging to Group II oxidise primary amines but do not act on secondary amines.

A similar classification of amine oxidases, also based on differences in inhibitor specificities, was proposed by Zeller et al.[211,212] According to Zeller, the enzymes attacking aliphatic compounds with terminal amino groups can be separated by means of semicarbazide into two groups: Group I. Semicarbazide-resistant MAO's, with only one kind of receptor in their active center. Group 2. Semicarbazide-sensitive DAO's with two types of receptors in their active center.

Group 1 of the semicarbazide-resistant amine oxidases includes the classical MAO,[20,87] which is characterized by its location in mitochondria, its oxidative action on monoamine substrates, its inability to deaminate histamine and short-chain diamines, its sensitivity to octanol and certain heavy metals, and its resistance to carbonyl reagents, to hydrazine and semicarbazide, as well as to carbon monoxide. A very characteristic property of this enzyme is its action on primary as well as secondary amines.

The rather heterogeneous group 2 of semicarbazide-sensitive amine oxidases includes the classical histaminase DAO, the beef and sheep plasma spermine oxidase, plasma benzylamine oxidase and mescaline oxidase. Zeller[211,212] stresses that in his classification scheme semicarbazide must not be replaced by the term carbonyl reagent, since many carbonyl reagents, such as monosubstituted alkyl and aryl hydrazines, act on both enzyme groups efficiently, whereas only monosubstituted acyl hydrazines and hydrazine itself solely inhibit enzymes of Group 2.

Zeller[212] believes that both groups of amine oxidases represent "whole families of homologous enzymes", and share many properties such as having some substrates in common.[208]

In this paper we shall, of course, only be referring to classical monoamine oxidase [monoamine oxidase: O_2 oxidoreductase (deaminating) E.C.1.4.3.4], an agent not sensitive to carbonyl reagents and not inhibited by semicarbazide.

B) Occurrence

Monoamine oxidase has been found in all

classes of vertebrates so far examined: mammals, birds, reptiles, amphibians and teleosts.[14] The enzyme occurs in many different tissues, particularly in glands, plain muscle, and the nervous[18] system. In man, the parotid and submaxillary glands seem to be the richest source of MAO.[167] A high MAO activity is always shown by the[14] liver, but the kidney, intestine, aorta, stomach and pancreas may also contain rather high concentrations of the enzyme.[2,13,14] MAO occurs in the human heart[113,142] and lung.[112] Low MAO activity has been encountered in skeletal muscle,[18] but this observation has not been confirmed. The enzyme has been encountered in the male sex organs.[2,13,202] In the uterus,[13] MAO activity is high, and related to the functional state of the organ. The human placenta,[117] as well as that of other mammals,[170] has been found to contain MAO. Erythrocytes,[178] blood plasma,[9] and blood platelets[135] also show some MAO activity.

MAO is found widely and rather evenly distributed in the central nervous[18] system, with the hypothalamus showing somewhat greater MAO activity than other parts of the brain. MAO is located in brain mitochondria mainly at synaptic nerve endings. It is present in the nerve cells and capillary walls, but not in glial cells or in nerve fibers.

In invertebrates, Blaschko[19,22] and co-workers demonstrated a wide occurrence of MAO, with a very high activity, in the haptopancreas of two species of cephalopids, Sepia officinalis and Octopus vulgaris. The finding of MAO in molluscs is of particular neurophysiological interest, since

Welsh[182] has suggested that serotonin, one of the main substrates of MAO, acts as a neurotransmitter in these lower forms.

The enzyme also occurs in plants.[106,184,185]

About 70-80% of classical intracellular MAO is located in mitochondria,[10,183] with the enzyme tightly bound to the mitochondrial membrane fraction, particularly the outer mitochondrial membrane,[181] which is rich in phospholipid, and which, according to Schnaitman,[181] can be dispersed preferentially by digitonin. All attempts to separate MAO from the insoluble particulate matter have been so far unsuccessful.[73]

Partially purified MAO has been prepared in various laboratories by treatment of the mitochondrial membranes with detergents, or with ultrasonic waves, or by disruption of the mitochondria by homogenization and subsequent treatment with Triton X-100.

Unlike other mitochondrial enzymes, MAO is very stable. Heating for 10 minutes at 50 degrees, however, destroys 50% of its activity. Its pH optimum depends on the purity and the origin of the enzyme.[186]

C) Prosthetic Groups

1) Pyridoxal

Since mitochondrial MAO does not discriminate in its action between primary amines and their N-methylated derivatives as substrates, and is not inhibited by carbonyl reagents, it has been generally felt that this enzyme probably does not require any pyridoxal as a prosthetic group.[186]

A direct indication of the absence of pyridoxal from intracellular MAO has recently been provided by Tipton,[172,173] who subjected a purified enzyme preparation of hog brain MAO to pronase digestion, followed by acid hydrolysis, and determined the ability of the acid hydrolysate to reactivate streptococcus faecalis L-tyrosine apodecarboxylase in the presence of ATP.[27,160] No detectable amounts of pyridoxal phosphate were encountered in this purified preparation of hog brain. This finding supports earlier reports indicating that pyridoxal phosphate is not involved in MAO activity.[186] It should be pointed out, however, that early reports were based on indirect evidence such as lack of sensitivity of MAO to carbonyl reagents or the absence of effect of a pyridoxine-deficient diet on the MAO activity in rat liver.

2) <u>FAD</u> (Flavin adenine dinucleotide)

The inhibition of MAO by quinacrine, a known inhibitor of flavoenzymes,[111,188] as well as studies made on riboflavin-deficient rats[89,187] suggested that MAO may have a flavin prosthetic group.

Nara et al[129] and Erwin and Hellerman[52,54] independently reported the occurrence of a flavin in purified preparations of beef liver and of beef kidney mitochondrial MAO, but both groups of workers failed to identify the flavin fully.

Yasunobu et al[96,127,128] provided further support for the assumption that mitochondrial MAO is a flavin dinucleotide enzyme by discovering that purified beef liver mitochondrial MAO preparations contained FAD covalently attached to the enzyme, and suggested that the mitochondrial MAO holds 3 moles of FAD per mole of enzyme, based on the assumed molecular weight of MAO of 300,000.

Tipton[172] was able to isolate a fluorescent material that proved to be FAD from purified preparations of hog brain mitochondrial MAO. Fluorescent material could be liberated from the purified MAO either by heating the enzyme solution at 100 degrees for 12 minutes, or by treatment of such a solution with trichloroacetic acid. The flavin component from supernatants of boiled MAO extracts was identified as FAD in 2 ways, i.e. either by ascending chromatography, or by titration with the FAD-specific D-amino acid apo-oxidase. From reactivation experiments with samples of the purified supernatant from boiled MAO, a molecular weight of 120,000 per molecule of FAD was calculated.

The relative ease with which flavin could be removed from the hog brain enzyme was not in agreement with results obtained with beef liver and beef kidney MAO. This observation led Nara et al[189] to the assumption that the flavin may be covalently bound to the enzyme. Tipton[172] found that the ease of extraction of the flavin group seemed to depend on the degree of purification since FAD could not be extracted from an only partially purified preparation of hog brain MAO by techniques which were successful in quantitatively extracting it from the purified preparation.

Tipton,[172] who applied a technique employed by Swoboda,[168] for the removal of FAD from glucose oxidase, was able to split MAO into apomonoamine oxidase and FAD.

Preparations of the apo-oxidase, which had no detectable enzyme activity, and showed negligible fluorescence, could be partly reactivated by incubation with FAD, but not with FMN.

Tipton's observation that chlorpromazine competitively inhibits the reactivation of monoamine apo-oxidase by FAD agrees well with that of Gabay and Harris[62], who studied the competitive inhibition of the reactivation of D-amino acid apo-oxidase by a number of phenothiazines, and suggested that phenothiazines may act by inhibiting flavoenzymes[62]. Tipton[172] regards the production of the hog brain MAO apoenzyme and its specific reactivation by authentic FAD as a decisive proof of the involvement of this cofactor in the activity of MAO.

Harada, Mizutani and Nagatsu[85] found the fluorescence spectrum of beef brain mitochondrial MAO to be similar to that of FAD. On heat denaturation of the enzyme with zinc sulphate, a yellow fluorescent compound was released. Thin-layer chromatography confirmed that the yellow fluorescent material was FAD.

3) Copper

Plant, beef plasma and mold amine oxidases have been shown to be copper proteins.

Review of the literature makes it apparent that there is considerable controversay as to whether copper is a prosthetic group, is involved in the actual structure of the enzyme, or even required for activity.

In order to demonstrate that a specific

protein is a metal protein, according to Erwin and Hellerman[52], one has to show 1) a direct proportionality between copper content and specific activity 2) what known chelating agents inhibit the enzyme 3) that copper is the only metal bound, in analysis of the most purified preparation 4) that copper is not lost during purification and dialysis.

After extensive investigations of beef liver mitochondrial MAO preparations, Yasunobu[96,127,128,129] and his colleagues concluded that this enzyme contains copper, mainly in the cupric state, which is essential for activity, and found a direct proportionality between copper content and the specific activity of the enzyme. These authors also made the interesting observation that, in contrast to the DAO type of amine oxidase, which is claimed to be copper-pyridoxal phosphate dependent, the beef liver mitochondrial MAO seems to be the only known example of an enzyme requiring for its activity both copper and flavin as prosthetic groups.

Nara[128], Gomes and Yasunobu, in their electron-paramagnetic resonance (EPR) studies on beef liver mitochondrial MAO, were able to demonstrate that the EPR spectrum of this enzyme differed considerably from that of hog kidney diamine[124] oxidase and of beef plasma[192] amine oxidase. Whereas the copper bonds in the very similar EPR spectra of the DAO and beef plasma amine oxidase showed considerable ionic character, the EPR spectrum of beef liver mitochondrial MAO is quite different, and resembles that reported for cytochrome oxidase in which the copper-protein bond is covalent[33]. According to Nara, Gomes[128] and Yasunobu, further differences in the copper-protein bonds of the beef liver and beef plasma amine oxidases are indicated by the fact that cuprizone is a competitive inhibitor of the plasma amine oxidase, but a mixed inhibiter (i.e. partially competitive and partially noncompetitive) of the beef liver enzyme. That cuprizone is also a mixed inhibiter of human plasma amine oxidase has been reported by McEwen[120].

Gabay and Valcourt[63], who obtained from rabbit liver a highly purified mitochondrial MAO preparation, studied the effects of metal chelating agents on this enzyme, and suggested that cupric or other metal ions may be involved in the enzyme function. These authors felt, however, that it was not necessary to accept with certainty that a divalent metal ion is a functional component of the catalytically active center since the metal ion may be involved in the maintenance of an effective structure of the enzyme, as is the case with many oxidative enzymes[60,165].

In contrast to the above findings, Erwin[52,54] and Hellerman, as well as Lagnado and Sourkes[111] failed to detect the presence of significant concentrations of copper in mitochondrial preparations of bovine kidney and rat liver MAO respectively, indicating that copper is not required for MAO activity. These authors found no observable correlation between the inhibition of the enzyme activity by appropriate chelating agents and the ability of these agents to chelate copper, and proposed other interpretations for the inhibition produced by certain of these agents. Erwin and Hellerman found no correlation between copper content and the specific activity of the enzyme. Actually, the final step in their purification of the enzyme resulted in a large decrease in copper content, with concomitant increase in specific activity. The copper content of their purified preparation (0.15 ug/mg) differed markedly from the values reported by Yasunobu et al and Sourkes.

Similarly, Tipton[172] obtained very low copper values for purified hog brain MAO preparations, which seemed to suggest that there were considerably fewer copper atoms in these preparations than there were molecules of enzyme. Moreover, they found that the cupric ion failed to reactivate MAO. The latter finding, however, is in contrast to the work of Coq and Baron[40], who found that inhibition of rat liver mitochondria by prolonged dialysis was reversed by the addition of cupric ions. Obviously more fundamental work is required to elucidate the function of copper in the activity of amine oxidases.

Harada, Mizutani and Nagatsu[85] analyzed beef brain mitochondrial MAO for copper spectrophotometrically, and found that copper concentrations in the enzyme decreased during the purification, and that the preparation after the electrophoresis had no detectable amount of copper (less than 0.01 ug/mg protein). The inhibitory effects of copper chelating agents such as cuprizone or diethyl-dithiocarbamate on the purified enzyme were very weak.

4) Presence of an SH group

Friedenwald and Hermann[61] were the first to suggest that MAO possessed a sulfhydryl group essential for its activity, and that the SH group might possibly be involved in an electron transfer. These authors found that the enzyme was inhibited by p-chloromercuribenzoate, but that a complete reversal of inhibition was obtained by adding glutathione or cysteine in the presence of cyanide. Later work by Barron[8] et al showed that cyanide had reacted with the p-chloromercuribenzoate, and that this apparently accounted for the complete reactivation. Since glutathione reactivated the enzyme in the absence of cyanide, and since MAO was also found to be inhibited by organoarsenicals as well as by iodoacetate, it was concluded that a sulfhydryl group was essential for its activity.[110] The inhibition of MAO activity by mercaptide-forming reagents was confirmed in experiments with partially purified enzyme preparations.[6,70,71,75]

Erwin and Hellerman[52] also claimed that the sulfhydryl groups were essential for activity in MAO.

Nara et al[127,128] found that their purified beef liver mitochondrial MAO preparations contained sulfhydryl groups that are not vicinal.

Gabay and Valcourt[63] recently reported that their highly purified rabbit liver mitochondrial MAO preparations were very sensitive to the action of p-chloro-mercuribenzoate, obtaining an almost 100% inhibition of the enzyme by 10 uM concentrations of this compound. This result is in marked contrast to the findings of Nara et al,[128] who observed an inhibition of only 37.5% by 5 mM p-chloro-mercuribenzoate. Gabay and Valcourt thought that this difference was possibly explainable by the fact that their rabbit liver preparation had been subjected to column chromatography. Gorkin[75] also suggested that repeated application of column chromatography causes a distinct increase in the sensitivity of highly purified MAO preparations to inhibitors.

In the case of beef liver MAO it was first reported that the sulfhydryl groups are not essential for activity[67]. Subsequent investigations, however, have demonstrated that some of the sulfhydryl groups are essential[99] for activity.

Inhibition of beef brain mitochondrial MAO by p-chlormercuribenzoate and N-ethylmaleimide was reported by Harada, Mizutani[85] and Nagatsu, with N-ethylmaleimide found to be a less potent inhibitor than p-chlormercuribenzoate.

D) Substrate specificity

Primary and secondary amines are readily oxidized by MAO provided that the substituent in the secondary amine is a methyl[25] group. The ability to act on methylated amines can be used as a criterion for the presence of a "true" MAO. The rate of oxidation of secondary derivatives is usually very high, sometimes even higher than that of primary amines. The tertiary amines are oxidized more slowly[24], the oxidation rate being higher in some species than in others[144].

The MAO substrates comprise aliphatic amines and the more important naturally occurring amines, carrying cyclic substituents[25]. In the homologous series of normal primary aliphatic amines, $CH_3(CH_2)_nNH_2$, the maximal rates are achieved with amylamine and hexylamine. The lower oxidation rate of amines with longer chains is probably due to their low solubility and their tendency to form micelles. MAO does not act on methylamine, but in some species ethylamine is slowly oxidised.

Whereas the typical substrates of DAO, the tetra and pentamethylene diamines (putrescine and cadaverine) are not attacked by MAO, the long-chain diamines, from heptamethylene diamine onward, are oxidised by this enzyme. The diamine most rapidly attacked by MAO is the compound with 13 methylene groups.[28] Competition experiments have shown that not only does the rate of oxidation of the diamines increase with the number of methylene groups, but also their affinity for MAO. The reason for this behavior may be that in the short-chain members of this series the second amine group probably interferes with the attachment of the amine to the enzyme. With the increasing intramolecular distance between the two amine groups, the disturbing action of the second amine group on the enzyme-substrate reaction becomes less effective.[17,18,25]

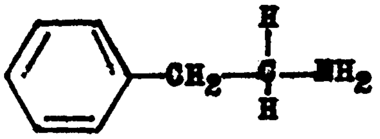
Pletscher[139] et al arranged a series of amines that are substrates of MAO according to the decreasing rate of the enzymic reaction: dopamine, tyramine, 3-methoxytyramine, tryptamine, 5-hydroxytryptamine, phenylethylamine, normetanephrine, metanephrine, epinephrine, norepinephrine, 1,4-methyl histamine, and kynuramine. Aromatic amines, such as aniline, with the amine group directly attached to the benzene ring are not attacked by MAO.[20] The reaction rate increases with the number of methylene groups in the side chain.[167]

The ability to serve as a substrate of MAO is lost when a methyl group is introduced in the alpha-position to the amine group of an aliphatic or aromatic amine.[2,13] With an increasing number of methoxy substituents in the benzene ring the affinity of such compounds for MAO decreases.[12,13,166,183]

E) - Active Site

Many properties of the active center of amine oxidases have been studied by Zeller[209] and his colleagues with the help of specifically selected substrates and inhibitors. The active center of amine oxidase seems to extend over several Angstrom[208] units, and its structure may be asymmetrical, possibly because it consists partly of L-amino acids.[95,137,140,209]

In his studies on the active center of MAO, Zeller[148,213,214] pointed out the existence of a striking structural analogy between the molecules of the optimal substrates of MAO, for example, phenylethylamine and of its most active inhibitors (trans-2-phenylcyclopropylamine). Accordingly, the most suitable substrate or inhibitor of MAO is characterized by a two-carbon chain containing an alpha-hydrogen atom and an alpha-amine group, and substituted in the beta-position by an aromatic ring.



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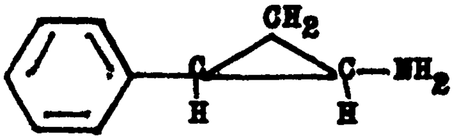
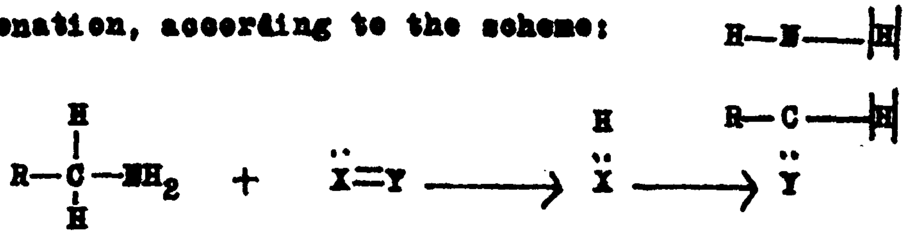


II

Whereas the MAO substrate I contains a second alpha-hydrogen atom in its side chain, in the MAO inhibitor molecule II, the second alpha-hydrogen atom is substituted by an alkyl group.

Zeller[214] suggests that one alpha-hydrogen is essential for establishing the enzyme-substrate complex or the enzyme-inhibitor complex, whereas the second alpha-hydrogen of the substrate participates in the process of dehydrogenation, according to the scheme:



where X-Y is part of the active center, in which X serves as an acceptor for the proton derived from an alpha-hydrogen[148] atom and Y for the alpha-carbon. The two hydrogen atoms H are being received by a hydrogen acceptor.

In agreement with McEwen's[125] findings with human liver MAO, it is thought that beef liver MAO has an electrophilic site which binds the $-NH_2$ group of the substrate, and a hydrophobic site which binds the R-side chain of the substrate. The active site is believed to contain FAD which accepts the electrons from the substrate, and is reduced to $FADH_2$. In addition, the enzyme contains about[67] 7-SH groups if it is assumed that the molecular weight is 100,000.

About[39] 75 compounds were tested recently with beef liver MAO, and the type of inhibition produced with respect to benzylamine and oxygen were studied. These studies demonstrated that the active site of the enzyme is very hydrophobic. The enzyme is inhibited by a large variety of aromatic compounds, aliphatic alcohols and aldehydes, and other hydrophobic compounds. Included in this list are metal chelating agents which appear to be inhibiting the enzyme without reacting with any metal; artificial electron acceptors, which explains the inability of these compounds to replace oxygen as the electron acceptor; and tranquilizers.

F) - Inhibitors

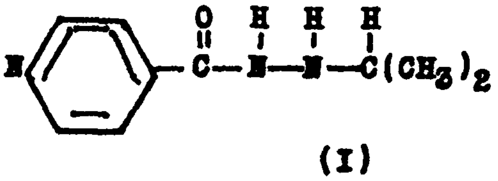
Since Zeller[204,205] and colleagues reported on the strong in vitro and in vivo inhibition of MAO activity by the anti-tuberculosis drug iproniazid in 1952, the list of compounds synthesized and tested as potential MAO inhibitors has grown enormously. Detailed aspects of both in vitro and in vivo inhibition of MAO activity have been well-documented in many reviews and symposia[50, 186, 138, 105,150,29,216,217,26,139,159,147].

Werle[186] distinguishes 2 main classes of MAO inhibitors: 1) the group of competitive inhibitors, comprising hydrazine derivatives, members of the B-phenylisopropylamine series, the harmala alkaloids, and choline-p-tolyl ether 2) the group of noncompetitive inhibitors, including the amidines.

1) Competitive MAO inhibitors.

a) Iproniazid (1-isonicotinyl-2-isopropyl-hydrazine)

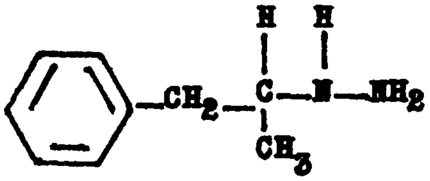
I is the best studied inhibitor of MAO.



(I)

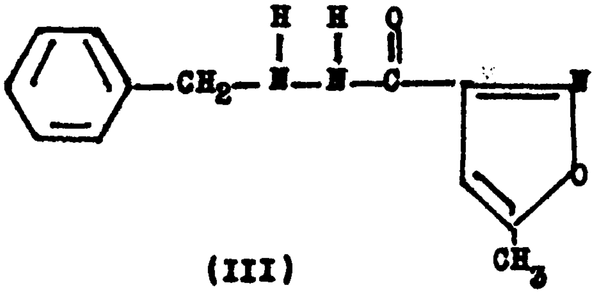
In vivo, iproniazid is probably first hydrolyzed to yield isonicotinic acid and isopropylhydrazine, the latter compound being a stronger inhibitor of MAO than iproniazid[25,215]. On incubation with erythrocytes, iproniazid loses its ability to inactivate MAO, possibly due to the presence in the erythrocyte membrane of an inactivator of iproniazid.[147]

b) Pheniprazine, B-phenylisopropylhydrazine (II) inhibits MAO in vitro about 50 times as strongly as iproniazid. This may be due to the presence of the benzene ring in its structure since isopropylhydrazine does not display this selectivity for the brain MAO.



(II)

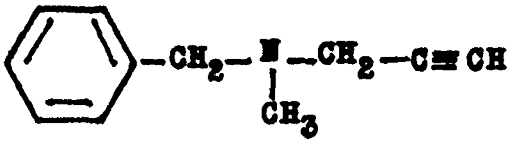
c) Isocarboxazid, (1-benzyl-2-(5-methyl-3-isoxazolyl carbonyl)-hydrazine (III) is, in vitro as well as in vivo, a much stronger inhibitor than iproniazid, and is less toxic than the latter.



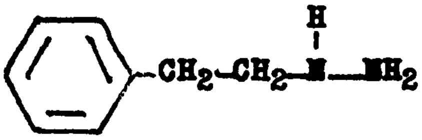
(III)

d) Pargyline (N-methyl-N-2-propynyl benzylamine), (IV), phenelzine (B-phenylethylhydrazine), (V), and tranylcypromine (2-phenylcyclopropylamine), (VI), are also potent MAO inhibitors.

