STUDIES ON THE MULTIPLICITY OF MONOAMINE OXIDASE

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THESIS

Submitted to the School of Graduate Studies,
in partial fulfilment of the requirements
for the degree of Doctor of Philosophy.

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF OTTAWA

DECEMBER 1973

My deepest gratitude and appreciation to Dr. A. D'Iorio who directed this work with great interest, patience and understanding.

The valuable criticisms, the stimulating discussions and the encouragement of all the colleagues in Dr. D'Iorio's laboratory are acknowledged with thanks. In particular, I would like to express my gratitude to Dr. L. Sierens and Dr. C. Kandaswami with whom I had the opportunity to collaborate.

I also wish to thank Mrs. B. Betz and Mrs. R. Keren-Paz for their valuable help and skillful assistance in performing some of the experiments.

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INTRODUCTION

Monoamine oxidase [MAO; monoamine: oxygen oxidoreductase (deaminating) EC 1.4.3.4] is the enzyme or enzymes which catalyse the deaminative oxidation of a series of monoamines such as aliphatic amines, serotonin, tryptamine, tyramine, catecholamines and other aromatic amines. Some of these substances have well recognized physiological, pharmacological, toxicological and clinical importance. The MAO inhibitors have had a wide usage in therapeutics, notwithstanding the fact that very little is known about their mechanism of action, except for their inhibition of MAO. This is especially true when these drugs are used in the treatment of mental disease (depression). Furthermore today the nature of the enzyme is very little understood in spite of the considerable amount of research in this field.

Though MAO has for a long time been considered a single enzyme, the question of whether MAO represents a group of enzymes or a single enzyme is of actual interest and the answer will contribute immensely to the understanding and modification of the action of pharmacologically active amines.

Indirect evidence of MAO multiplicity has been obtained on the basis of kinetic studies and effects of selective inhibitors or physical agents. The fact that the

enzyme is insoluble and tightly bound to the mitochondria made it difficult to study MAO by more direct methods such as electrophoresis or standard purification procedures. This and other general aspects of the enzyme are mentioned in the review of the literature.

The experimental part of this thesis is an attempt to study the multiplicity of MAO by means of electrophoretic procedures. In section I a procedure to detach the enzyme from the mitochondrial membranes is described. This procedure was carried out with the purpose of obtaining a preparation in which the MAO substrate specificity is similar to that of the original mitochondria. Interpretation of the results would be facilitated in the case when a fraction, specific towards a particular substrate, was isolated. Section II and III deal with the electrophoresis of a crude liver mitochondrial preparation on sucrose gradient electrophoresis and on polyacrylamide gel, respectively. With the electrophoresis of this crude preparation we hoped to obtain an idea of the pattern of migration of MAO activities with regard to different substrates and to have a preliminary basis for future experiments where attempts to purify the multiple MAO forms would be undertaken. Rat liver mitochondria was selected for these studies because it has been widely studied, is easily accessible and a relatively good source for the enzyme.

REVIEW OF LITERATURE

CHAPTER I: HISTORICAL

According to Blaschko (1) the first clear indication that amines are metabolized through deamination was given by Schmiedeberg in 1887 (2). The latter detected benzoylglycine in dog urine after administering an oral dose of benzylamine which was interpreted as occurring through the intermediate formation of benzoic acid. He also suggested that all the bases in which nitrogen is not directly linked to a benzene ring are broken down in the organism with the formation of ammonia. In 1889, Mosso (3) recovered over 90% of the benzylamine injected to a dog as hippuric acid in urine. Minkowski (4) showed the formation of benzoic acid from benzylamine after incubating the amine with minced rabbit tissues. Ewins and Laidlaw (5) reported the recovery of p-hydroxyphenylacetic acid when tyramine was perfused through the liver of cat or rabbit or through the uterus of rabbit. Subsequently the same authors (6) isolated indolacetic acid as the breakdown product of tryptamine in the perfused liver of the rabbit. In 1916 Guggenheim and Löffler (7) reported the conversion in vivo of β-phenylethylamine into phenylacetic acid. Three years later they obtained evidence for the

formation of isovaleric acid after perfusing rabbit liver with isoamylamine. In all the above reports the first reaction was assumed to be an hydrolytic one followed by oxidation (1).