(III)



(IV)



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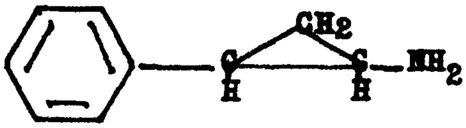
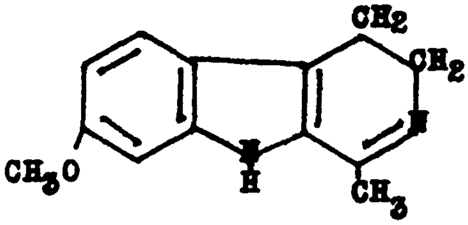


(VI)

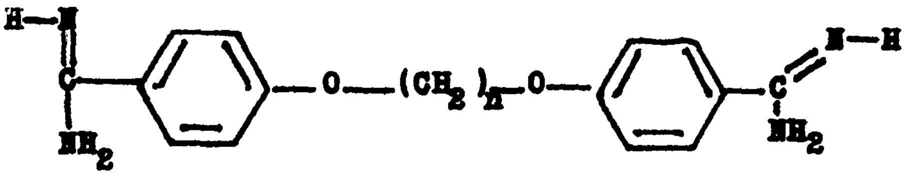
e) Another group of very strong MAO inhibitors is that of the harmala alkaloids. Harmaline, VII, is the most potent inhibitor of this series.[175]



(VII)

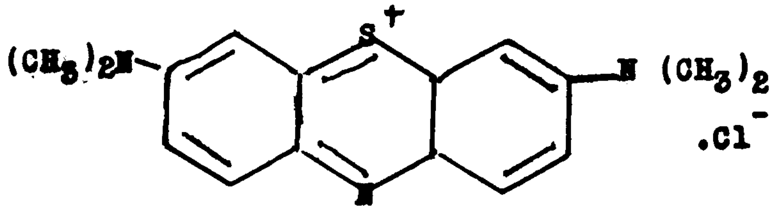
2) Noncompetitive MAO inhibitors.

This group comprises amidines, monoisothiourea derivatives, and di-isothiourea derivatives.



(VIII)

Methylene blue, (IX), is also a fast-acting reversible MAO inhibitor. Octyl alcohol also actively inhibits MAO.



(IX)

G) - <u>Mechanism of Inhibition</u>

From the following discussion it will be evident that more fundamental work is needed for the elucidation of the problem of MAO inhibition.

As previously mentioned, in all substrates of MAO the alpha-carbon atom must be unsubstituted. Amines in which one of the hydrogen atoms of the alpha-carbon atom is substituted are inhibitors of amine oxidase.[15,119,140]

In his study on liver mitochondrial MAO, Davison[49] observed that the inhibition of this enzyme by iproniazid is progressive, first order, and requires the presence of oxygen. Davison suggested that the first step in the inhibition reaction may be a dehydrogenation of iproniazid to 1-isopropylidene-2-isonicotinyl hydrazine in a manner similar to that postulated for the oxidation of the substrate.

In the interpretation of results obtained in in vitro inhibition experiments with MAO, Zeller[215] has outlined a few factors of importance. Thus, substrates protect MAO against the action of iproniazid, or of cis and trans-phenylcyclopropylamine only when they are added simultaneously with the inhibitor. The higher the substrate concentration, the less effective is the blockage of MAO, suggesting that iproniazid and other hydrazine derivatives are bound to the part of the active center with which the substrate usually combines. When the inhibitor is brought into contact with MAO before the addition of the substrate, the inhibition effect of the drug increases about tenfold. This reaction is independent of the substrate concentration. When the inhibitor reaches the active site, it apparently remains irreversibly attached to it. Accordingly, prolonged dialysis of the iproniazid-MAO[148,206] complex restores only a small fraction of the original MAO activity. Since the substrates almost instantaneously form a complex with the active site of MAO, they are able to protect those enzyme molecules that have not been inactivated by iproniazid. In experiments on the competition between substrate and inhibitor, the affinity of the substrate for MAO is the decisive factor. Thus, tryptamine[100] is known to be more strongly bound by MAO than tyramine, and, accordingly, MAO was found to be four to six times more resistant to an inhibition by 2-phenyl cyclopropylamine in the presence of tryptamine as substrate than is that of tyramine.

Green has pointed out the existence of many striking similarities between the inhibition of MAO by hydrazine derivatives and the decomposition of the latter, catalyzed by cupric ions. Both processes require oxygen, and both can be retarded or prevented by compounds capable of chelating with or being oxidized by cupric ions.

Davison[48] was the first to notice a decrease in inhibition when MAO and one of its inhibitors, iproniazid or isopropylhydrazine, were incubated under nitrogen instead of oxygen.

Gorkin[74] recently reported that the chelating agent cysteamine prevents the irreversible inhibition of MAO by iproniazid when added before the inhibiter. On the basis of these and many similar observations, Green[79] advanced the hypothesis that the inhibition of MAO by hydrazine derivatives may result from a copper-catalyzed liberation of free radicals near the active center of the enzyme, with the copper constituting part of the enzyme. That metal ions may be involved in the structure of particles containing mitochondrial MAO has been suggested by Gorkin[177] and his colleagues.

H) - Multiple forms of MAO

There have been many reports concerning the multiple forms of mitochondrial MAO. Some investigators have reported that there is more than one enzyme with different substrate specificities in the rat liver[70,72,76,86,143,200]. Others have demonstrated the presence of three separable isoenzymes in rat liver mitochondria on polyacrylamide-gel electrophoresis[98,198], and four such active bands in the whole liver homogenate. Isozymes of mitochondrial monoamine oxidase have been described in rat brain[200] and chick[155] brain. The latter authors suggested the possible existence of multiple forms (isoenzymes) of the enzyme MAO, and showed that MAO derived from adult chicken brain existed in multiple forms as measured by their gel electrophoretic technique, and that these multiple forms are different from those seen in embryonic and neonatal chick brains. Two active fractions of the pig brain mitochondrial monoamine oxidase have been separated on Sephadex G-200 by Tipton,[173] who postulated that the first one eluted is a lipid complex or a tetramer of the major component.

Yasunobu[196] et al, who found three active components on polyacrylamide gel electrophoresis of purified beef liver MAO believed that the enzyme was broken down into fragments in which the enzyme exists in different polymeric states. Raglund,[143] who separated at least 3 different MAO's in rat and rabbit liver by means of gel filtration, and at least 2 in beef liver, using 2% cutscum as detergent, felt that MAO should not be considered a series of isoenzymes with multiple substrate specificity but rather a series of homologous specific amine oxidases with closely related structural coenzyme and purification characteristics. He believed that the name, monoamine oxidase, is ambiguous and should be replaced by the more specific terms, tyramine oxidase, serotonin oxidase, etc.

Harada[85] et al showed that beef brain MAO exhibited two components on continuous-flow electrophoresis, - a slow-moving major component (enzyme 1), showing a higher specific activity, and a fast-moving minor peak (enzyme 2), with a lower specific activity, with the major component having a stronger color. The enzymes 1 and 2 did not appear to be different molecular weight forms, since both components had the same molecular weight as judged by Sepharose 6B gel filtration.

Shih and Eiduson[156] assayed brains, hearts and livers from newborn and adult rats for MAO, using gel electrophoretic techniques. Their results suggest that each of the tissues possesses multiple forms (isoenzymes) of MAO, and that these forms are different for the various tissues. In order to assess whether these multiple forms of MAO have different substrate specificities beyond those already shown under the conditions of the method, neonatal tissues (brain, heart and liver) were assayed for MAO active bands, using three different substrates for the enzyme; 5-hydroxytryptamine, benzylamine and tryptamine. In the case of neonatal brain, when tryptamine was used, 2 bands appeared. When 5-hydroxytryptamine was used as substrate, only one of the bands appeared. If substrate affinities of the upper bands are compared with those of the lower bands appearing in the gel runs, it appears that tryptamine and benzylamine are more readily utilized as substrate for the slower moving enzyme forms, whereas the lower bands have little affinity for benzylamine although they utilize tryptamine and 5-hydroxytryptamine as substrates. Since the technique of gel staining may lead to artifacts, Shih et al[34,179] incubated a radioactive substrate with segments of the gel to check the authenticity of stained bands presumed to be MAO.

Another explanation for the multiple forms of MAO, according to Raglund[143], is that the elution profiles do not actually represent 3 different MAO activities per se, but represent the separation of other factors differentially influencing activity with the 3 substrates. Raglund stated that such factors could be other enzymes acting upon the specific substrates or products, specific inhibitors, or specific activators.

Still another possibility is that the three components belong to the same enzyme protein in the mitochondria, and are derived by an ability of the detergent to alter the structure of the protein.

And lastly, as mentioned by Shih et al,[156] their data do not indicate whether the multiple forms seen in the various tissues are not themselves artefacts of the entire method. Against this, however, is the fact that homogenization of tissue and solubilization of the enzyme by different methods (Triton X-100), lubrol or sonic oscillation) resulted in the same qualitative band patterns, suggesting that these multiple forms may indeed be real and not an artefact of the method. This is supported by the work of Kim and D'Iorio[98] that different methods of solubilizing the enzyme result in similar patterns of MAO active bands.

That MAO may be a single enzyme with multiple active sites has also been considered.

No work on subunits has been reported in the literature to date.

I) - Formal Reaction Mechanism

As mentioned previously, there are two types of amine oxidase, which can be differentiated most readily on the basis of the prosthetic groups present in the enzyme. The plasma type requires copper and possibly coavalently linked pyridoxal phosphate, while the mitochondrial enzyme requires covalently attached FAD. Both enzymes, however, catalyze the same general reaction.

McEwen[120,121] investigated the effect of pH on $K_m^{RNH_2}$ and V_m for the human and rabbit plasma amine oxidases, and showed that there are two types of enzymes, - the human enzyme, which acts on the unionized amine, and the rabbit type, which shows a more complicated pH dependence. In addition, there is a report that a Schiff base intermediate is formed during the reaction,[36] which can be trapped anaerobically by the addition of sodium borohydride.

Reed and Swindell[145] reported that in the reaction catalyzed by the beef plasma amine oxidase, 1 mole of benzaldehyde is produced anaerobically, and that the K_m for oxygen is dependent on the amine concentration. These studies suggest that a ping-pong mechanism is at work.

Since enzymes from different sources appear to have different substrate specificities and spectra, and there is no evidence to indicate that all the copper-pyridoxal phosphate amine oxidases proceed by the same mechanism, one should, for the time being, consider these amine oxidases from different sources as separate entities.

In regard to the mechanism of beef plasma amine oxidase, Yasunobu[130,131] et al proposed the following mechanism on the basis of chemical studies and initial velocity and product inhibition patterns.

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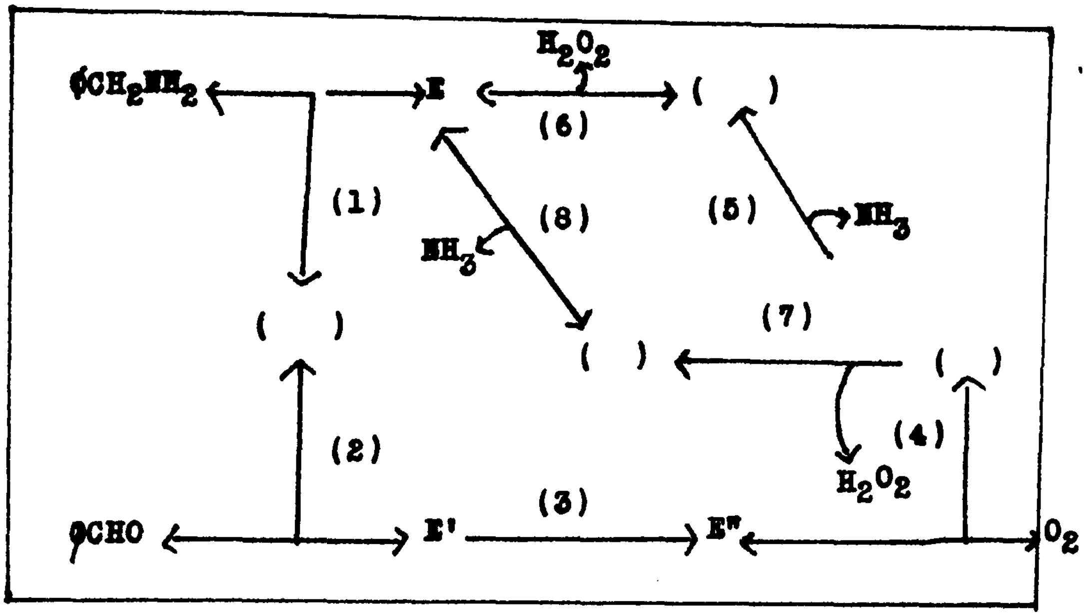


Proposed mechanism for reaction catalyzed by beef plasma amine oxidase

Although the reaction catalyzed by beef plasma amine oxidase is a ternary-ternary reaction and quite complicated, the fact that this enzyme operates by a ping-pong mechanism was demonstrated by both kinetic and chemical studies. Since the initial velocity studies yielded a series of parallel lines with respect to both substrates, the authors concluded that this was indicative of a ping-pong mechanism as originally pointed out by Alberty.[1,37] Yamada and Yasunobu[193] showed that the pink color of the native enzyme is bleached by the anaerobic addition of the substrate, and that the addition of oxygen subsequently restores the pink color. In agreement with the report of Reed and Swindell,[145] 1 mole of benzaldehyde was produced per mole of enzyme in the absence of oxygen, - a verification of the double-displacement nature of the reaction. The double-displacement character of the reaction predicts the noncompetitive behavior of the first product, benzaldehyde, toward the amine substrate. The similar pattern observed with respect to oxygen suggests the presence of at least two enzyme-substrate complexes between the step involving the liberation of benzaldehyde and that involving the entry of oxygen as substrate. This amounts to assuming an isomerization step, as commonly found in enzyme mechanisms[55]. This additional step prevents competition between benzaldehyde and oxygen for the same enzymic species, and would predict the strictly noncompetitive behavior observed.

The product NH_3 and H_2O_2 demonstrate identical inhibition patterns, competitive with respect to benzylamine and uncompetitive with respect to oxygen. The pathway through 5 and 6 would yield competitive behavior for H_2O_2 and noncompetitive for NH_3 with respect to the amine; that through steps 7 and 8 would yield the inverse result.

According to Tipton[173], there is accumulating evidence that MAO from different species and tissues proceeds by different mechanistic routes such as to warrant separate investigations on each enzyme.

Tipton[173] published evidence that pig brain MAO proceeds by a ping-pong mechanism.

Smith et al[161] demonstrated by the use of isotopic oxygen that the oxygen is converted to H_2O_2, and that the oxygen in the product, aldehyde, arises from the solvent water. In addition, these investigators proposed that the true form of the substrate is the un-ionized species, RCH_2NH_2, and suggested a mechanism very similar to that proposed for the enzyme D-amino acid oxidase. Belleau[11] showed that there is a 3-point attachment of substrate to enzyme by the use of various deuterium-labelled substrates.

In regard to beef liver mitochondrial MAO, Oi[130,131] recently showed that this enzyme also proceeds by a ping-pong mechanism. When benzylamine was used as the substrate, one mole of benzaldehyde was produced anaerobically per FAD present in the enzyme. According to this mechanism, the hydrated enzyme reacts with benzylamine in a Theorell-Chance mechanism to produce benzaldehyde, ammonia and $E\text{-}FADH_2$. In the second step, $E\text{-}FADH_2$ reacts with O_2 to give rise to the intermediate $E\text{-}FADH_2O_2$. In the third step, this complex breaks down to give rise to E-FAD and H_2O_2. In the last step, E-FAD reacts with water to give the hydrated enzyme, $E\text{-}FADH_2O$.

The rate equation is:

$$\frac{1}{v_0} = \frac{1}{V}\left[K_m^{amine}/(\text{amine}) + K_m^{O_2}/(O_2) + K_m^{H_2O}/(H_2O) + 1\right]$$

$$K_m^{amine} = k_{+3}/k_{+1}\ ,\ K_m^{O_2} = (k_{-2}+k_{+3})/k_{+2} \text{ and } K_m^{H_2O} = k_{+3}/k_{+4}$$

where the k values are rate constants for the various steps mentioned above.

So far no mechanism has been proposed for human liver MAO.

J) Methods for the Measurement of MAO activity

Current interest in the function of biogenic amines, and in the enzymes involved in their metabolism has been stimulated by the recent development of simple and sensitive methods for their chemical detection.

Quantitative assay methods have been

described based on the uptake of oxygen[30,46,158,186] by a variety of techniques, including manometric, diffusion and spectrophotometric, the liberation of ammonia[44], concomitant analyses of oxygen uptake and ammonia evolution[45], the disappearance of substrate[162,181], and the production of intensely colored dinitrophenylhydrazine derivatives in alkaline solution of the aldehyde product formed in the reaction between MAO and the substrate.[78]

The manometric method is relatively insensitive and complicated by secondary reactions. Measurement of ammonia liberated may provide a reliable and sensitive assay under certain conditions. Both of these methods, however, require a prohibitive amount of purified enzyme.

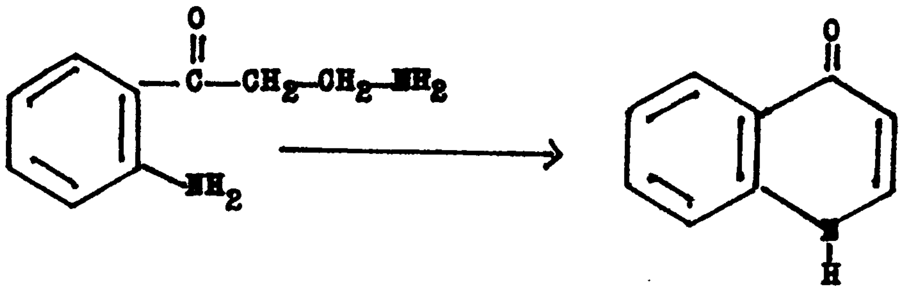
Most of the methods mentioned above have proved to be too insensitive for determination of MAO in organs with low enzyme activity such as dog heart, or in small amounts of tissue. Modern radiometric, fluorometric, spectrophotometric and histochemical procedures have been recently published for the estimation of MAO, some of which will be described in some detail.

a) Spectrophotometric assay of MAO, utilizing the disappearance of the substrate benzylamine.

Tabor[169] and associates were the first investigators who followed the action of a plasma amine oxidase (spermine oxidase) by a spectrophotometric method, utilizing benzylamine as a substrate. The product of the reaction, benzaldehyde, displays a very high extinction coefficient at 250 nm, and can easily be detected spectrophotometrically.

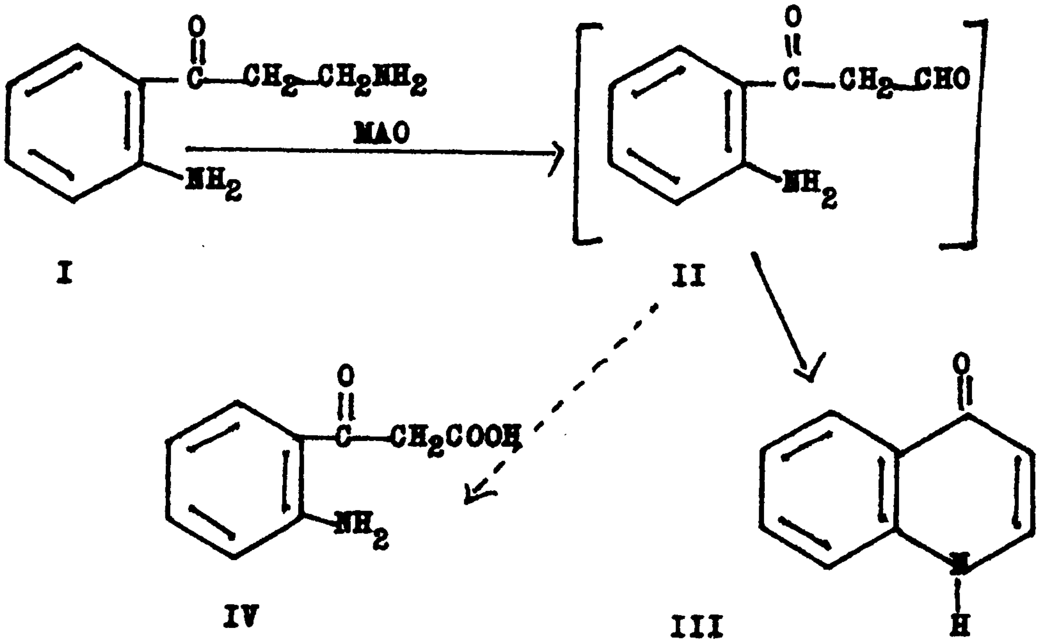
A rapid spectrophotometric method for the determination of MAO and MAO inhibition, according to Zeller, Ramachander and Zeller,[218] is based on the high degradation rate of m-iodobenzylamine, and on the strong absorbance at 253 nm of the m-iodobenzaldehyde formed during the action of MAO on m-iodobenzylamine. This primary reaction product does not appear to undergo further oxidation by oxidoreductases. The choice of m-iodobenzylamine as a substrate of MAO was based on the studies of E.A. Zeller[216] on the occurrence of eutopic and dystopic complexes in the MAO reaction, which indicated that, with the exception of fluorine, the introduction of any substituent in the meta position led to much better substrates than benzylamine itself. By far the highest reaction rates, as expressed by maximum velocity (V), were obtained with meta-iodobenzylamine.

2) Disappearance of kynuramine, or the appearance of 4-hydroxyquinoline. In 1955 Makino and his colleagues[118] showed that mouse liver homogenates convert kynuramine, the decarboxylation product of kynurenine, to 4-hydroxy-quinoline, which these authors identified by its paper chromatographic and spectral properties.



Kynuramine 4-hydroxyquinoline

A spectrophotometric assay of MAO based on the rate of disappearance of kynuramine was devised by Weissbach et al.[181] According to Weissbach et al, MAO probably deaminates kynuramine (I) to the corresponding aldehyde (II), which may either condense to 4-hydroxyquinoline (III) or undergo further oxidation to the corresponding acid (IV).



Intramolecular (nonenzymic) condensation of the amino aldehyde (II) proved, however, to be faster than further oxidation of the aldehyde to the corresponding acid (IV).

In their kinetic studies on the effect of various inhibitors on purified beef liver mitochondrial MAO, Barbato and Abood[6] compared the spectrophotometric assay of MAO by Weissbach et al[181] with that of Tabor and co-workers[169], which utilizes benzylamine as substrate. Barbato and Abood[6] found that kynuramine was a better substrate for the spectrophotometric MAO estimation than benzylamine, particularly when working with carbonyl reagents, such as potassium cyanide, which can react with the benzaldehyde formed, or when using the inhibitor phenanthroline, whose extinction coefficient at 250 nm is extremely high. Furthermore, carbonyl reagents have no effect when kynuramine is used, evidently because the intramolecular reaction between the aldehyde group and the amino group of kynuramine is much more rapid than the reaction of the aldehyde group with the carbonyl reagents.

Zeller[218] and his colleagues have criticized the spectrophotometric kynuramine method of Weissbach et al[181] for the following reasons: upon the action of MAO, kynuramine is deaminated, and the resulting aldehyde condenses intramolecularly to form 4-hydroxyquinoline, a substance with a much lower molar extinction coefficient at 360 nm than the starting product. Since the initial optical readings with 0.1 mM kynuramine are relatively high, small differences in optical density may be of doubtful value.

b) Fluorometric assay

The average fluorescence measuring instrument will permit the determination of quantities of fluorescent material as small as 0.1 to 0.001 ug/ml. With the more sensitive instruments, and with compounds having a high absorption and a high quantum yield of fluorescence, a millimicrogram (nanogram) of a fluorophor can emit sufficient fluorescence to allow measurement.

1) Rate of indoleacetic acid formation.

This is a sensitive fluorometric assay of MAO activity in vitro[115] based on the formation of indoleacetaldehyde on incubation of tissue homogenates with tryptamine, and the oxidation of indoleacetaldehyde to indoleacetic acid, a fluorescent compound, on addition of aldehyde dehydrogenase and nicotinamide-adenine dinucleotide (NAD). The activity of the aldehyde dehydrogenase preparation is checked by measuring the rate of $NADH_2$ formation during incubation with acetaldehyde.

2) Rate of formation of 4-hydroxy quinoline formation[107]. This is a rapid microfluorometric determination of MAO which is a fluorometric adaptation of the MAO assay of Weissbach[181] et al. Instead of determining the disappearance of kynuramine, Krajl monitors fluorometrically the appearance of 4-hydroxyquinoline which arises from the spontaneous cyclization of the intermediate aldehyde, formed by the enzymic oxidative deamination of kynuramine. Unlike many methods for the determination of MAO, which are based on the assay of the acid produced by further oxidation of the relevant aldehyde by a second enzyme, an aldehyde dehydrogenase, this microfluorometric assay does not depend on the tissue sample containing aldehyde dehydrogenase.

3) Measurement of hydrogen peroxide formed in oxidase reactions by coupling it to the formation of a fluorophor with homovanillic acid in the presence of peroxidase[83]. Tissue preparations are incubated with amine substrate, homovanillic acid, and horseradish peroxidase, and the hydrogen peroxide evolved is measured fluorometrically. This permits the use of a number of substrates and continuous monitoring of the enzyme reaction. The method is comparable in sensitivity to the radiometric methods for amine oxidases, and is relatively simple to perform.

4) Combined paper chromatographic-fluorescent methods. Studies on the cellular localization and turnover of

biogenic amines have been impeded by the lack of methods for the adequate separation and discrimination of these amines, as well as of their precursors and metabolites. Recently, thin-layer chromatography has been successfully applied to the rapid isolation and identification of minute amounts of tissue biogenic amines. Nanogram amounts of these amines can be detected by exposing the thin layer to an o-phthalaldehyde spray or paraformaldehyde gas, whereby the biogenic amines are converted into highly fluorescent[4,84] derivatives. These methods have served as a basis for the development of simple radiometric assays of amine oxidase activity.

c) Radioisotopic methods.

1) Wurtman and[189] Axelrod devised a simple, sensitive and specific assay of MAO, utilizing the measurement of deaminated ^{14}C-metabolites of ^{14}C-tryptamine. The deaminated radioactive material, ^{14}C-indoleacetic acid is extracted by shaking with toluene, and identified by ascending paper chromatography. The indoleacetic acid is separated from tryptamine in a butanol: acetic acid: water (4:1:1) system, and from indoleacetaldehyde in an isopropyl alcohol: ammonia: water (8:1:1) system.

2) A radioisotopic assay for MAO determination, according to Otsuka and[134] Kobayashi, is based on the formation of a radioactive anisole-soluble end-product from the substrate ^{14}C-tyramine. After the enzyme incubation, the end-product is extracted into anisole, the aqueous phase is frozen, and the anisole, containing both the radioactive end-product and phosphor, is poured into a counting vial for assay

in a liquid scintillation spectrometer.

d) Histochemical demonstration of MAO

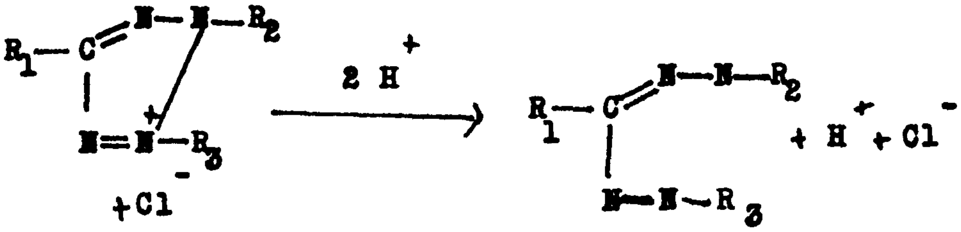
Several techniques for the histochemical demonstration of MAO activity have been reported.

1) Demonstration of aldehyde groups. Oster and Schlossman[132] were the first to demonstrate MAO activity based on the visualization of aldehyde groups in the oxidized amines. Tissues were incubated in the presence of tyramine and the resulting aldehyde groups were demonstrated by Schiff reagent. The results were unreliable as the aldehyde product of the reaction, being water-soluble, tended to diffuse prior to the staining reaction. Koelle and Valk,[102, 103] in 1954, and Eder,[51] in 1957, attempted to "trap" the aldehydes with 2-hydroxy-3-naphthoic acid hydrazide. These authors treated tissue sections with hydrazine and incubated these in the presence of tryptamine and 3-hydroxy-2-naphthoic acid hydrazide in pyrophosphate buffer containing a high concentration of sodium sulfate. The aldehyde formed by the action of MAO condenses with the hydrazide, and the condensation product is then converted to a bluish-purple pigment by coupling with tetrazotized o-dianisidine. Despite its validity, this method has not been frequently used because it is so laborious.

2) Pigment formation: In fresh frozen sections incubated with tryptamine-HCl at pH 7.4, Blaschko and Hellmann[21] in 1953 observed the formation of a dark pigment. Later, Arioka and Tanimukai[3] in 1957 modified this technique by using serotonin as the substrate. The initiative role of MAO in the pigment formation is accepted, but the role

and structural relation of other oxidative systems that may be operationally parallel or even coupled with amine oxidase in the pigment formation and the diffusibility of the pigment intermediates has not been determined. The application of this technique is therefore limited.

3) Tetrazolium methods: Appropriate water-soluble tetrazolium salts can be reduced to water-insoluble formazans in connection with the MAO reaction.



Production of formazan in living cells was discovered by Kuhn and Jerchel[108] in 1941. Tetrazolium salts are colorless, water-soluble, toxic, and are deployed as redox indicators. Formazans are water insoluble compounds. In the histochemical reaction the tetrazolium salt acts as a hydrogen acceptor. The p-nitro group in the N_2-phenyl ring increases the readiness with which the tetrazolium salts accept H from various dehydrogenase systems. Reduction is facilitated by alkaline pH.

In 1953 Francis[57] demonstrated the deposition of formazan in frozen sections incubated with tyramine at pH 7.4 in the presence of neotetrazolium chloride. By substituting tryptamine for tyramine, and employing Nitro-BT for neotetrazolium as electron acceptor in Francis' method, Glenner, Burtner and Brown[64,65] obtained more satisfactory and reproducible results. Under these conditions there was a distinct localization of the formazan at tissue sites during an hour of incubation.

In human brain, Robinson[146], in 1966, used Nitro-BT as electron acceptor according to the method of Glenner et al[64,65], and obtained an accurate localization of the enzyme by rapid production of discrete formazan deposits.

4) And finally, Graham and Karnovsky[77] have succeeded in demonstrating MAO activity in the liver and kidney of rats and guinea pigs by a coupled peroxidation-oxidation technique. In the presence of peroxidase, its substrate, 3-amino-9-ethyl-carbazole has been found to be oxidized by the hydrogen peroxide generated during the oxidation of tryptamine, catalyzed by MAO. The resulting insoluble red oxidation product is apparently deposited at sites of MAO activity. This histochemical coupled oxidation method of MAO estimation may be useful as an alternative technique to the tetrazolium method since it depends on an entirely different type of chemical reaction.

K) - Biological Importance of MAO

The metabolic function of MAO in the animal body is to break down potent biogenic amines by oxidative deamination. This reaction is followed by the further oxidation of the aldehyde formed to the corresponding carboxylic acid. The identification and quantitation of such acid urinary constituents has helped considerably in the elucidation of the biological function of MAO in man and other mammals[25].

The biological role of MAO is not only confined to the inactivation of biogenic monoamines. Barondes[7] demonstrated that many different MAO substrates and their aldehyde metabolites stimulate in vitro glucose-1-^{14}C oxidation to $^{14}CO_2$ by beef anterior pituitary slices. The effect of the aldehyde metabolites is not blocked by MAO inhibitors. Similarly, Pastan and Field[136] have reported on an in vitro stimulation of glucose oxidation in thyroid by serotonin. The unexplained effects of MAO inhibitors on carbohydrate metabolism in vivo[42] may be due to interference by the inhibitors with some yet unknown function[75] of MAO.

Oxidative deamination and o-methylation are the two enzymic pathways of catecholamine metabolism in the peripheral sympathetic system,[5] and also occur in the central nervous system.[18]

Spector[164] and his co-workers presented evidence that MAO is the principal enzyme in the inactivation process of nor-epinephrine and serotonin in the brain. These authors also suggested different physiological roles for MAO and COMT. Whereas MAO appears to be responsible for the metabolism of the biogenic monoamines in tissues, COMT is involved particularly in the inactivation of exogenous, extra-neuronal, circulating catecholamines.[94,164]

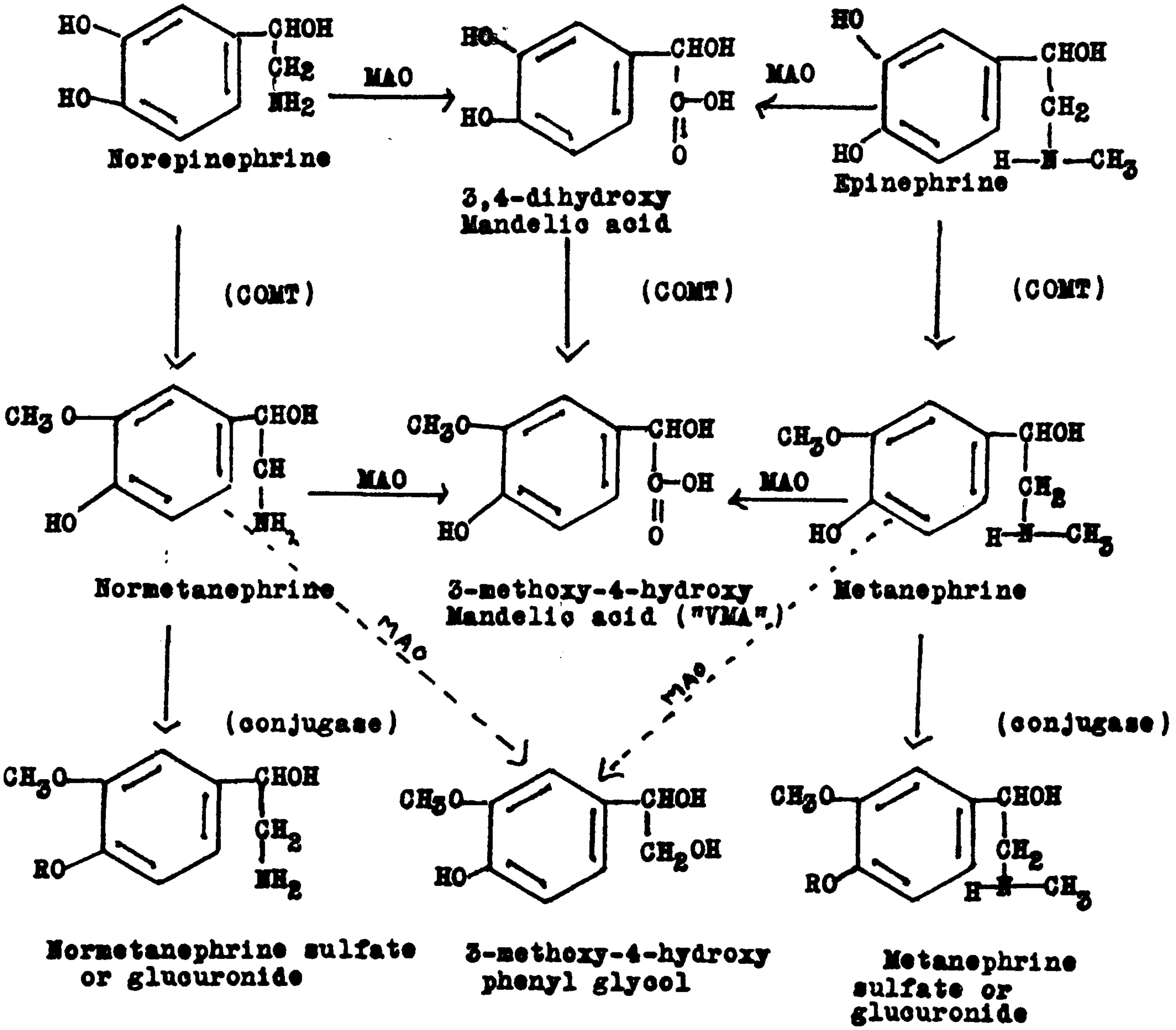
The two enzymes of major importance in the initial steps of metabolic transformation of catecholamines in the mammal are MAO and catechol-o-methyl transferase[20,105] (COMT). Yet it is unlikely that either of these enzymes is of importance in the termination of the effects of adrenergic impulses in relation to other processes such as re-uptake by the axonal terminals, diffusion, temporary absorption to plasma proteins and reflex adjustments. As Blaschko has pointed out, the narrow spatial and temporal limitations of the action of the transmitter that are essential for efficient functioning at most sites of cholinergic transmission probably do not apply to adrenergic transmission at peripheral autonomic effectors.

Both MAO and COMT, which are widely distributed throughout the body, including the brain, have the highest concentrations in the liver and the kidney. There are, however, distinct differences in their cytological location. Whereas MAO is associated chiefly with mitochondria, including those within the terminals of adrenergic fibers, COMT is confined largely to the soluble cytoplasmic fraction and apparently has no selective association with adrenergic nerves.

From a variety of studies in which isotopically labelled catecholamines were used, it appears that most of the epinephrine and norepinephrine that enter the circulation from the adrenal medulla or from exogenous administration, or that is released rapidly from adrenergic fibers is first methylated by COMT to metanephrine or normetanephrine respectively. The norepinephrine that is released slowly, either by drugs such as reserpine or by nerve impulses of low frequency, is probably initially deaminated by the MAO of intra-axonal mitochondria to the corresponding aldehyde, and then converted rapidly at extraneuronal sites to 3,4-dihydroxymandelic acid. In either case, most of the metabolite resulting from attack by the initial enzyme is then converted by the other to the common product, 3-methoxy-4-hydroxymandelic acid, generally but incorrectly called vanillyl mandelic acid (VMA), which constitutes the major metabolite of catecholamines excreted in the urine. The corresponding product of the metabolic degradation of dopamine, which contains no hydroxyl group in the side chain, is homovanillic acid (HVA). In the human brain, and throughout the body of the rat and certain other species the aldehyde oxidation products of MAO are reduced rather than oxidized, resulting in the ultimate formation of 3-methoxy-4-hydroxyphenylglycol.

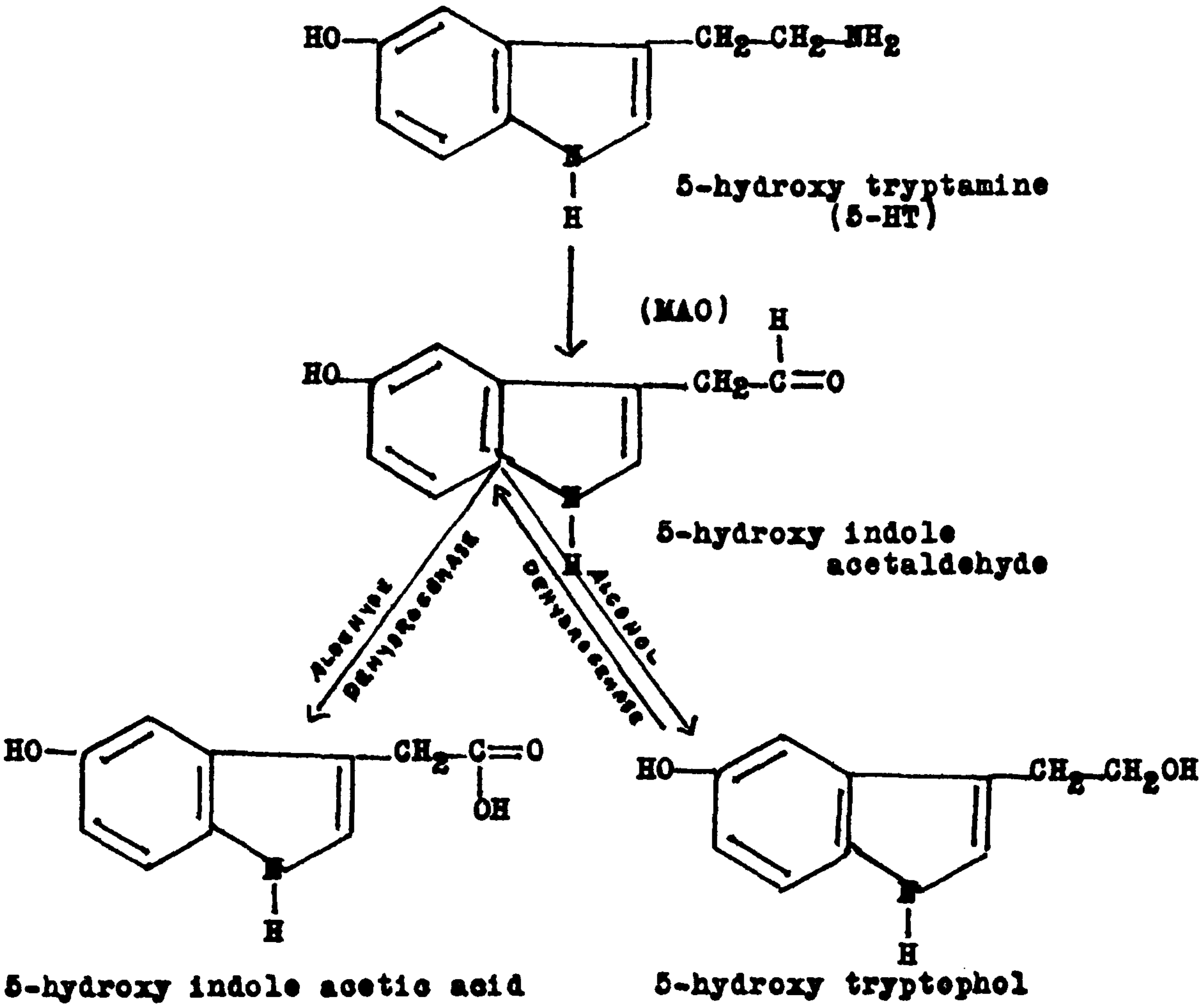
69

Steps in the metabolic disposition of catecholamines



The metabolism of serotonin (5-hydroxytryptamine) varies somewhat from one species to another, but in man, most of it undergoes oxidative deamination by MAO to form 5-hydroxy-indole acetaldehyde, which is promptly degraded, mainly by further oxidation to 5-hydroxyindole acetic acid by aldehyde dehydrogenase.

<u>Metabolic degradation of 5-hydroxytryptamine</u>[69]



L) - Present Status of purification of MAO from various tissues and extracellular sources of various species

Work on the chemistry of amine oxidase has been hampered by the great difficulty in purifying the enzyme from tissues because of its insolubility. Although amine oxidase has been crystallized from extracellular sources such as beef plasma[192] and hog plasma[27], obtained in a highly pure form (450-fold) from rabbit serum[121] and human plasma[120] (5000-fold), and crystallized from a species of fungus (aspergillus[195] niger), it has been much more difficult to purify the enzyme from tissues because of difficulty in solubilizing the enzyme. Cotzias[43,44] and Dole in 1951, and Hawkins[88] in 1952 explained this behavior of MAO by demonstrating the intimate adherence of this enzyme in most organ tissues to the insoluble structures of mitochondrial membranes. It should be mentioned, however, that Weissbach, Redfield and Udenfriend[180] were able to show that guinea-pig liver, unlike other organs and other species, contains MAO in a soluble form. This unique finding was confirmed by Oswald[133] and Strittmatter.

Procedures used to solubilize the enzyme have achieved[6,63,81,196] varying degrees of success. Partially purified MAO has been prepared by various workers by treatment of the mitochondrial membranes with various detergents such as Triton-X, digitonin, cutscum, cholic acid, non-ionic detergents, such as Nonion NS-210, and Tergital-NPX, or with ultrasonic waves, or by disruption of the mitochondria by homogenization and subsequent treatment with Triton X-100. A criterion of solubility, according to Nara, Gomes and Yasunobu[128], is that the enzyme precipitates to the bottom of the tube after the addition of ammonium sulfate, and that the enzyme can be dialyzed against buffer that does not contain any detergent.

Barbato and Abood[6] purified MAO only 20-fold from bovine liver mitochondria. Guha and Krishna Murti[81] achieved an 180-fold purification from rat liver mitochondria. Youdim and Sourkes[197] purified mitochondrial MAO from rat liver 208-fold as compared with the crude homogenate. Nara, Gomes and Yasunobu[128] first reported a 58-fold purification of beef liver mitochondrial MAO. Later Yasunobu et al[67], using the method that is employed in this paper for human liver mitochondrial MAO, isolated three amine oxidase components from beef liver, with the three active fractions having specific activities of 1000-2000,2000-3000, and 6000-8000, respectively. All components were bright yellow, and showed a pH optima near 9.2. None of the components was inhibited by aldehyde reagents, and all components showed similar substrate specificities. The sedimentation coefficients were 14.4 and 20.6, and the molecular weight about 405,000 and 1,280,000 respectively for components 1 and 2. Component 1 contained 4, and component 2 contained 12 FAD or FAD-like substance per mole of enzyme. Components 1 and 2 contained 24 and 106 moles of phospholipid per mole of enzyme respectively. Their studies indicated that a part of the multiplicity of the enzyme reported by other workers is due to the isolation of the enzyme in different molecular weight forms. Components 1 and 2 exhibited peaks at 410 nm and 450 nm, and a shoulder at 480 nm. The ratio of the absorbance at 280/450 nm was 1.1-1.2. The amino acid composition of the enzyme was determined.

Gabay and Valcourt[63] purified rabbit liver monoamine oxidase 270-fold. Erwin and Hellerman[54] outlined a simple preparative procedure for obtaining bovine kidney cortex mitochondrial MAO. R. Sen et al[153] purified MAO from rat heart tissue 89-fold.

There are only a few reports dealing with the purification of brain MAO. Nagatsu[126] was the first to demonstrate that MAO can be extracted from beef brain mitochondria by sonication in the presence of a detergent. Green and Van Niel[80] prepared a partially purified monoamine oxidase (15 fold) from beef brain mitochondria in a soluble stable form by treating the mitochondria with a combination of Triton X-100, sonication and centrifugation at 100,000xg for 60 minutes. The soluble enzyme is concentrated with the Amicon ultrafiltration apparatus. These authors state that further purification by standard techniques of Sephadex and DEAE cellulose chromatography, ammonium sulfate and organic solvent fractionation have thus far been unsuccessful.

Tipton[173] purified MAO from pig brain, with the preparation having a specific activity of 2760. His technique involved a simple, if rather tedious method of repeated sonication, freezing and thawing, and exposure to low pH and alcohol fractionation in the absence of a detergent. The purified enzyme appeared to be homogeneous by cellulose-acetate electrophoresis, and had a molecular weight of approximately 102,000. It was inhibited by iproniazid, and also by p-chloro-mercuribenzoate. Further studies suggested that the enzyme contained FAD as a prosthetic group. The pH optimum was found to be about 7.2, which compares well with values reported by Guha[81,82] for the enzyme purified from rat liver mitochondria, but does not agree with the work of Hare,[87] who found a considerably higher optimum pH for enzyme preparations from the same source.