The earliest mention of oxidative deamination was made by Hare in 1928 (8). She showed that tyramine incubated with rabbit liver extract resulted in an increased oxygen consumption and concluded that the oxidation of tyramine took place in the presence of an enzyme contained in the liver extracts. This author also reported that the reaction liberated ammonia and hydrogen peroxide and postulated the formation of an aldehyde (8, 9). In 1937 Pugh and Quastel (10) described a similar amine-oxidizing system that acted on aliphatic amines which was distinct from the α-amino-acid-oxidizing system. In a later report (11) they mentioned a common amine oxidase system concerned with the oxidation of tyramine, β-phenylethylamine and indolethylamine, and proposed the term amine oxidase for this kind of oxidase. In the same year Kohn (12) suggested the identity between the enzyme described by Hare and the adrenaline oxidase reported by Blaschko et al. (13). Blaschko, Richter and Schlossmann (14) concluded that the adrenaline oxidase described by them, the tyramine oxidase of Hare and the aliphatic amine oxidase of Pugh and Quastel were identical. This conclusion was based on the similarities

of the chemical reactions they catalysed, their distribution in the animal kingdom and their behavior towards inhibitors as well as the competition between different substrates (adrenaline-tyramine, isoamylamine-tyramine and tyramine-tryptamine). In 1938 Zeller, (15) proposed the name monoamine in order to distinguish this enzyme system from diamine oxidase (EC 1.4.3.6). In 1940, Zeller et al. (16, 17) attempted to classify the amine oxidases into two groups, the monoamine oxidases and the diamine oxidases, on the basis of their substrate specificities. The significance of this classification was questioned when it was demonstrated that long chain aliphatic diamines were substrates for monoamine oxidase and not for diamine oxidase (18). Futher complications arose when other monoamine oxidases were described. In 1947, Steensholt (19) reported a rabbit liver amine oxidase that acted on mescaline, histamine and cadaverine. It was inhibited by diamine oxidase inhibitors. Other mescaline-oxidizing enzymes were described in the hog liver and hog kidney cortex with properties similar to a monoamine oxidase rather than a diamine oxidase (20, 21). The amine oxidase of beef plasma was found to be active on spermine, spermidine, benzylamine and several aliphatic monoamines; tyramine and mescaline were poor substrates (22, 23). This amine oxidase was inhibited by iproniazid and isoniazid. In some species the plasma amine

oxidase acted more specifically on benzylamine rather than on spermine and spermidine (24, 25). Kolb (26) described another amine oxidase in hog serum which deaminated histamine, but with little effect on cadaverine. Buffoni and Blaschko (27) found that its best substrate was benzylamine and that it was inhibited by carbonyl reagents. Kobayashi (28) reported yet another amine oxidase with similar properties to MAO that metabolizes histamine and cadaverine. Hill and Mann (29) purified an amine oxidase from pea seedling which was sensitive to carbonyl reagents. This enzyme deaminated aliphatic diamines as well as aliphatic monoamines, phenylalkylamines, histamine, spermidine and agmatine. Histamine a classical substrate for diamine oxidase is also a weak substrate for monoamine oxidase (30) and its N_1-methylated derivate is a good substrate for the latter enzyme (31, 32). In view of these observations new classifications appeared. In 1959, Blaschko et al. (33) classified the amine oxidases on the basis of their sensitivity to carbonyl reagents. Two groups of enzymes were described, one which was inhibited by carbonyl reagents, group I, and the other which was not, group II. Group I included diamine oxidase (34), histaminase (35, 36), spermine oxidase (22), benzylamine oxidase (26), mescaline oxidase (37), pea seedling amine oxidase (38) and the polyamine oxidase of Neisseria perflava (39). In group II the classical monoamine oxidase and the mouse liver histaminase

were included (28). Blaschko et al. (33) explained that it was not their intention to replace names like histaminase, amine oxidase or diamine oxidase by new ones. They wanted to point out that when one of these names is used, "the term is applied to what is in fact not one entity but a collection of enzymes of differing properties". The differences among the analogous enzymes of different species could make the identification of new identities dependent on individual preference. Other complication could be the difference in organ specificity in the same species. In accordance with Blaschko et al, (33) it may be possible that some of the identities listed may be superfluous.

On similar basis Zeller (40) and Zeller et al. (41) proposed another classification. The enzymes attacking aliphatic compounds with terminal amino groups are separated by means of semicarbazide in two groups:

a) Semicarbazide-resistant monoamine oxidases with one type of receptor in their active center.

b) Semicarbazide-sensitive diamine oxidases with two types of receptors.

To the semicarbazide-resistant enzyme group belonged the classical monoamine oxidase which he described as a group of enzymes with small differences. On the other hand, the group of semicarbazide-sensitive amine oxidases, which was considered heterogeneous, included classical diamine oxidase (35, 40, 42), beef and sheep plasma spermine oxidase (22, 23), benzylamine oxidase (24) and mescaline oxidase (37). Zeller (40) mentioned that the ultimate solution to the problem of naming the different amine oxidases should come from a reliable insight into the structure of the active center of these enzymes.