In regard to the purification of monoamine oxidase from human body fluids or tissues, McEwen[120] purified MAO from human plasma 5000-fold. In 1968 Youdim et al[199] prepared soluble MAO from human placental mitochondria by subjecting the mitochondria to sonication, followed by treatment with Triton X-100, ammonium sulfate fractionation, and column chromatography on Sephadex G-200 and DEAE-Sephadex. A product with a specific activity of 3500, showing a 400-fold purification over the original homogenate may be obtained by this method. When subjected to polyacrylamide-gel electrophoresis, the human placental MAO showed two bands of enzyme activity, whereas solubilized and partly purified MAO from the rat liver, prepared by a similar method, showed 3 bands of enzyme activity.

And lastly, McEwen et al[122] partially purified human liver mitochondrial monoamine oxidase by solubilizing the mitochondria and treatment with Triton X-100 and ammonium sulfate precipitation. Their final preparation had a specific activity of 44, with fold purification of 8. These authors stated that neither Triton X-100 nor sonically disrupted preparations could be purified further by the procedures developed for the beef or rat liver mitochondrial monoamine oxidases.

CHAPTER II

MATERIALS AND METHODS

Tissues

Normal human liver was obtained from patients who had died from accident, suicide or homicide within 3-24 hours after death.

Chemicals and reagents

The calcium phosphate gel, hydroxylapatite, tryptamine hydrochloride, sodium salt of cholic acid, glycine and tris buffer (hydroxymethyl)-aminomethane, serotonin and hydrazine were purchased from Sigma Chemical Company, St. Louis, Mo. DEAE-cellulose was obtained from Bio-Rad. Sephadex G-25 and Sephadex G-200 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. P-nitro-tetrazolium blue (nitro-BT) was obtained from General Biochemicals. Monobasic and dibasic potassium phosphate were obtained from Fisher Scientific Co., Fair Lawn, N.J. The Edman reagent (phenylthiocyanate) was obtained from Eastman Organic Chemicals (Division of Eastman Kodak, Rochester, N.Y.). PTH-amino acid standards were purchased from Pierce Chemical Co. Dimethyl allyl amine (coupling buffer in Edman procedure) was obtained from Pfaltz Bauer, Inc., Flushing, N.Y. Trifluoroacetic acid was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. Vacuum dialysis bags and apparatus were purchased from Schleicher and Schuell, Inc., Keene, New Hampshire. The polyacrylamide-gel electrophoresis apparatus was supplied by Canal Industries Corp. (Bethesda, Maryland).

Instrumentation

The enzyme assays and protein determinations were carried out, using a Beckman model DU spectrophotometer. A Radiometer pH meter, made in Copenhagen, was employed. The Beckman model L ultracentrifuge was used for preparative procedures. The Sorvall Model RC-2B refrigerated centrifuge was used for all other centrifuge runs. The spectrofluorometric determinations were obtained in the Aminco-Bowman spectrophotofluorometer.

Analytical procedures

Protein estimation. The protein concentration was determined by the method of Lowry[116] et al, using bovine serum albumin as a standard.

Enzyme assay. The enzyme was assayed spectrophotometrically with benzylamine hydrobromide as the substrate[169]. A unit of enzyme activity is defined as the amount of enzyme catalyzing an increase of 0.001 absorbance unit per minute at 25°. The assay mixture contained 0.1 ml of enzyme, 0.5 ml of benzylamine hydrobromide containing 20 uM/ml (final concentration of 3.3 uM/ml), and 2.4 ml of 0.05 M phosphate buffer, pH 7.4 in a total volume of 3 ml.

The protein stain reagent contained 1% amido black in 7% acetic acid.

Discontinuous polyacrylamide-gel disc electrophoresis was performed according to the method of Davis[47], and employing apparatus and reagents from Canalco, Bethesda, Maryland. A concentrated tris-glycine buffer at pH 8.3 was used. (Tris-6.0 grams, glycine-28.8 grams, with water to 1 liter, pH 8.3).

[146] The enzyme stain for the polyacrylamide-gel electrophoresis contained tryptamine, 112 mg; sodium sulfate, 18 mg; nitro-BT, 22.5 mg; 0.1 M phosphate buffer, pH 7.4, 22.5 ml, and distilled water to a total volume of 90 ml.

Ultracentrifugation. Sedimentation velocity was performed in a Spinco model E analytical ultracentrifuge at 59,780 rpm in a synthetic boundary cell at a temperature of 20°. Photographs were taken at 16 min. intervals. The movement of the schlieren peaks was measured by a Nikon Shadowgraph Model 6C micro-comparator. The sedimentation coefficient was calculated by the method described by Schachman.[149]

All buffers used for the purification of the enzyme were KH_2PO_4, K_2HPO_4 buffers, pH 7.4 or 7.6 at the indicated concentrations. Sørensen's glycine-sodium hydroxide buffer (0.1 M glycine; 0.1 M NaCl) was made up as follows: 7.505 g glycine plus 5.85 g NaCl to 1 liter with distilled water, adjusted to pH's in the range 9-12 with 0.1 M NaOH.

TLC plates (20x20 pre-coated silica gel with fluorescent indicator) were obtained from Mann Research laboratories, NYC.

Methods

Unless otherwise noted, all operations were performed at 4° in the cold room.

Human liver MAO was purified by two different methods.

Method #I: an original method which does not depend on the isolation of mitochondria, and in which vacuum dialysis, during which the enzyme separates out as a yellow precipitate, is an important step in purification.

All previous methods of obtaining the enzyme from animal tissues, and even from human liver have been based upon the isolation and subsequent solubilization of mitochondria. In purifying this enzyme from human tissues such as liver, however, one is faced with special problems not encountered in animals, where normal tissues can be obtained easily at any time after death, and where the animals' habits, diet, etc. can be carefully controlled. It is not generally appreciated that one cannot isolate intact mitochondria by the method of Hogeboom and Schneider[152] more than a few hours after the tissue is removed from its host because not only cellular debris, but the entire homogenate not infrequently sediments at speeds as low as 200xg. Since autopsies, even in accident cases, are often not performed for 12-24 hours after death, and since some of these cases are addicts on drugs which may interfere with the procedure of obtaining mitochondria, with considerable loss of enzyme, methods of obtaining the enzyme from human tissues by isolation and subsequent solubilization of mitochondria are not practicable.

To obviate this difficulty, and to employ a much simpler method of obtaining the enzyme, a procedure was devised (Method #I) of isolating the enzyme by subjecting the whole liver homogenate to high speed centrifugation to effect complete sedimentation of all solid particles, and, at the same time, to get rid of the hemoglobin in the supernatant, and then extracting the sediment, which contains most of the enzyme, with the detergent Triton X-100, followed by the usual techniques of ammonium sulfate precipitation, column chromatography with DEAE-cellulose and Sephadex G-200. The final step of purification, namely, vacuum dialysis, is a most important procedure in purification, as the high molecular weight enzyme, or possibly the aggregated enzyme, settles out as a yellow powder. The supernatant, which may contain protein impurities, can be removed, and the process repeated, if necessary, several times, after reconstitution of the enzyme with phosphate buffer.

Method #II: by the classical method of obtaining the enzyme by solubilization of mitochondria, using the procedure employed by Yasunobu et al[67] for the purification of beef liver MAO.

By both methods, the same result was obtained, namely, a preparation showing three active bands on polyacrylamide disc electrophoresis. By Method #I a single peak was obtained in the ultracentrifuge.

Since monoamine oxidase is quite insoluble, and requires a detergent for solubilization, a considerable amount of preliminary work was done, testing out the efficacy of various detergents used by previous investigators, such as cutscum, cholic acid, digitonin, non-ionic detergents, such as Nonion NS-210, Triton X-100 and Tergital-NPX, with the finding that digitonin is the most effective solubilizing agent. Although a great deal of preliminary work was done, using digitonin, this substance was eventually abandoned because it was difficult to keep in solution, and tended to settle out in purified preparations. Attempts to solubilize the enzyme by means of sonication resulted in almost complete loss of activity. Since monoamine oxidase is associated with the mitochondrial membrane, the butyl alcohol method of extraction was tried, but was unsuccessful as this substance caused denaturation of the enzyme. Sodium pyrophosphate buffer, with its marked dissociating effect on hydrogen bonds and salt linkages, as well as its chelating effect on many divalent cations, and protective effect, was not found to be superior to 0.1 M potassium phosphate buffer.

Purification of the Enzyme

Method #I

Step 1 - Homogenization of human liver (from Case #5)

90 grams of presumably normal human liver were washed with distilled water to clean off surface blood, cut into small pieces with scissors, and put through a meat grinder before homogenization with 6 volumes (w/v) of 0.1 M phosphate buffer, pH 7.4 in a Waring blender for 1½ minutes.

Step 2 - Centrifugation of 1: 6 homogenate

The homogenate was then centrifuged in the Model L ultracentrifuge, using Rotor 30, at 24,000 rpm (approximately 50,000xg) for 1 hour. The supernatant, which contained very little enzyme, and most of the hemoglobin, was discarded. This procedure was repeated, washing the sediment with 500 ml of 0.1 M phosphate buffer, pH 7.4, to free it from additional hemoglobin by pressing the particles against the side of a beaker with a rubber spatula, and again centrifuging in a similar manner, with discarding of the supernatant.

Step 3 - Extraction of the sediment with 1.5% Triton X-100. The enzyme was then extracted from the sediment by adding 0.1 M phosphate buffer, pH 7.4 to a fixed volume of 200 ml, and then adding 16 ml of a 20% solution of Triton X-100 (made up in 0.1 M phosphate buffer, pH 7.4), gently and briefly homogenizing with a Waring blender for ½ minute, and then by hand, in batches, with a Potter-Elvehjem glass homogenizer for 20 strokes, gently stirring the solution with a magnetic stirrer for 1½ hours, and then centrifuging at 15,000 rpm (about 20,000xg) for 1 hour in a Beckman Model L ultracentrifuge. The slightly opalescent amber supernatant was carefully removed from the sediment by means of a long 18 gauge spinal needle and syringe. The sediment was extracted three more times with the Triton X-100 in a similar manner, and the supernatants from the 2nd and 3rd extractions pooled.

Step 4 - 40% ammonium sulfate precipitation of enzyme in Triton X-100 extract. To the pooled Triton X extracts, solid ammonium sulfate was added to 40% saturation, and the pH of the solution adjusted to 7.4 by the addition of 5N NH_4OH. After stirring for 30 minutes, the solution was centrifuged for 20 minutes at 10,000 (16,300xg) in the Sorvall RC-2B. The tan precipitate (enzyme) floated to the surface, and was collected by removing the liquid under the precipitate with a syringe and long 18 gauge spinal needle. The precipitate was dissolved in a minimal amount of 0.1 M phosphate buffer, pH 7.4, producing a somewhat turbid solution.

The enzyme solution was passed through a Sephadex G-25 column (4.5x45 cm) in order to remove excess ammonium sulfate, eluting with 0.01 M phosphate buffer.

Step 5 - DEAE-cellulose chromatography. (For elution pattern, see Figure 1).

The eluate was applied to a DEAE-cellulose column (2.2x45 cm) which had been equilibrated with 0.01 M phosphate buffer, pH 7.4. After the addition of enzyme to the column, the column was treated with approximately 500 ml of starting buffer, followed by approximately 500 ml of 0.1 M phosphate buffer, pH 7.4. The enzyme was then eluted from the column, using a linear gradient of Triton X-100. Gradient elution (900 ml of 0.1 M phosphate buffer, pH 7.4 in the mixing chamber, and 900 ml of 0.2% Triton X-100 in 0.1 M phosphate buffer, pH 7.4 in the reservoir) was used to elute the enzyme. Fractions of 8 ml were collected in a Buchler fraction collector at a flow rate of approximately 0.5 ml/min.

Step 6 - 40% ammonium sulfate precipitation of enzyme in pooled fractions after DEAE-cellulose chromatography. The combined active fractions (185 ml) from tubes 65-88 were concentrated by the addition of solid ammonium sulfate (40% saturation), and centrifugation performed at 10,000 rpm (16,300xg) for 20 minutes. After centrifugation, the enzyme, which floated on the surface of the solution, was dissolved in a minimal amount of 0.1 M phosphate buffer, pH 7.4.

Step 7 - Sephadex G-200 chromatography (For elution pattern, please see Figure 2). The dissolved ammonium sulfate precipitate was placed on a Sephadex G-200 column (2.2x45 cm) equilibrated with 0.05 M phosphate buffer, pH 7.4. The enzyme was then eluted from the column by means of 0.05 M phosphate buffer, pH 7.4. Fractions were collected in a Buchler fraction collector by the drop method (120 drops per tube). As can be seen, the activity is eluted between fractions 21-40, with maximum activity in tubes 28-35.

Step 8 - Vacuum dialysis of pooled fractions after Sephadex G-200, removal of supernatant and reconstitution of enzyme with 0.05 M phosphate buffer, pH 7.4.

Vacuum dialysis was performed on the pooled tubes from Sephadex G-200 chromatography, and the solution dialyzed against 0.01 M phosphate buffer, pH 7.4, with apparatus kept in an ice water bath. As dialysis progressed, the enzyme came out of solution and settled as a bright yellow powder at the bottom of the dialysis bag. When the solution had been concentrated down to 10 ml, the vacuum was discontinued, and the solution permitted to settle in the dialysis bag in the dialysis solution for 24 hours. The supernatant was then carefully drawn off by means of a spinal needle or very fine Tygon tubing attached to a hypo syringe, and the yellow enzyme powder at the bottom of the tube dissolved in 9 ml of 0.05 M phosphate buffer.

<u>The purification procedure is summarized in Table I.</u>

Method #II

Step 1 - Mitochondrial homogenate (from Case #9)

1200 grams of presumably normal human liver were washed with distilled water to clean off surface blood, cut into small pieces with scissors, and put through a meat grinder before homogenization with 9 volumes of 0.25 M sucrose in a Waring blender for 1½ minutes. The mitochondria were then isolated according to the method of Hogeboom and Schneider, washed until colorless with 0.01 M phosphate buffer, pH 7.4, collected by centrifugation (5000 rpm for 15 minutes), and resuspended in 0.1 M phosphate buffer, pH 7.4. The suspension was homogenized in batches for 2 minutes in a glass Potter-Elvehjem homogenizer, with a total volume after homogenization of 500 ml.

Step 2 - Extraction and first ammonium sulfate fractionation

To the mitochondrial homogenate (500 ml), 40.8 ml of 20% Triton X-100 (made up in 0.1 M buffer, pH 7.4), and 42.3 grams of ammonium sulfate were added so that the final concentrations were 1.5% and 15% saturated, respectively. After the addition of the ammonium sulfate, the pH was adjusted to approximately 7.4 with 5N NH_4OH. The mixture was stirred with a magnetic stirrer gently for 1.5 hrs. and then centrifuged for 45 minutes in a Sorvall RC-2B at 11,000 rpm or (20,000xg), discarding the precipitate.

Step 3 - 0.15-0.40 saturated ammonium sulfate

To the yellow supernatant (420 ml), 76.3 grams of solid ammonium sulfate (40% saturation) were added, and the solution was adjusted to pH 7.4 by the addition of 5N NH_4OH. After stirring for 30 minutes, the solution was centrifuged for 20 minutes at 11,500 rpm (20,000xg). The tan precipitate (enzyme) floated to the surface, and was collected by removing the liquid under the precipitate with a syringe and long spinal needle. The precipitate was dissolved in a minimal amount of 0.1 M phosphate buffer, pH 7.4. The solution containing the dissolved tan-yellow precipitate (211 ml) was turbid at this stage.

Step 4 - Second ammonium sulfate fractionation

After adjusting the protein concentration to 10 mg/ml by the addition of 0.1 M phosphate buffer, pH 7.4, 8.1 ml of 20% sodium cholate (0.4 mg cholate per mg of protein) were added. After determination of the ammonium sulfate concentration by the Nessler procedure, 35.9 grams of ammonium sulfate were added to the solution (25% saturation). The material was then centrifuged for 30 minutes (11,500 rpm or 20,000xg), and the precipitate discarded.

To the filtrate, after checking the concentration of ammonium sulfate, 33.6 grams of ammonium sulfate (40% saturation) were added, and the solution stirred for 30 minutes. Centrifugation for 30 minutes was then carried out at 10,000 rpm (16,300xg). The reddish-brown precipitate (color due to cytochrome C impurity), floated to the top, and the liquid phase was discarded. The precipitate was dissolved in a minimal amount of 0.1 M phosphate buffer, pH 7.4, and the solution stirred for 30 minutes. (Total volume - 244 ml).

Step 5 - Calcium phosphate gel eluate after ammonium sulfate

The reddish-brown solution was then dialyzed against 6 liters of 0.01 M phosphate buffer, pH 7.4, (with change at end of 2 hours, a second change at the end of three hours, and then overnight dialysis). The solution was turbid at the beginning of dialysis, possibly due to the high concentration of ammonium sulfate. The enzyme solution was then adjusted to a protein concentration of 10 mg/ml by the addition of 0.01 M phosphate buffer, pH 7.4. A total of 235 ml of calcium phosphate gel (33.5 mg/ml dry weight, with CaP gel: protein ratio of 3:1) were added to the enzyme solution. The mixture was stirred gently for 15 minutes and then centrifuged for 20 minutes at 8500 rpm (11,700xg). The precipitate was saved for further studies.

The resulting yellow supernatant was treated with calcium phosphate gel, with CaP: protein ratio of 1:1, stirred for 15 minutes, and centrifuged at 9000 rpm (13,200xg) for 15 minutes. Lastly, the supernatant was treated with CaP gel: protein ratio of 4:1, then stirred for 45 minutes, and centrifuged for 20 minutes at 10,000 rpm (16,300xg). There was no activity in the yellow supernatant.

The gel obtained by centrifugation was eluted successively after stirring for 15-30 minutes with 200 ml each of 0.1 M and 0.2 M phosphate buffer, pH 7.6, and each centrifuged for 15 minutes at 9000 rpm (13,200xg). Approximately 400 ml of combined eluate was obtained and concentrated by the addition of 90.4 grams of ammonium sulfate (40% saturation). After centrifugation for 30 minutes at 10,000 rpm (16,300xg), the floating, frothy yellow precipitate was dissolved in 60 ml of 0.1 M phosphate buffer, pH 7.4.

Step 6 - DEAE-cellulose chromatography after Sephadex G-25
(For elution pattern, see Figure 3)

The enzyme solution was passed through a Sephadex G-25 column (4.5x45 cm) in order to remove excess ammonium sulfate, eluting with 0.01 M phosphate buffer. The eluate (100 ml) was applied to a DEAE-cellulose column (2.2x45 cm) which had been equilibrated with 0.01 M phosphate buffer, pH 7.4. After the addition of enzyme to the column, the column was treated with approximately 500 ml of starting buffer, followed by approximately 500 ml of 0.1 M buffer. The enzyme was then eluted from the column, using a linear gradient of Triton X-100. Gradient elution (900 ml of 0.1 M phosphate buffer, pH 7.4 in the mixing chamber, and 900 ml of 0.2% Triton X-100 in 0.1 M phosphate buffer, pH 7.4 in the reservoir) was used to elute the enzyme. Fractions of 8 ml were collected in a Buchler fraction collector at a flow rate of approximately 0.5 ml/min. The combined active fractions (360 ml) from tubes 43-88 were concentrated by the addition of 81.4 grams of solid ammonium sulfate (40% saturation), and centrifugation performed at 10,000 rpm (16,300xg) for 20 minutes. After centrifugation, the enzyme, which floated to the surface, was dissolved in a minimal amount of 0.1 M phosphate buffer, pH 7.4. The solution was desalted on a column of Sephadex G-25, equilibrated with 0.01 M phosphate buffer, pH 7.4 (with eluate-75 ml).

Step 7 - Hydroxylapatite chromatography (<u>For elution pattern, see Figure 4</u>).

The slightly brownish-yellow enzyme solution (75 ml) was then absorbed onto an hydroxylapatite column (2.9x19 cm), equilibrated with 0.01 M phosphate buffer, pH 7.4. The column was then washed with about 200 ml of the same buffer. Fractionation of the enzyme was achieved by stepwise elution with the following buffers:

Fraction 1 - 0.01 M phosphate buffer, pH 7.4 (100 ml)
This fraction with zero enzyme activity is not shown on the figure.

Fraction 2 - 0.1 M phosphate buffer, pH 7.6
(100 ml)

Fraction 3 - 0.2 M phosphate buffer, pH 7.6
(100 ml)

Fraction 4 - 0.2 M phosphate buffer, pH 7.6
plus 0.15% potassium cholate
(100 ml)

Fractions of 6.2 ml were collected at a flow rate of 0.3 ml/min. The fractions were collected separately. The active tubes from Fraction 2 (by far the most active fraction) were pooled and subjected to further study.

A summary of the steps in the purification of human liver monoamine oxidase by Method #II is shown in Table II.

CHAPTER III

RESULTS AND DISCUSSION

Comment on purification procedures

Human liver mitochondrial monoamine oxidase was partially purified by McEwen et al[122] in 1968 by solubilizing the mitochondria with Triton X-100, sonification and ammonium sulfate precipitation. The final specific activity of their preparation was 44.

This paper describes the purification of the enzyme by two different methods, - an original and simple method (Method #I), and the more conventional procedure of obtaining the enzyme by solubilization of mitochondria as used by Yasunobu et al[67] for the purification of beef liver MAO (Method #II). In both cases, the end result is the same, namely, the production of three active bands on polyacrylamide disc electrophoresis. The pure enzyme obtained by Method #I showed a single peak in the analytical ultracentrifuge. The final specific activity of the enzyme produced by Method #I was 550, with fold purification of 42. By Method #II, the final specific activity was 1010, with a fold purification of 12. On hydroxylapatite chromatography, the main fraction (fraction 2), with highest specific activity of 1010, was eluted with 0.1 M phosphate buffer, pH 7.6, whereas fractions 3 and 4 were obtained with 0.2 M phosphate buffer, pH 7.6 and 0.2 M phosphate buffer, pH 7.6 plus 0.15% potassium cholate, respectively. The specific activity of fraction 3 was 600, and of fraction 4 was 177. No enzyme activity was found in fraction 1, obtained by elution with 0.01 M phosphate buffer, pH 7.4.

Method #I was found to be much simpler to perform, and about seven times as efficient. In this regard, it is of interest that, using Method #II, 1200 grams of liver have to be processed to obtain 3000 units of enzyme (2.97 mgs protein), whereas by Method #I, 1430 units of enzyme and 2.6 mgs of protein are obtained from only 90 grams of liver. Furthermore, this new procedure eliminates the cumbersome treatment with calcium phosphate gel, with the attendant loss of 300,000 units of enzyme in one step.

Contrary to the usual practice of performing Sephadex G-200 chromatography initially, followed by DEAE-cellulose chromatography, the reverse procedure is used. The purpose of this is to remove most of the Triton X-100 on Sephadex G-200 chromatography to render the enzyme as insoluble as possible in order to facilitate sedimentation on vacuum dialysis.

And lastly, the above new purification procedure takes advantage of the marked insolubility of the enzyme by using vacuum dialysis, during which the pure enzyme settles out as a yellow powder.

Properties of the enzyme

Sedimentation Study

The results of a sedimentation velocity run are presented in Figure 5. The sedimentation coefficient of the pure enzyme obtained by Method #I was found to be 6.78 at a temperature of 20, with protein concentration of 0.75 mg/ml in 0.05 M phosphate buffer, pH 7.4. In order to obtain good ultracentrifuge patterns, the protein concentration must be kept below 2 mg/ml as the enzyme tends to aggregate and precipitate when the concentration is higher.

Electrophoresis

Polyacrylamide-gel disc electrophoresis was performed on the pure enzymes obtained by Methods #I and #II.

One tube was stained for protein with 1% amido black in 7% acetic acid, and the other tube, run concomitantly, stained specifically for the enzyme, using a method hitherto only employed for the histochemical demonstration of monoamine oxidase[146], i.e. using tryptamine as a substrate and nitro-BT as electron acceptor, with rapid production of purple-pink formazan. The tubes to be stained for enzyme were incubated at 37° for 1 hour. (The reaction can be accelerated by heating at a higher temperature).

Method #I - Electrophoretic patterns

a) 7.0% standard gel at pH 9.5. Under these conditions, 3 distinct active bands were found only in the stacking gel, with a heavy band at the origin, a lighter band close to the origin, and a fairly heavy band at the junction between the stacking and running gel. Only 2 bands were seen with the protein stain, - one at the origin, and the other at the junction between the stacking and running gels. (See Figure 6 Ia).

b) 2.5% gel at pH 9.5. Three bands were again seen in the enzyme preparation, - a heavy one at the origin, and at the junction between the stacking and running gel, and a much lighter one far down into the running gel, almost at the end of the tube. Only 2 bands, - one at the origin, and the other at the junction between the stacking and running gels were seen with the protein stain. (See Figure 6Ib)

Method #II - Electrophoretic patterns

a) 7.0% standard gel at pH 9.5. Three distinct active enzyme bands were found only in the stacking gel, with a heavy band at the origin, a band at the junction between the stacking and running gel, and a smaller band halfway between these two. (See Figure 6IIa).

b) Two weeks later, using a 3.5% gel at a running pH of 9.5, a band was seen at the origin, one at the junction between the stacking and running gel, and now only a smudge in between, suggesting that perhaps aggregation of the enzyme was taking place with time. (See Figure 6IIb).

c) 2.5% gel, at pH 9.5. Two light bands were seen, one at the junction between the stacking and running gel, and the second moving 3 mm into the running gel. There was also a large dark band at the origin in the stacking gel. (See Figure 6IIc).

There was complete denaturation of the protein and no enzyme stain produced with a running pH of 4.3 and a 5.0% gel.

A single component was observed when components 1 and 2 of beef[67] liver MAO were analyzed by starch zone electrophoresis. Component 1 showed a single band when examined by disc electrophoresis at pH 9.0. Considerable difficulty was observed with component 2, however, when examined by the latter two methods as it tended to aggregate rapidly, and partially precipitated out of solution.

Beef brain monoamine[85] oxidase showed 2 components on continuous-flow electrophoresis, - a slow-moving major component (enzyme 1), showing a higher specific activity, and a fast-moving, minor peak (enzyme 2), with a lower specific activity, with the major component having a stronger color. The enzymes 1 and 2 did not appear to be different molecular weight forms, since both components had the same molecular weight as judged by Sepharose 6B gel filtration.

A major and a minor component of pig[172,173] brain mitochondrial MAO was separated on Sephadex G-200 chromatography.

In the above study with human liver MAO, although 3 active bands were seen on the purified enzyme preparations obtained by both methods, as can be seen, there were some differences in the positions of the active bands, a finding perhaps due to the different age of the enzyme preparations, to greater aggregation, etc. The above results suggest that on electrophoresis the enzyme is broken down into fragments in which it exists in different aggregated states. Other possibilities are that the bands represent isozymes, or that the three components belong to the same enzyme protein in the mitochondria, and are derived by an ability of the detergent to alter the structure of the protein.

pH optimum

The effect of pH on the reaction velocity at 27° was investigated over the pH range of 5.45-11.80, using 3 different buffer systems (0.5 M phosphate buffer, from pH 5.40-7.80; 0.2 M tris (hydroxymethyl)-amino-methane from pH 7.8-9.0; and Sorensen's 0.1 M glycine-sodium hydroxide buffer from pH 9.0-12.00). The standard reaction mixture consisted of 0.3 ml of buffer, 0.1 ml of enzyme, and 0.5 ml of benzylamine hydrobromide (final concentration-3.3 umoles/ml) in a total reaction mixture of 3.0 ml adjusted with water. In this experiment, the pH of the buffer was varied as shown in Figure 7.

The enzyme exhibited optimal activity at pH 8.65, with no activity below pH 5.45 or above pH 11.80.

Review of the literature reveals that the above results differ considerably from those reported for MAO in various organs and various species, with the exception of bovine kidney[54] MAO, which exhibits optimal activity at approximately pH 8.5. The pH optima of components 1 and 2 of purified beef[67] liver MAO was found to be about pH 9.1-9.2, which is in agreement with the value reported earlier by Hare[87] (1928), and are different from those reported by other workers.[128] These authors also investigated the effect of pH on the stability of the enzyme, with the enzyme most stable at pH 7, and least stable at pH 8.9. The maximal activity of purified MAO from rabbit[63] liver is obtained at pH 8.4, with the substrate kynuramine, and about 9.2[63] with another substrate, m-iodobenzylamine. The optimum pH of rat heart MAO was reported to be 7.0[153], and the pH optimum to be approximately 8.8 for beef brain[85] MAO.

Michaelis constant

Using a reaction mixture consisting of 0.3 ml of 0.5 M phosphate buffer, pH 7.4, 0.1 ml of enzyme, and an aliquot of substrate (benzylamine hydrobromide) varying from 0.05-0.80 ml in a total volume of 3.0 ml adjusted with water, with assay time 20 minutes, the Michaelis constant determined by the method of Lineweaver and Burk[114], was found to be $1.7x10^{-3}$M. The velocity was expressed as the change in absorbance at 250 nm during a reaction time of 20 minutes. <u>The results are illustrated in Figure 8</u>.

According to Yasunobu et al[196], the K_m for beef liver MAO, using benzylamine as a substrate at pH 7.4 was $2.2x10^{-4}$M. The K_m value for beef plasma MAO[192] for benzylamine was $1.5x10^{-3}$M at pH 7.2, with K_m value varying with pH as well as ionic strength. Oswald and Strittmatter[133] found the K_m for benzylamine for rat liver to be $1.8x10^{-3}$M.

McEwen et al[122] obtained a K_m for benzylamine for human liver MAO at pH 7.4 of $4x10^{-4}$M. These authors[121,122] found that Michaelis constants depend upon the pH value of the determination, and that the effect of pH upon apparent Michaelis and inhibitor constants indicates that the non-protonated species of these substances interact with the enzyme active center, which contains an electrophilic binding site for substrate and inhibitor.

In regard to the K_m for natural substrates such as tyramine, tryptamine, octopamine, serotonin and the catecholamines, Oswald and Strittmatter[133] found the K_m to be $8.5x10^{-4}$M for tyramine and $1.4x10^{-3}$M for serotonin for rat liver; $1.6x10^{-3}$M for tyramine and $2.7x10^{-3}$M for serotonin for guinea pig liver. These authors also found the K_m to be $3.2x10^{-4}$ M with tyramine and $1.9x10^{-3}$ M with serotonin for guinea pig kidney.

<u>Inhibition study</u>

1) Carbonyl reagents. There was no

inhibition by cyanide, semicarbazide, hydroxylamine or aminoguanidine.

2) Thiol inhibitors

Reaction mixtures consisting of 0.3 ml of 0.5 M phosphate buffer, pH 7.4, 0.1 ml enzyme, 0.2 ml inhibitor (0.05 M N-ethyl maleimide or NEM, with final concentration of 3.3×10^{-3}M or 0.03 M iodoacetic acid, with final concentration of 2×10^{-3}M), and water-1.9 ml were prepared. After incubation at 23° in a water bath for the periods shown in Figure 9, 0.5 ml of benzylamine were added to each sample, and the enzyme activity determined spectrophotometrically over a 10 minute period. For each time interval, enzyme samples were incubated as above, with 0.2 ml water substituted for the inhibitor, and the activity was compared with samples containing inhibiter.

To determine whether there was any reaction between either inhibitor and the substrate, benzylamine, reaction mixtures containing 0.3 ml of 0.5 M phosphate buffer, pH 7.4, 0.5 ml benzylamine, 0.2 ml inhibitor (either NEM-3.3×10^{-3}M or iodoacetic acid 2×10^{-3}M final concentrations) and water-2 ml were prepared, and the change in optical density at 250 nm determined over a 10 minute period.

Although there was virtually no reaction between the iodoacetic acid and benzylamine, there was considerable reaction between the NEM and benzylamine, as reflected in an average change in optical density of 0.050/min.

A final concentration of 2×10^{-3}M iodoacetic acid caused 48% inhibition of the enzyme at 110 minutes. The enzyme was not inhibited at all by 4.1×10^{-4}M of iodoacetic acid. (See Figure 9).

Although a final concentration of $3x10^{-3}$ M of NEM caused 65% inhibition at 111 minutes, the results with this inhibitor were not considered valid because of the above reaction with the substrate.

The inhibition by thiol reagents suggests that human liver MAO has one or more essential SH groups.

Temperature-activity curve

After bringing a reaction mixture consisting of 0.1 ml of enzyme and 2.4 ml of 0.05 M phosphate buffer, pH 7.4 to the requisite temperature, the substrate, 0.5 ml of benzylamine hydrobromide, was added, and the enzyme activity determined over a 10 minute period at various temperatures from 0-80°.

As can be seen from Figure 10a, there was no activity at 0°. Maximal activity occurred at 60°, after which there was a progressive decline, with loss of all activity above 80°. At 65°, 70° and 75°, the activity was destroyed after 5 minutes.

After assaying its activity at room temperature (23°), the enzyme was pre-incubated for 15 minutes in a mixture consisting of 0.1 ml enzyme and 2.4 ml of 0.05 M phosphate buffer, pH 7.4 at various temperatures ranging from 70°-23°C, cooled rapidly to 23°, and then assayed as above by adding 0.5 ml benzylamine hydrobromide. (See Figure 10b). Human liver MAO was found to be completely inactivated at 70°, 70% denatured at 50°, 47% at 37°, and only 5% at 23°.

This is in sharp contrast to the observations of Yasunobu et al[128], who found that when beef liver MAO was pre-incubated at temperatures between 25-30° for 15 minutes, cooled at 25°, and then assayed at pH 7.4, the enzyme was inactivated at temperatures above 30°.

Oswald and Strittmatter found that when rat liver was incubated for 10 minutes at 55°, then quickly chilled and assayed for MAO with benzylamine, there was 40.5% of the original activity at 55°, and only 0.8% at 60°.

For an Arrhenius plot, please refer to Figure 10e.

Stability of enzyme (See Figure 11)

As depicted in curve A, the frozen ammonium sulfate precipitate, dissolved in 0.1 M phosphate buffer, pH 7.4, lost 17% of activity at the end of 20 days, and about half its activity at the end of 2 months. A 1:20 dilution of the original liver homogenate in 0.1 M phosphate buffer, pH 7.4, when kept at 4°, lost 42% of its activity at the end of 20 days (curve B). A 1:20 dilution of the original homogenate in 2M ammonium sulfate showed a slower decline in activity than a similar preparation in 0.1 M phosphate buffer, pH 7.4, with 30% loss at the end of 20 days (curve C). At room temperature, a 1:20 dilution of the original homogenate in 0.1 M phosphate buffer, pH 7.4 showed rapidly diminishing activity, with eventual complete loss at the end of 2 weeks (curve D).

From the above findings, it is clear that human liver MAO is a stable enzyme.

Prosthetic groups of human liver
Monoamine Oxidase

A. Pyridoxal

Since the enzyme is not inhibited by carbonyl reagents, as one would expect, it does not contain pyridoxal as a cofactor as shown by the following experimental evidence:

According to the method of Rabinowitz and Snell, 1 ml of highly purified enzyme was hydrolyzed with 180 ml of 0.055 N HCl in an autoclave under 20 lb. pressure for 5 hours to ensure maximal release of pyridoxal, if present, from the apoenzyme. After adjusting the pH to 7.4, 0.1 ml of 0.03 M KCN in 0.2 M sodium phosphate, pH 7.4, was added to a 3 ml aliquot of the above solution, according to the method of Bonavita et al[31] in order to form the fluorescent cyanohydrin derivative of pyridoxal. After incubating for 48 hours at 25°, and employing similarly treated reagent blanks, with final concentration of 2.5 ug/ml (8×10^{-5}M), the fluorescence of the solution was read in an Aminco-Bowman spectrophotofluorometer with activation at 358 nm and emission at 430 nm.

If pyridoxal was present, one would expect to obtain the fluorescent cyanohydrin derivative. Failure to form this compound was experimental evidence that human liver monoamine oxidase does not contain pyridoxal as a cofactor.

B. FAD

Human liver monoamine oxidase is a flavo-enzyme, as shown by the following experimental evidence:

When a highly purified enzyme preparation in 0.05 M phosphate buffer, pH 7.4 was excited at 450 nm, there was no emission at 530 nm as there was with a standard synthetic preparation of FAD (20 ug/ml).

When, however, the enzyme was incubated for 10 minutes at 40° with 1% sodium dodecyl sulfate (lauryl sulfate) and the solution centrifuged for 3 minutes at 3000 rpm, the fluorescence emission spectrum of the supernatant was similar to that of the above preparation of synthetic FAD under the same experimental conditions, showing that human liver MAO is a flavoenzyme and possibly bound firmly to the apoenzyme. (See Figure 12a).

When the holoenzyme was reduced with benzylamine or sodium dithionite under anaerobic conditions by flushing through with nitrogen for 20 minutes, with stirring of the sample at 2°, and the enzyme then treated as above with sodium dodecyl sulfate, the fluorescence at 520 nm was depressed, as shown in Figure 12a. The decrease in fluorescence produced by benzylamine and sodium dithionite was about 40%. Since it has been well-established that reduced flavins (FAD and FMN)[190] do not fluoresce at 520 (530 nm) when activated at 450 nm, the persistence of fluorescence, although diminished, could have been due to the fact that the quenching was not complete.

The fluorescence activation spectrum of human liver monoamine oxidase and of FAD alone were determined by activation at 517 nm. (See Figure 12b).

To determine the number of milligrams of FAD/milligram of protein, 1% sodium dodecyl sulfate was added to 1.2 ml of a highly purified enzyme preparation containing 1.14 mgs protein, and the solution incubated at 40° for 10 minutes. The preparation was then centrifuged for 3 minutes at 3000 rpm, and the supernatant carefully removed by means of a syringe and long spinal needle, measured, and brought to a volume of 3 ml. The optical density of this solution was read at 375 nm, and the amount of FAD/ml calculated from an FAD calibration curve. (See Figure 14). From this, the number of milligrams of FAD/mg of enzyme protein was calculated, and found to be <u>.071 mgs FAD/mg protein,</u> or approximately <u>9 moles of FAD/100,000 grams of protein</u>, assuming a molecular weight of FAD of 785.56 grams.

Pig brain mitochondrial[172,173] MAO contains FAD as cofactor, which is dissociable from the protein. Beef liver mitochondrial[67] monoamine oxidase has firmly bound flavin as cofactor, as well as pig kidney[54] mitochondrial MAO.

<u>Absorption spectrum</u> (<u>See Figure 13</u>)

The absorption spectrum of a highly purified preparation of the enzyme was determined. From Figure 13 it can be seen that there is a protein peak at 280 nm, and a minor peak at 375 nm, probably due to FAD. The reason for the absence of a characteristic peak at 450 nm due to FAD is unclear.

<u>Absorbance index</u>, ($E^{1\%}_{1cm}$) of the enzyme was calculated as follows:

$Ecl =$ optical density (or absorbance)

where

$c =$ concentration in grams/100 ml

$l =$ length of light path $= 1$ cm.

Since the OD of a purified enzyme solution having a protein concentration of 0.26 mg/ml (0.026 grams/100 ml) was .323,

$$E^{1\%}_{1cm} = \frac{.323}{.026 \times 1cm} = 12.4 = \text{Absorbance index}$$

The molar absorbance index could not be determined as the molecular weight of the enzyme is not known.

The spectrum of beef[196] liver MAO reveals a maximum at 410 nm, a plateau at about 450 nm and a shoulder around 480 nm. Yasunobu[196] et al found the ratio of absorbance at 280/450 nm to be 1.1-1.2

Amino Acid Composition

A 5 mg sample of the lyophilized enzyme[125] was placed in a Carius combustion tube, and suspended in 1 ml of constant boiling HCl, redistilled from reagent grade. One crystal of phenol was added, and the solution was degassed repeatedly until there were no more bubbles. The tube was then sealed under vacuum, and hydrolyzed for 20 hours at 110 degrees. After opening the tube, the solution was taken down to dryness with nitrogen in a 40° water bath. The residue was taken up in a known volume (1.3 ml) of 0.2 M sodium citrate buffer, pH 2.2, and placed on the amino acid analyzer. (0.5 ml were added to each column).

Below is the amino acid composition of the enzyme (based upon a value of 1 for cystine)

aspartic acid----4.2
threonine--------2.6
serine-----------2.7
glutamic acid----4.4
proline----------2.4
glycine----------4.6
alanine----------3.8
cystine---------------1.0
valine----------------3.3
methionine-------------.76
isoleucine------------2.4
leucine---------------5.4
tyrosine--------------1.7
phenylalanine---------2.6
lysine----------2.6
histidine--------.91
arginine--------2.4

From the above it can be seen that leucine and glycine are present in the greatest amount.

C-terminal group

2 mg of the lyophilized enzyme was subjected to hydrazinolysis in a vacuum sealed tube for 24 hrs. at 80°, according to the procedure outlined by Fraenkel-Conrat and C. M. Tsung.[56] Following removal of the hydrazine by high vacuum drying overnight, the hydrazinolysate was redissolved in the appropriate buffer, and put through an amino acid analyzer, using standard elution procedures. The C-terminal group was found to be glycine.

N-terminal group

Repeated attempts to determine the N-terminal group, using a modification of the Edman[53] procedure on 5 mgs of the lyophilized enzyme, were unsuccessful. The PTH-amino acid

(continued on next page)

derivatives were chromatographed on special thin-layer silica gel plates with fluorescent indicator, and compared with similar plates run with known PTH-amino acid derivatives. On at least two occasions a very faint but unmistakable fluorescent spot corresponding to the position of leucine was identified. This result could not, however, be confirmed by hydrolyzing the PTH-derivatives and running them through the amino acid analyzer.

Recent work by Yasunobu[196b] on the N-terminal group of beef liver MAO has shown that the N-terminal group is attached to a phospholipid moiety, which must first be removed by treatment of the enzyme with sodium dodecyl sulfate, followed by the use of an appropriate organic solvent. Even with the use of this method, the results have not been definitive.

In conclusion, the properties of human liver MAO as outlined above can best be compared with those of monoamine oxidases from different species and tissues by means of a tabular summary as shown below.

COMPARATIVE DATA ON MONOAMINE OXIDASE
FROM
VARIOUS TISSUES AND BODY FLUIDS OF DIFFERENT SPECIES

	Human Liver	Beef liver	Rabbit liver
Degree of purification	122 Final spec. act. of 44 * Spec.act-1010 " - 550*	67 Spec.act. 6000-8000	63 270-fold
Sedimentation coefficient	6.78* (0.05 M phosphate buffer, pH 7.4)	14.4 (comp.1) 20.6 (comp.2) (0.1 M phosph. buffer, pH 7.4	

* refers to this thesis

	Human Liver *	Beef liver	Rabbit liver
Findings on electrophoresis	3 active bands on polyacryl. disc electroph.	Single comp. when comp 1 & 2 analyzed by starch zone electroph. Comp. 1-single band on disc electrophor.	
pH optimum	8.65*	[67] 9.1-9.2	[63] 8.4 (kynuramine) 9.2 (m-iodobenzylamine)
pH at which enzyme is most stable		[67] 7.00	
pH at which enzyme is least stable		8.9	
Michaelis constant	1.7x10^{-3} M* at pH 7.4 (benzylamine)	2.2x10^{-4} M (benzylamine)	K_M-77 uM (pH 7.4) (kynuramine)
Prosthetic groups	* .071 mgs FAD/ mg. enzyme or approximately 9 moles FAD/ 100,000 grams protein	[67] 4 FAD's/mole (comp. 1) 12 FAD's/mole (comp. 2) Copper-0.17 ugs/ mg of protein	
Molecular weight		405,000 (comp.1) 1,280,000 (comp.2)	[197] 260,000
Absorption spectrum	* Protein peak at 280 nm, with minor peak at 375nm	Maximum at 410 nm, plateau at 450 nm, with shoulder at 480 nm [196a] Ratio of absorbance 280/450-1.1-1.2	Max. at 412 nm
Absorption coefficient	$E^{1\%}_{1cm}$ - 12.4*		

	Human Liver	Beef liver
C-terminal group	Glycine*	
N-terminal group	* Unsuccessfully determined by usual means, possibly because of presence of a phospholipid moiety masking N-terminal group (Ref.196b)	See under human liver MAO
Amino-acid analysis	* Leucine and glycine in greatest abundance	

	Rat liver	Pig brain	Aspergillus Niger
Degree of purification	81 180-fold 197 208-fold	173 spec.act. 2760	195 crystallized
Electrophoresis	3 bands of enzyme activ.	homogeneous on cellulose acetate elect. (2 bands if enzyme kept 3 or more weeks)	
Sedimentation constant	6.3 6.7 (197)		11.4
pH optimum	81,82 7.2	173 7.2 (acting on tyramine)	7.5 (for n-butylamine) 7.2 (for benzylamine) 7.8 (for histamine) 8.5(for putrescine)
Michaelis constant all at pH 7.0	8.5×10^{-4} M (tyramine) 1.4×10^{-3} M (serotonin) 1.8×10^{-3} M (benzylamine)		1.7×10^{-5} M (benzylamine) 1.2×10^{-3} M (putrescine)

	Rat liver	Pig brain	Aspergillus niger
Prosthetic groups		contains bound FAD	Pyridoxal phosphate (12.1 millimicromoles of Cu/mg. protein)
Molecular weight	290,000[197]	102,000	273,000 (252,000)-average
Absorption maximum			280 nm and 480-500 nm, with shoulder at 410 nm
Extinction coefficient ($E^{1\%}_{1cm}$)			11.8
Ratio of $\frac{E_{260nm}}{280nm}$ and $\frac{E_{480nm}}{280nm}$			0.61 and 0.015

	Beef brain[85]	Bovine kidney[54]	Rat heart[153]
Degree of purification	40-fold	spec.act. 12,600 Fold purific. 34.2	89-fold
Findings on electrophoresis	2 components (continuous flow)		
Michaelis constant			$4x10^{-4}$ M (tyramine)-pH 7.0
pH optimum	8.6[85]	8.5[54]	7.0[153]

	Beef brain	**Bovine kidney**	**Rat heart**
Molecular weight	400,000 (estimated)		
Absorption spectrum	Max.at 410nm, with broad shoulder at 480-490nm		Max-410nm
Prosthetic groups	1 mole of FAD/ 1.5×10^6 grams protein	1 mole FAD/ 100,000 grams protein 8-SH groups per 10^5 grams protein	

	Beef plasma	**Hog plasma**	**Human plasma**
Degree of purification	192 crystallized	27 crystallized Turnover no.-42 (No. of substrate/ min/mole of enzyme)	120 5000-fold
pH optimum	7.4-7.6 (benzylamine) 7.2-7.4 (kynuramine)		
Sedimentation constant	8.1-8.2 (acidic or alkal. pH) 9.23 (pH 7.0)	8.05 (pH 7.0)	
Michaelis constant	1.5×10^{-3} M (ref.192)- (benzylamine) (K_m varies with pH as well as ionic strength)	2×10^{-3} M (histamine) 2.5×10^{-5} M (benzylamine)	3.3×10^{-5} M (ref. 120)
Prosthetic groups	1-2 grams of copper/mole of enzyme. Pyridoxal phosphate	Copper Pyridoxal phosphate	
Molecular weight	265,000	195,000	

	Beef plasma	Hog plasma
Absorption spectrum	Max. at 410 nm (alk. pH) 480 nm (acidic pH)	peak at 280 nm; shoulder at 480 nm

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	Human placental	Rabbit serum
Degree of purification	199 spec. act.-3500 (400-fold)	121 450-fold
Findings on electrophoresis	2 bands of enzyme activity	
Absorption spectrum		Absorption maximum at 470 nm

TABLE I
PURIFICATION OF HUMAN LIVER MONOAMINE OXIDASE (Method #I)

Steps	Vol.(ml)	ACTIVITY (units/ml)	PROTEIN (mgs/ml)	Specific activty	TOTAL UNITS	TOTAL prot.	YIELD	FOLD purific.
1) Homogenization of 90 grams of human liver 1:6 (w/v) with 0.1 M phosphate buffer, pH 7.4	540	500	38.5	13	270,000	20,520	100%	1
2) Centrifugation of homogenate in Rotor 30 at 24,000 rpm for 1 hr. a) Supernatant b) Sediment	460 80	43 3125	20 140	2.1 22.3	19,780 250,000	9,200 11,200	92%	1.7
3) a) Extraction of the sediment with 1.5% Triton X-100 (4 extractions)	1stE-106 2ndE-120 3rdE-116 4thE-110	300 130 200 182	9 5 5 10	33.3 26 40 18.2	31,800 15,600 23,200 20,020	954 600 580 1100	14%	2.5
b) Pooling of 2nd and 3rd extractions	-236	164	5	32.8	38,704	1180		
4) 40% ammonium sulfate precipitation of enzyme in pooled Triton X-100 extracts after Sephadex G-25	130	270	4.5	60	35,100	585	13%	4.6
5) DEAE-cellulose chromatography	185	60	0.5	120	11,100	92.5	4.1%	9.2
6) 40% ammonium sulfate precipitation of enzyme in pooled fractions after DEAE-cellulose chromatography	20	450	3.0	150	9,000	60	3.3%	11.5
7) Sephadex G-200 Chromatography	20	105	0.3	350	2,100	6	0.7%	27
8) Vacuum dialysis of pooled fractions after Sephadex G-200 removal of supernatant and reconstitution of yellow precipitate	10	143	0.26	550	1,430	2.6	0.5%	42

TABLE II (Method #II)

PURIFICATION OF HUMAN LIVER MITOCHONDRIAL MONOAMINE OXIDASE

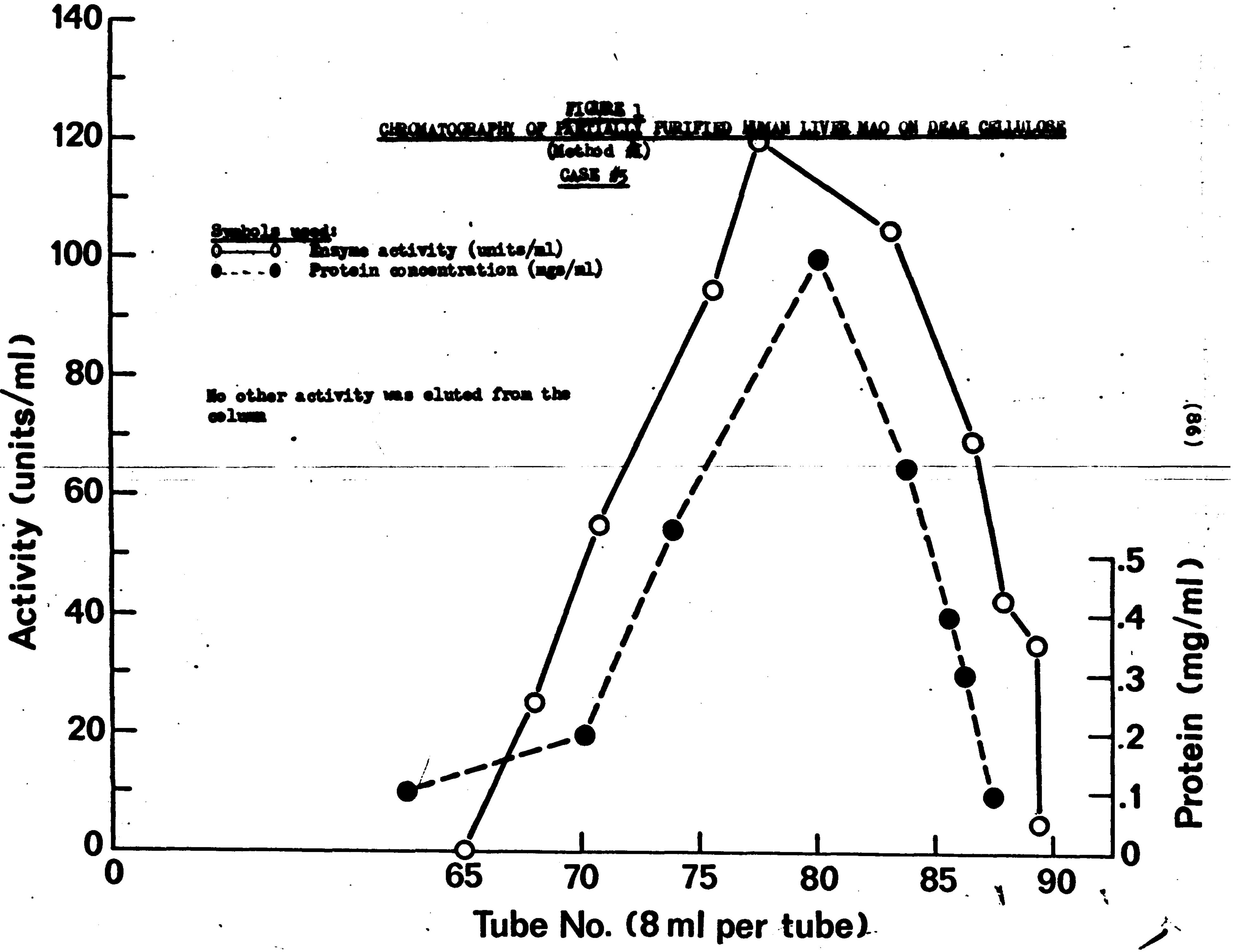
PROCEDURE	VOL. ML	ACTIVITY/ml	PROTEIN (mg/ml)	SPECIFIC ACTIVITY	TOTAL UNITS	TOTAL PROTEIN (mg/ml)	YIELD(%)	PURIFICATION
Mitoch.Homo	500	3,000	34.5	87	1,500,000	17,250	100	1
Triton X-100 plus 0.15 sat'd ammon. sulfate	420	3,600	16	225	1,512,000	6,720	100	2.6
0.15-0.40 sat. ammon. sulfate	211	3,200	18.2	167	675,200	4,051	45	1.9
0.25-0.40 sat. ammon. sulfate	244	1,400	10.9	128	341,600	2,660	23	1.5
CaP gel eluate after ammon. sulfate	100	180	0.96	187	18,000	96	1.2	2.2
DEAE-cellulose after Seph G-25	75	75	0.336	223	5,625	25.2	0.3	2.5
Hydroxylapatite 100 ml 0.01 M buffer, pH 7.4 (Fraction 1)	100	0	0.0700	0	0	7.0	0	0
100 ml 0.1 M buffer, pH 7.6 (Fraction 2)	100	30	0.0297	1010	3,000	2.97	0.2	12
100 ml 0.2 M, pH 7.6 (Fraction 3)	98	6	0.0100	600	588	.9	< 0.2	7
100 ml 0.2M, pH 7.6 plus chelate (Fraction 4)	99	2.5	0.0150	177	248	1.49	< 0.2	2

FIGURE 1

CHROMATOGRAPHY OF PARTIALLY PURIFIED HUMAN LIVER MAO ON DEAE CELLULOSE

(Method #1)

CASE #5



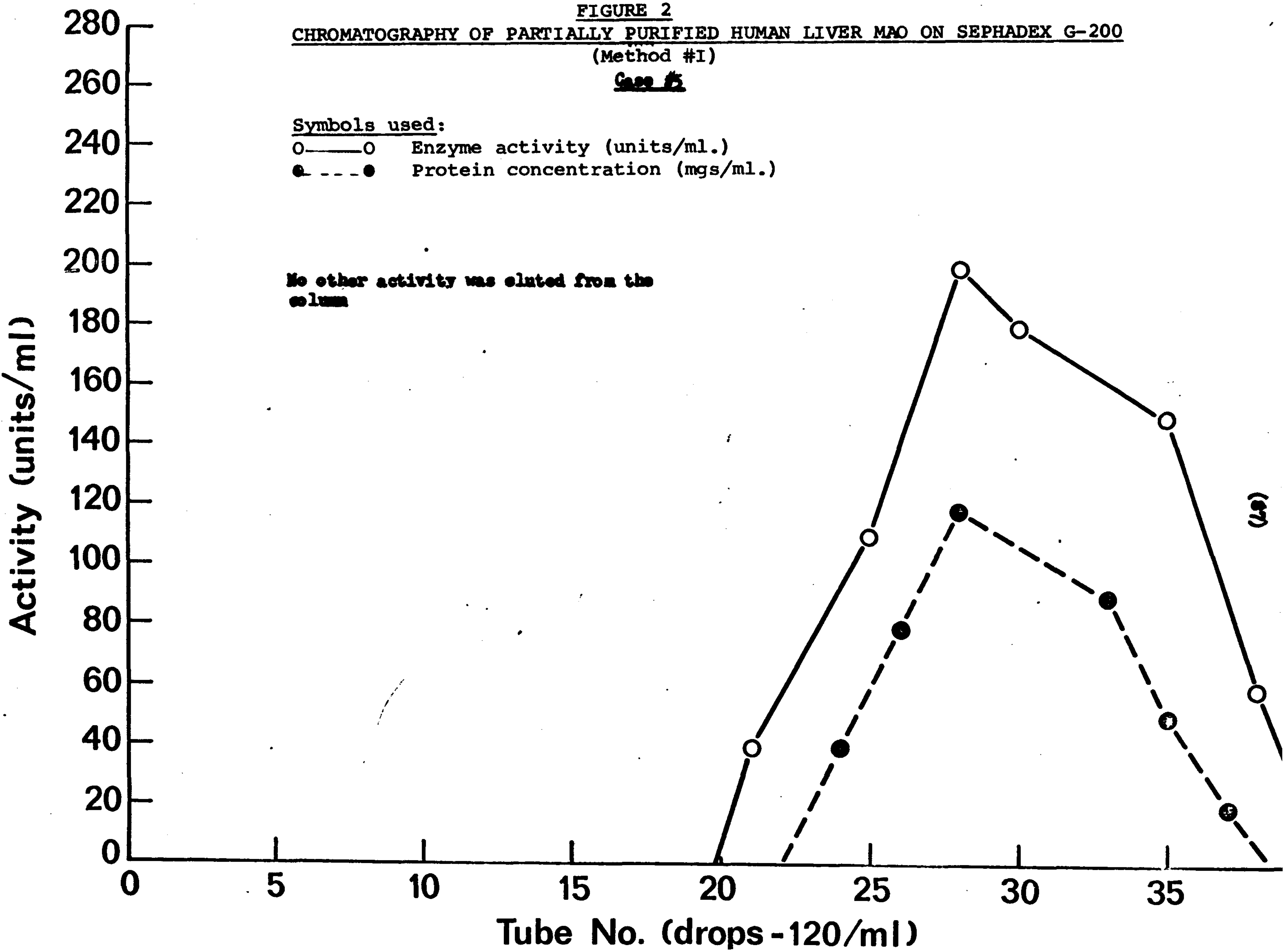


FIGURE 2

CHROMATOGRAPHY OF PARTIALLY PURIFIED HUMAN LIVER MAO ON SEPHADEX G-200

(Method #I)

Case #5

Symbols used:

O———O Enzyme activity (units/ml.)

●- - - -● Protein concentration (mgs/ml.)

No other activity was eluted from the column

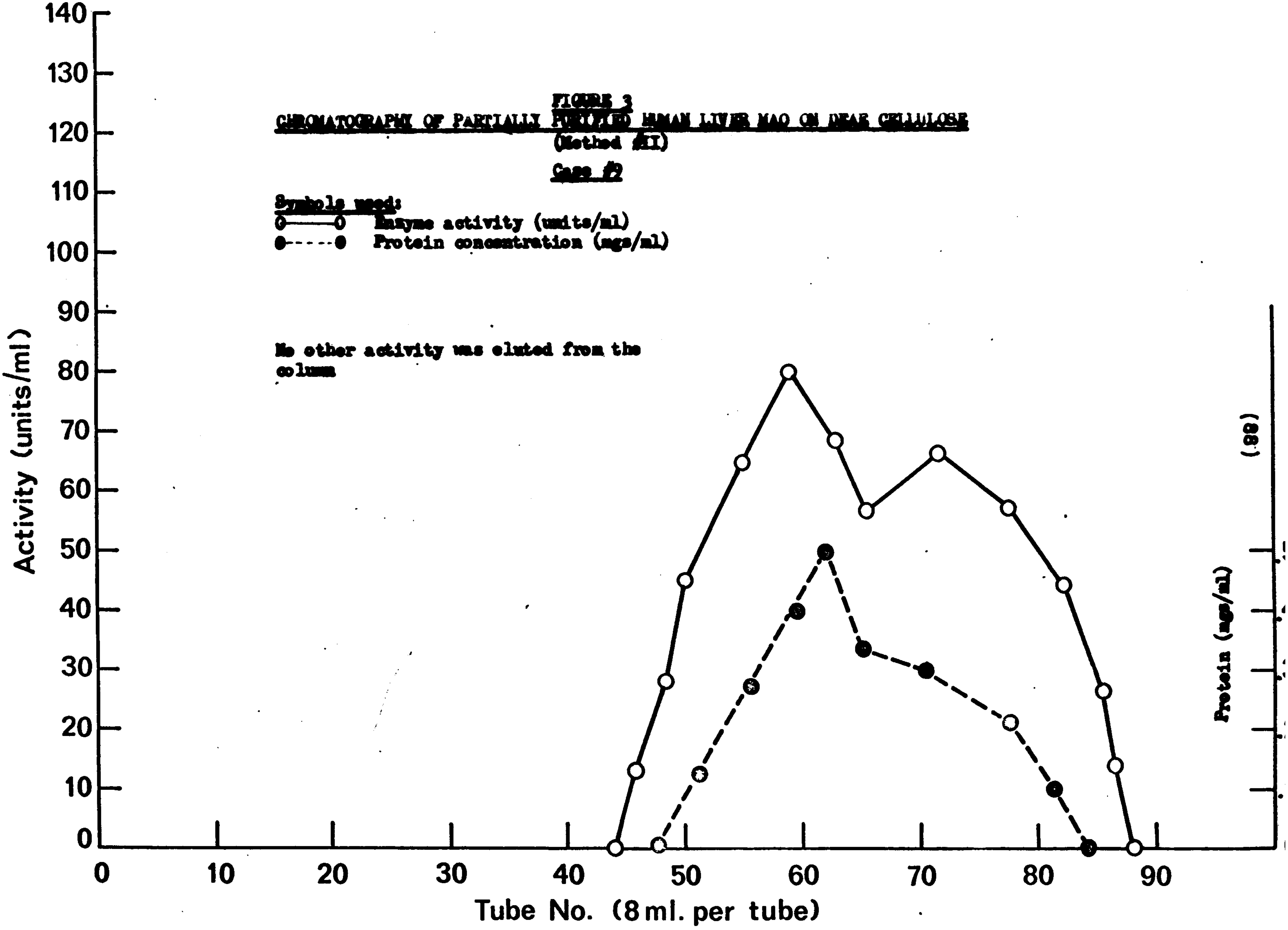


FIGURE 3
CHROMATOGRAPHY OF PARTIALLY PURIFIED HUMAN LIVER MAO ON DEAE CELLULOSE
(Method #II)

Case #2

Symbols used:
○——○ Enzyme activity (units/ml)
●- - - -● Protein concentration (mgs/ml)

No other activity was eluted from the column

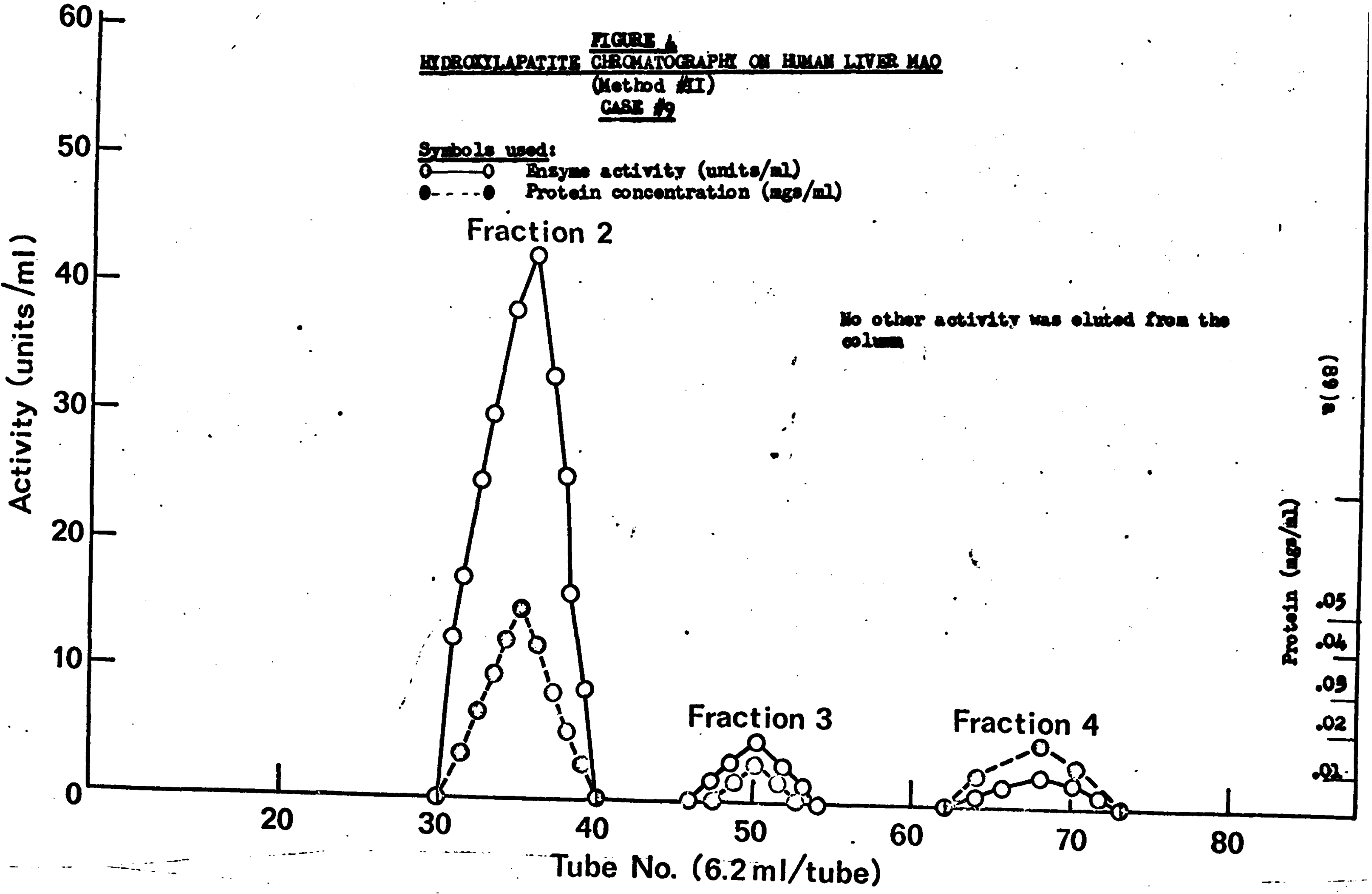


FIGURE 4
HYDROXYLAPATITE CHROMATOGRAPHY ON HUMAN LIVER MAO
(Method #II)
CASE #9

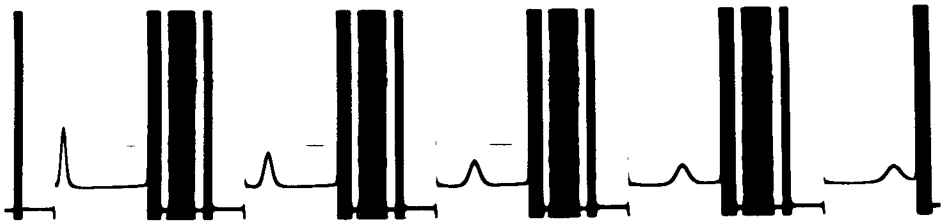
Symbols used:
○——○ Enzyme activity (units/ml)
●- - - -● Protein concentration (mgs/ml)

No other activity was eluted from the column

FIGURE 5

SEDIMENTATION PATTERN OF HUMAN LIVER MONOAMINE OXIDASE

(Enzyme concentration - 0.75 mg/ml in 0.05 M
phosphate buffer, pH 7.4)

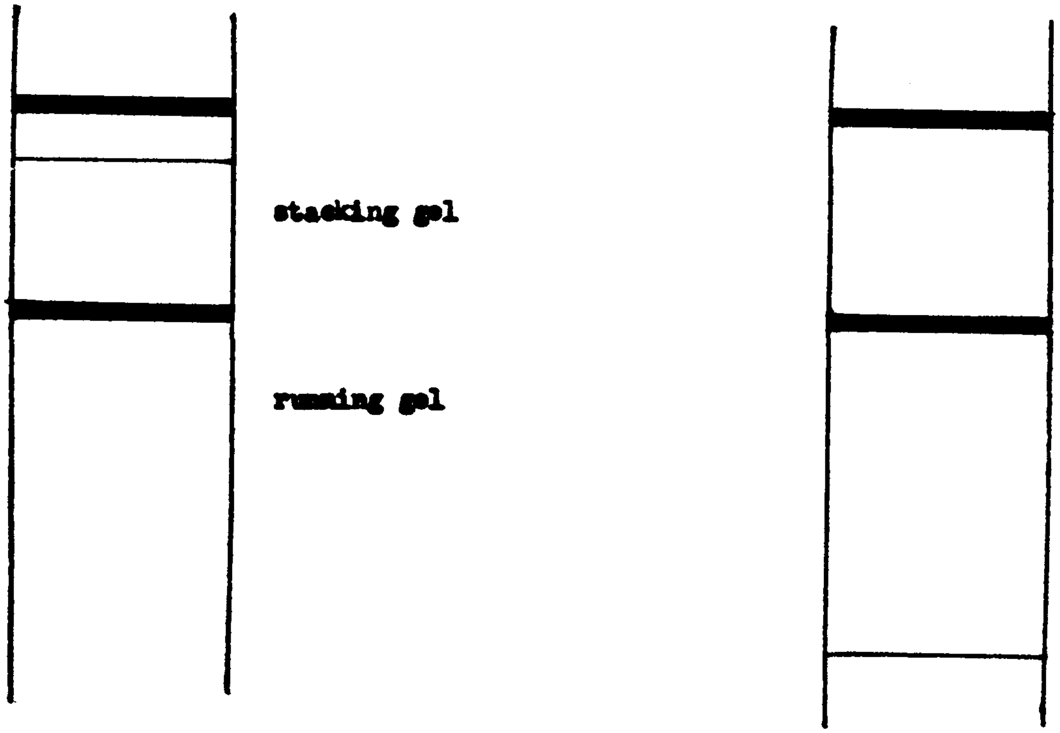


The study was made at 59,780 rpm at a temperature of 20°. Photographs were taken at 16 minute intervals after obtaining top speed.

FIGURE 6I
(Method #I)

POLYACRYLAMIDE-GEL DISC ELECTROPHORESIS OF HUMAN LIVER MONOAMINE OXIDASE

Active bands of the enzyme stained with tetrazolium nitro blue, employing tryptamine as a substrate



a
7.0% standard gel
at pH 9.5
(only 2 bands with
protein stain)

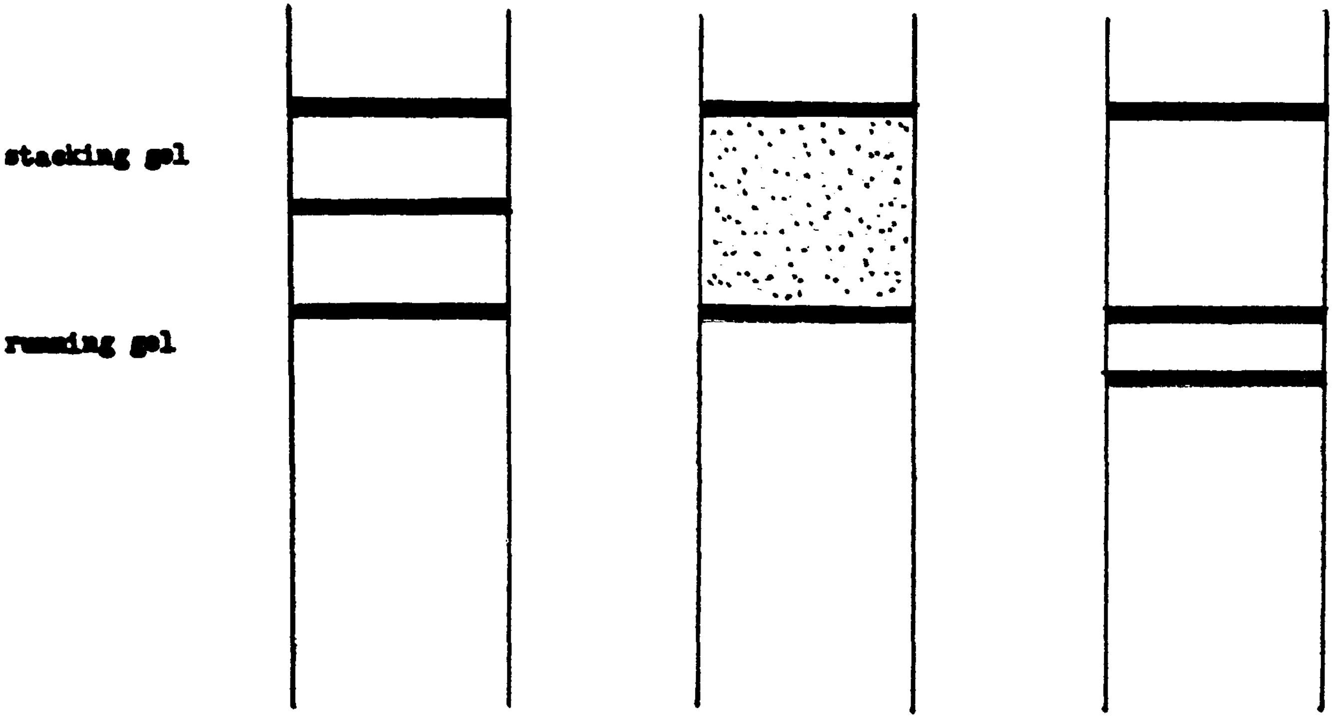
b
2.5% gel at pH 9.5

(only 2 bands with
protein stain)

FIGURE 6II
(Method XII)

POLYACRYLAMIDE GEL DISC ELECTROPHORESIS OF HUMAN LIVER MONOAMINE OXIDASE

Active bands of the enzyme stained with tetrazolium blue, employing tryptamine as a substrate



a
7.0% standard gel at pH 9.5
(3 corresponding bands with protein stain)

b
3.5% gel at pH 9.5-two weeks later
(3 corresponding bands with protein stain)

c
2.5% gel, at pH 9.5
(3 corresponding bands with protein stain)

FIGURE 7

THE EFFECT OF pH ON ACTIVITY OF HUMAN LIVER MAO AT 27°

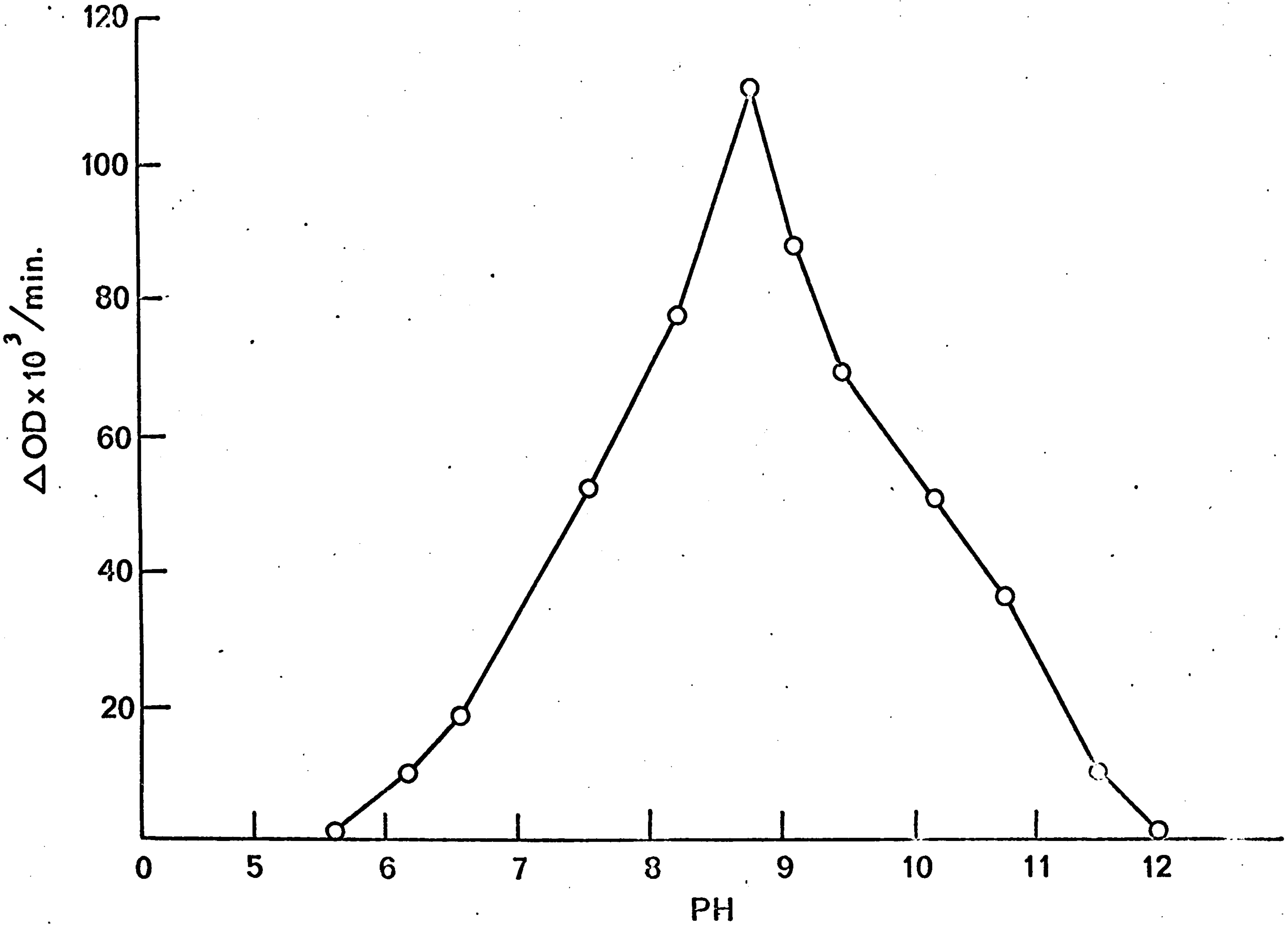


FIGURE 8

DETERMINATION OF MICHAELIS CONSTANT

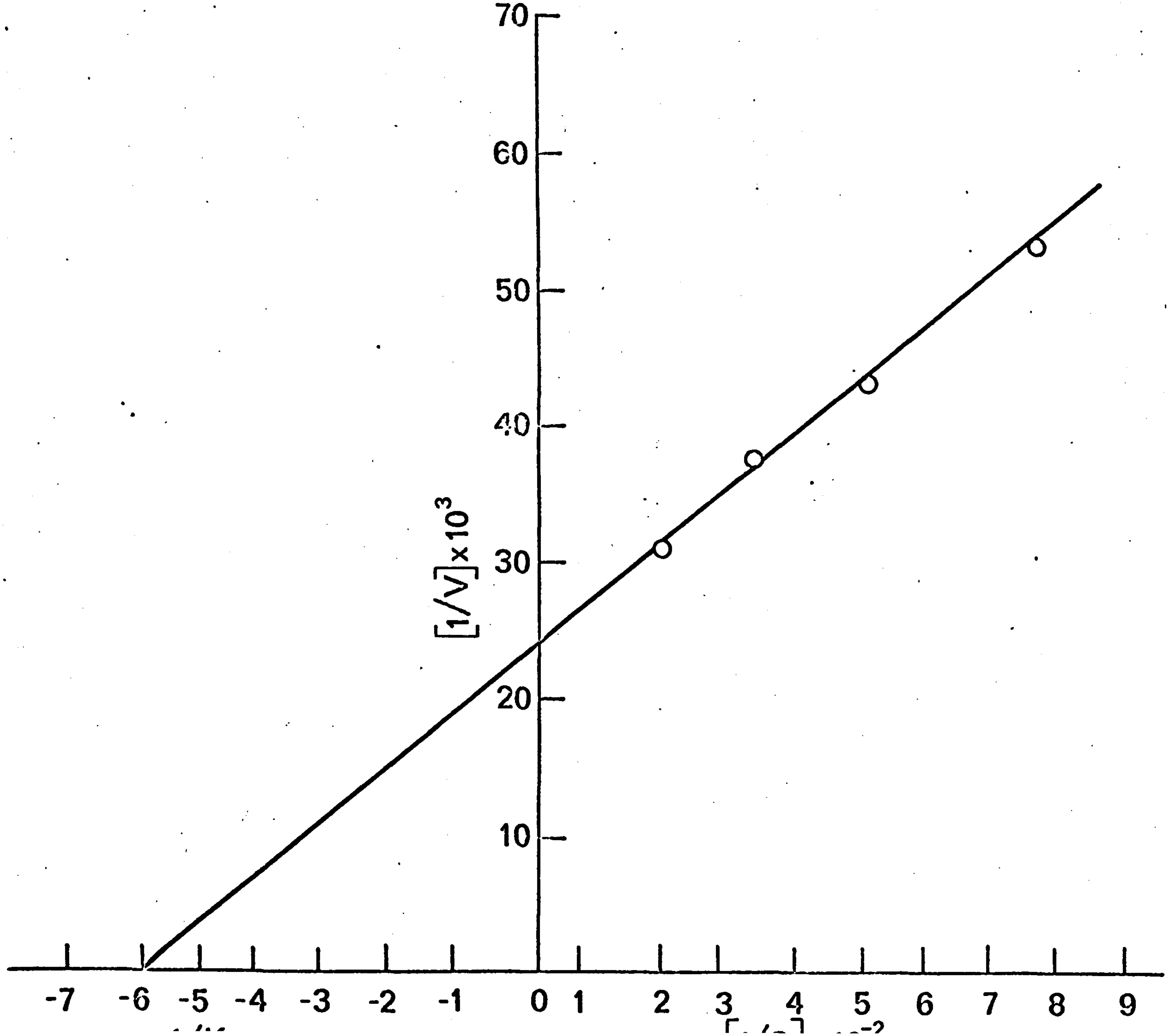


FIGURE 9

INHIBITION OF HUMAN LIVER MONOAMINE OXIDASE BY THIOL INHIBITORS

(Inhibition by iodoacetic acid)
Final concentration - 2×10^{-3} M

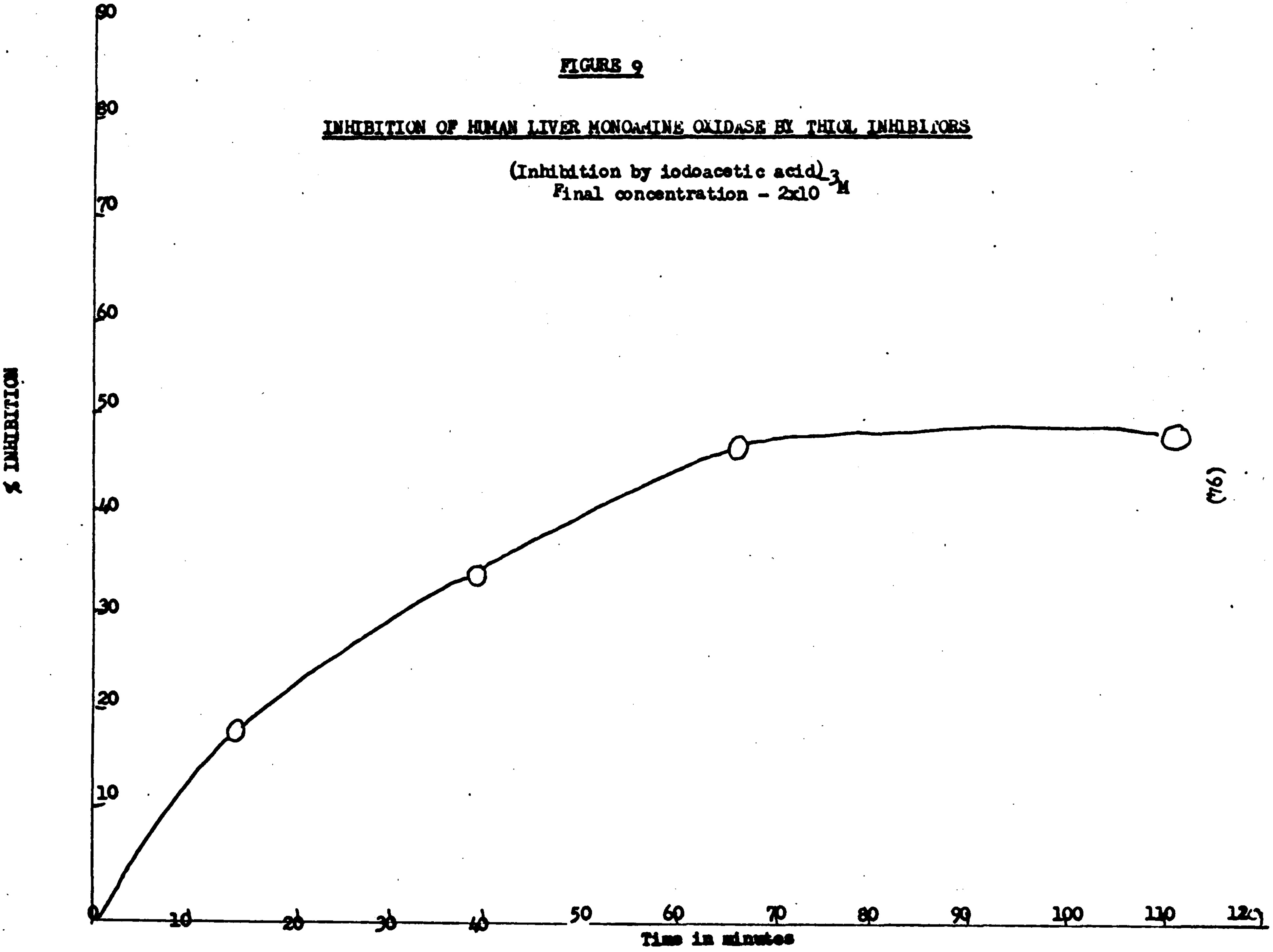


FIGURE 10a

TEMPERATURE-ACTIVITY CURVE OF HUMAN LIVER MAO

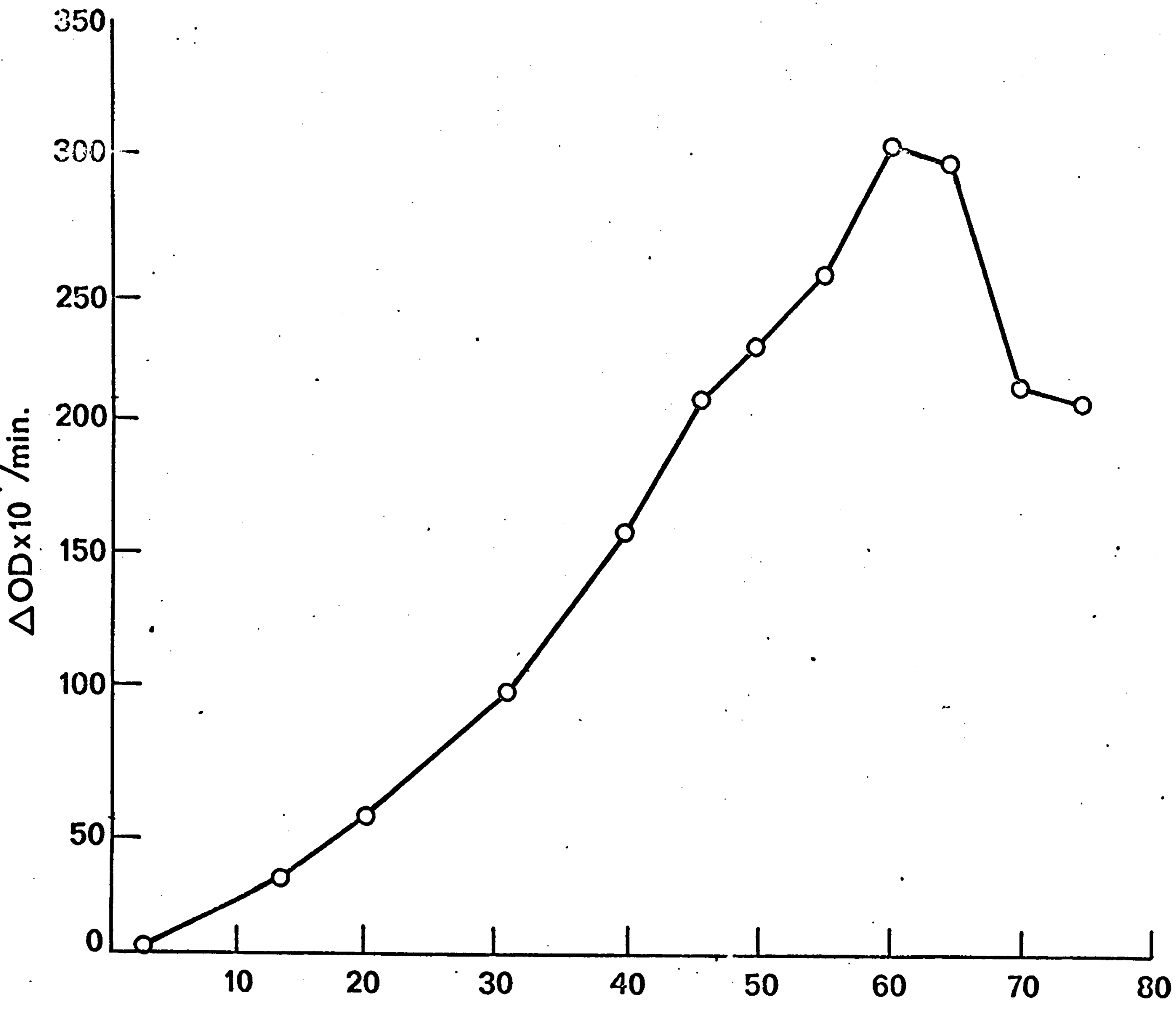


FIGURE 10b

TEMPERATURE-ENZYME DENATURATION CURVE

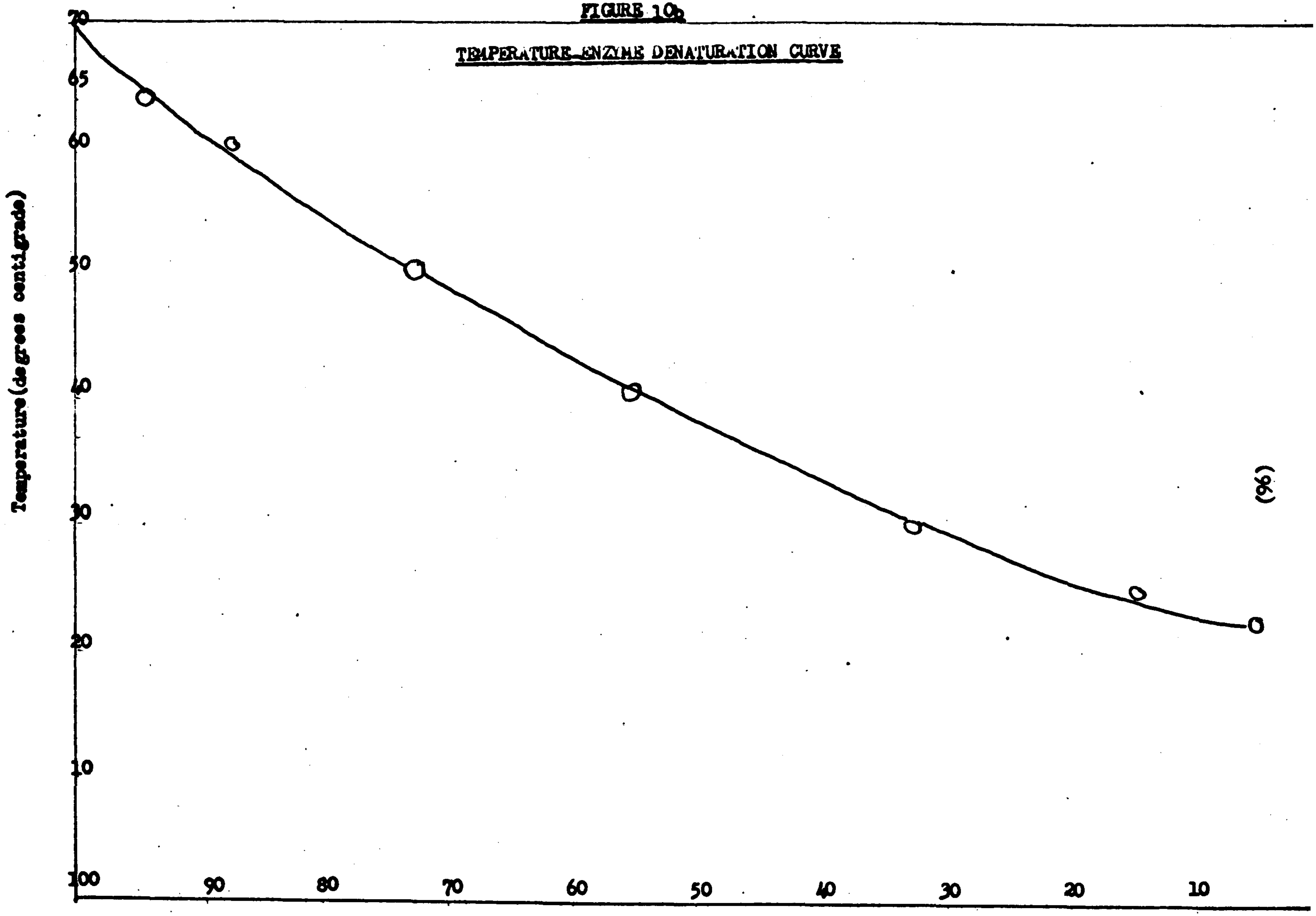
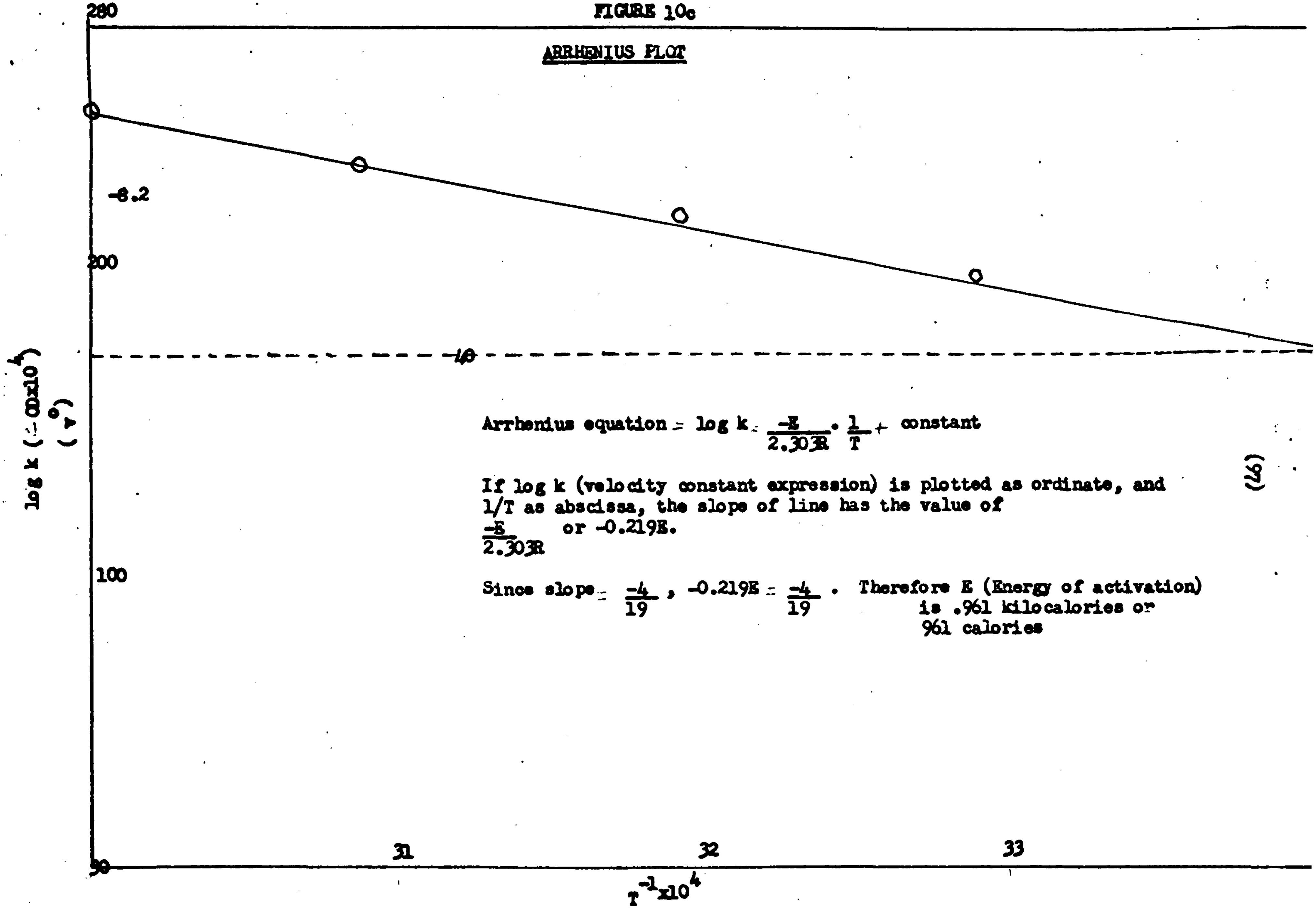


FIGURE 10c

ARRHENIUS PLOT



Arrhenius equation = log k = $\frac{-E}{2.303R} \cdot \frac{1}{T}$ + constant

If log k (velocity constant expression) is plotted as ordinate, and 1/T as abscissa, the slope of line has the value of $\frac{-E}{2.303R}$ or -0.219E.

Since slope = $\frac{-4}{19}$, -0.219E = $\frac{-4}{19}$. Therefore E (Energy of activation) is .961 kilocalories or 961 calories

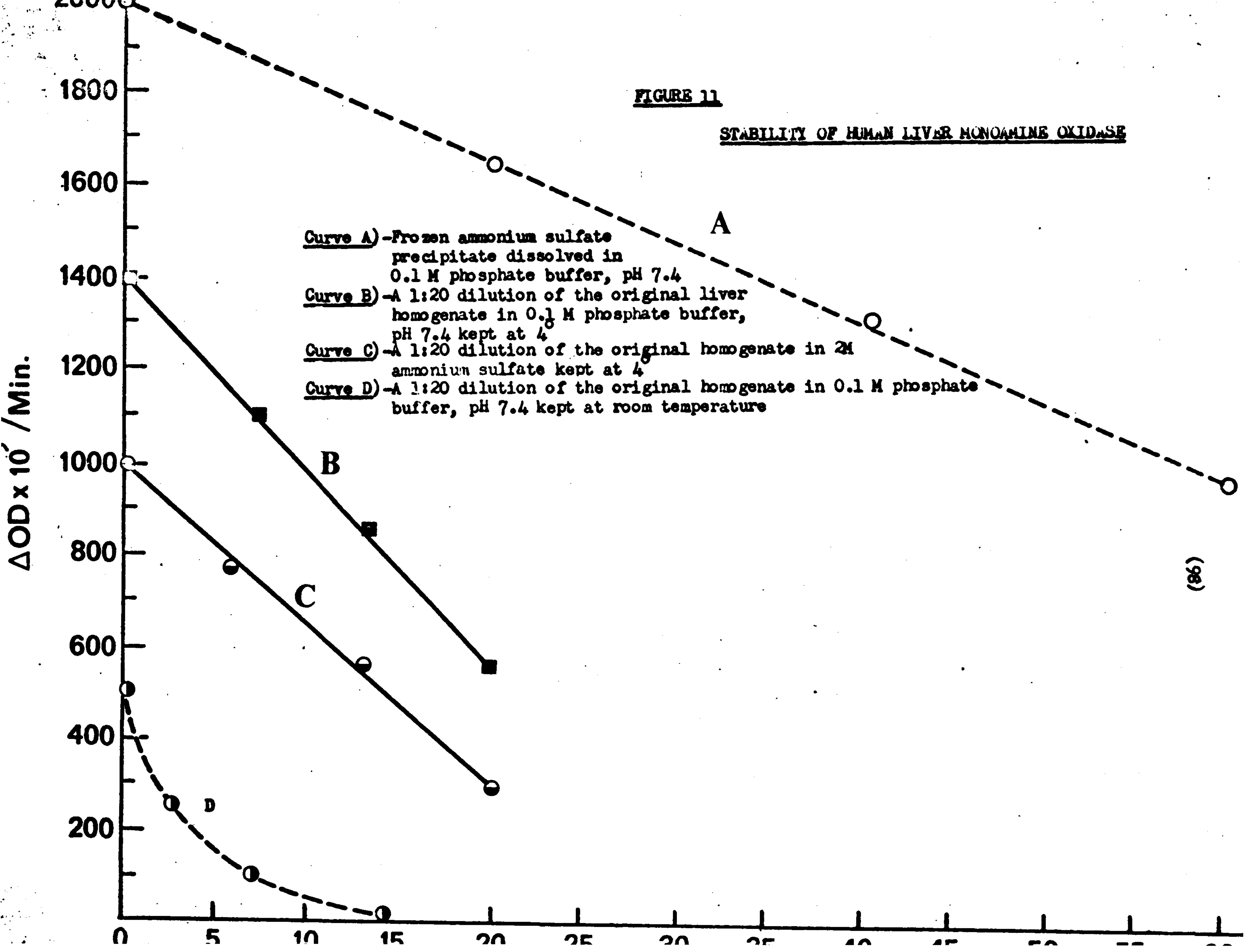


FIGURE 11

STABILITY OF HUMAN LIVER MONOAMINE OXIDASE

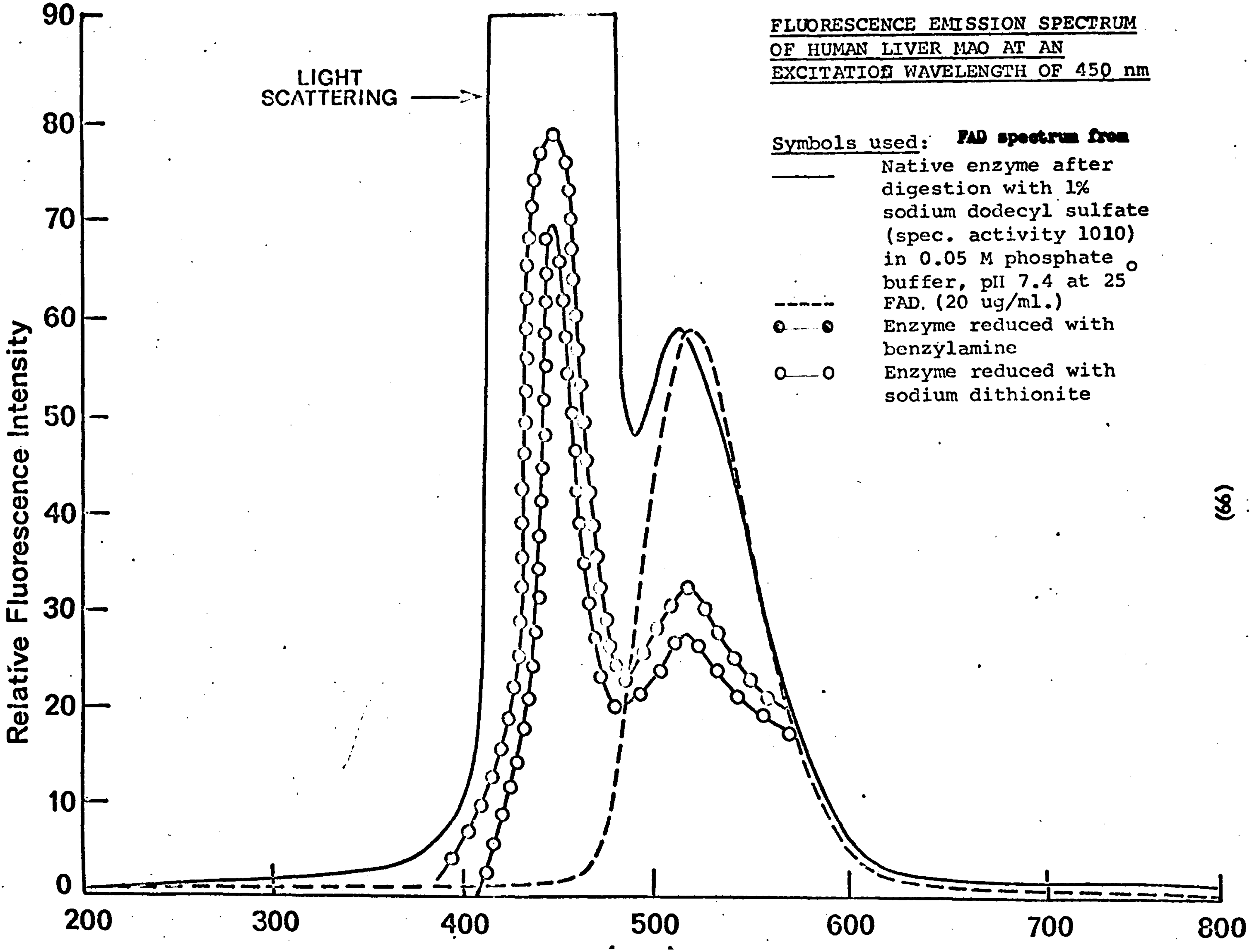
Curve A) -Frozen ammonium sulfate precipitate dissolved in 0.1 M phosphate buffer, pH 7.4

Curve B) -A 1:20 dilution of the original liver homogenate in 0.1 M phosphate buffer, pH 7.4 kept at 4°

Curve C) -A 1:20 dilution of the original homogenate in 2M ammonium sulfate kept at 4°

Curve D) -A 1:20 dilution of the original homogenate in 0.1 M phosphate buffer, pH 7.4 kept at room temperature

FIGURE 12a



FLUORESCENCE EMISSION SPECTRUM OF HUMAN LIVER MAO AT AN EXCITATIOΠ WAVELENGTH OF 450 nm

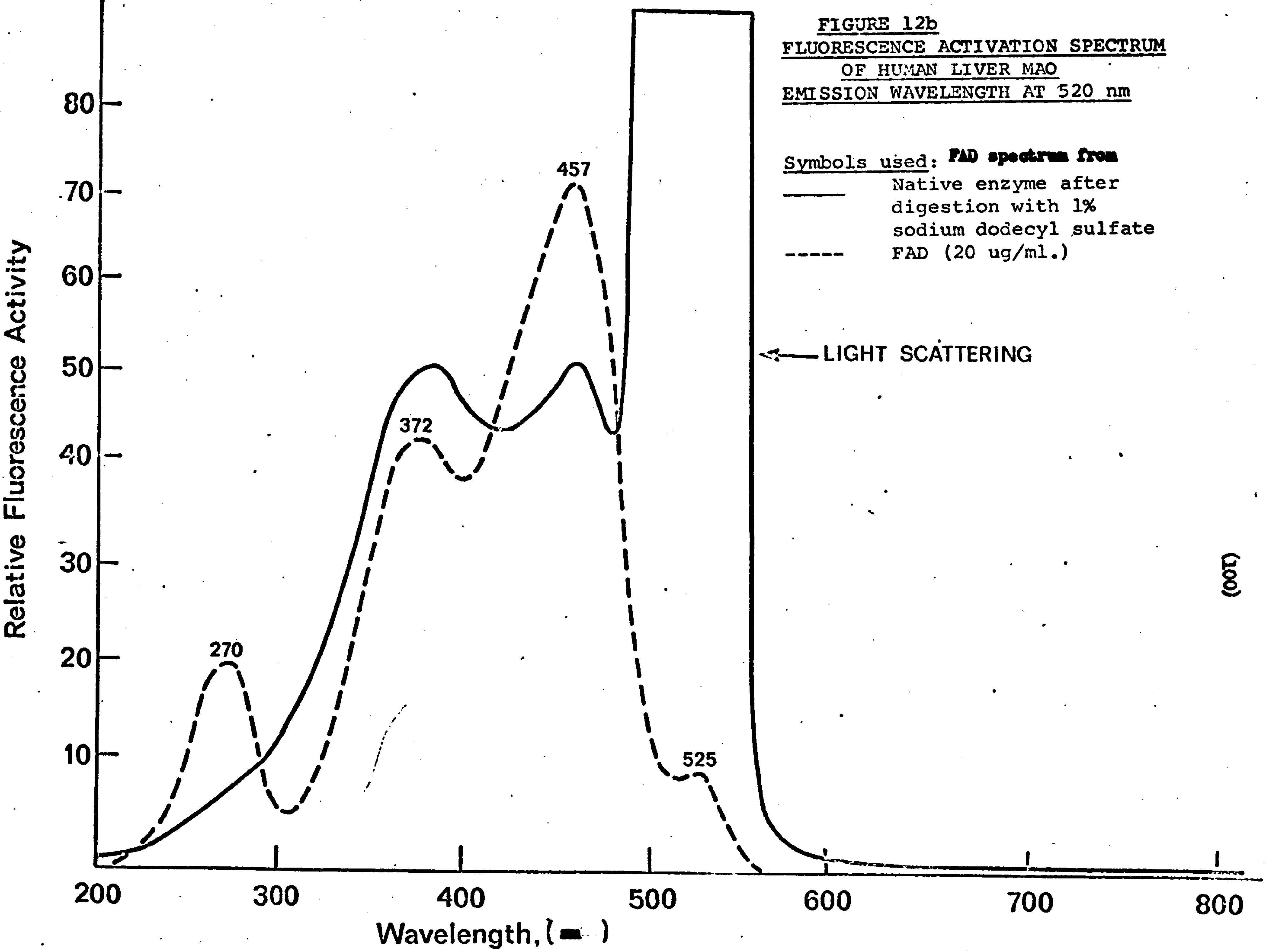


FIGURE 12b
FLUORESCENCE ACTIVATION SPECTRUM
OF HUMAN LIVER MAO
EMISSION WAVELENGTH AT 520 nm

FIGURE 13
ABSORPTION SPECTRUM OF HUMAN LIVER MAO

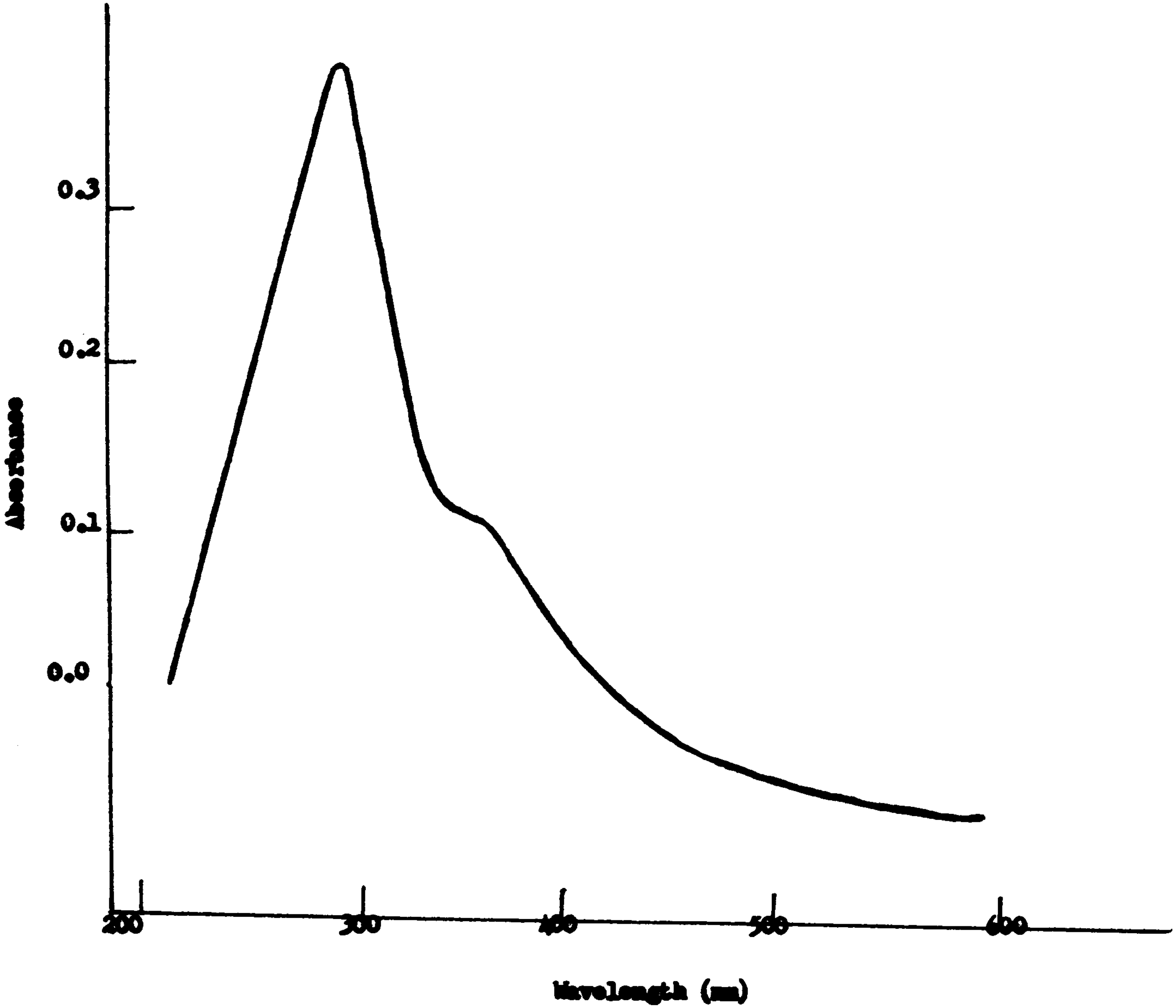
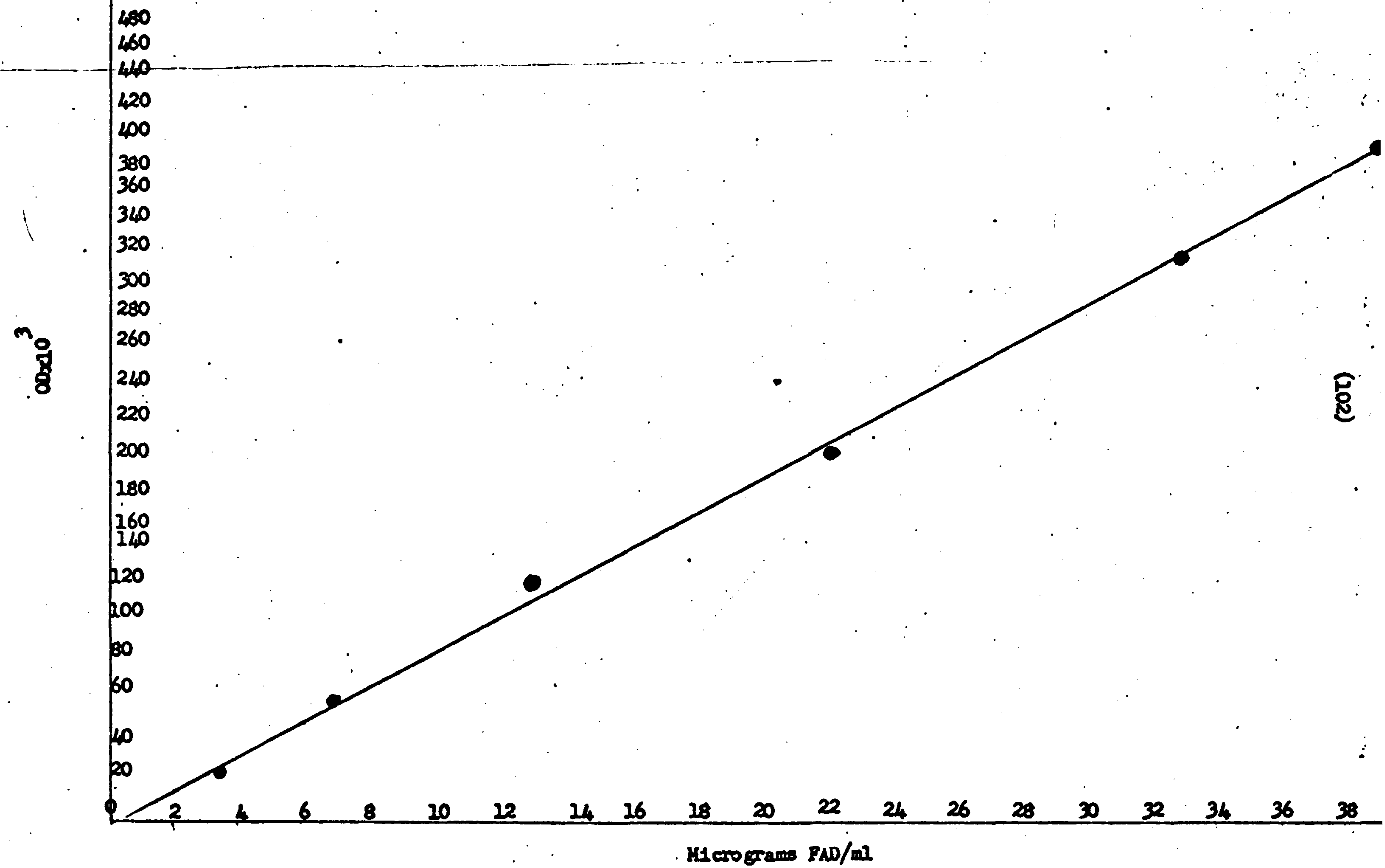


FIGURE 14

CALIBRATION CURVE FOR FAD



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