Up to the present a failure to get a clear picture of the amine oxidases continues in spite of the large amount of work accumulated. Recent reviews on this subject have appeared elsewhere (43-45). Some aspects of the recent advances made in the comprehension of the classical MAO (EC 1.4.3.4) will be discussed in subsequent chapters.

CHAPTER II: SOME PROPERTIES OF THE MAO CATALYSED REACTION

The amine oxidases are considered enzymes that oxidatively deaminate mono, di and polyamines with the stoichiometric formation of one molecule each of aldehyde, ammonia and hydrogen peroxide (43)

$$\text{(I)}\quad R\text{-}CH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O_2$$

The classical intracellular MAO is characterized by its localization in the particulate fraction, its oxidative action on monoamine substrates, its inability to deaminate histamine and short-chain diamines, its sensitivity to octanol and certain heavy metals, its resistance to cyanide ion, hydrazine, semicarbazide and carbon monoxide and its action on primary and secondary amines (43).

Since long the reaction products of the MAO enzymic reaction have been isolated by several authors (8, 12). The stoichiometry of the reaction was shown in accordance with equation I. In presence of catalase (EC.1.11.1.6)

$$\text{(II)}\quad 2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The overall reaction will thus be

(III) $R\text{-}CH_2\text{-}\overset{+}{N}H_3 + \frac{1}{2}\ O_2 \xrightarrow{\text{Catalase}} RCHO + \overset{+}{N}H_4$

When a secondary or tertiary amine is attacked, one molecule of a lower amine is released (46). Therefore equation III in a more general form, can be delineated as follows

(IV) $R\text{-}CH_2\text{-}\overset{+}{N}HR_2 + \frac{1}{2}\ O_2 \longrightarrow R\text{-}CHO + \overset{+}{N}H_2\text{-}R_2'$

where R'= hydrogen or an alkyl group.

In presence of catalase, H_2O_2 can be metabolized by a peroxidative reaction if there are hydrogen donors such as ascorbate, ferrocyanide, phenols, short-chain alcohols, formate, sodium nitrate, azide or hydroxylamine (47).

Aebi et al. (48) used the coupled oxidation of formate-^{14}C as an indicator of the relationship between mitochondrial MAO and catalase. They found in lyophilized mitochondria, without added catalase, that a peroxidase type of reaction occurred to an extent of 15-40%. The aldehyde can be further oxidized to the corresponding acid (40). The reactions catalatic and peroxidative catalyzed by catalase as well as the further oxidation of the aldehyde can alter the oxygen comsumption stoichiometrically. The oxygen uptake is a common method used to measure MAO activity (see p. 51,

this thesis). Thus it is necessary to evaluate the possible occurrence of each one of these reactions before interpreting any results obtained with this methodology, especially when non-purified preparations are used.

Pletscher et al. (49) in accordance with Mason (50) classified MAO as a 2 electron transferase: two electrons are transferred directly or indirectly to oxygen, which is reduced to hydrogen peroxide, while the aminogroup is oxidized to the iminogroup or the aldehyde. Earlier, in 1931 Hare-Bernheim (9) represented the possible stages of the MAO reaction in the following way:

$$\text{(V)}\quad R\text{-}CH_2\text{-}NH_2 + O_2 \longrightarrow R\text{-}CH{:}NH + H_2O_2$$

$$\text{(VI)}\quad R\text{-}CH{:}NH + H_2O \longrightarrow R\text{-}CHO + NH_3$$

Smith et al. (51) showed by means of isotopic oxygen that the oxygen, in the MAO catalyzed reaction, is converted to hydrogen peroxide and that the oxygen in the product arises from the solvent water. The production of aldehyde under anaerobic conditions have also been demonstrated (52,53). Thus Yasunobu and Oi (54) divided the MAO reaction into two phases, an anaerobic one where the aldehyde is produced, ammonia is liberated and the enzyme is reduced; and a second phase where the enzyme is reoxidized by molecular

oxygen with the production of hydrogen peroxide. These authors and Tipton and Spires (55) described the reaction as occurring through a ping-pong mechanism. Hellerman et al. (56) discussing the validity of equations (V) and (VI) observed "remains only the proof that the imine, elaborate as such at the active site, appears as the immediate reaction product". They claimed to have experimental evidence for the confirmation of this idea.

CHAPTER III: SUBSTRATE SPECIFICITY

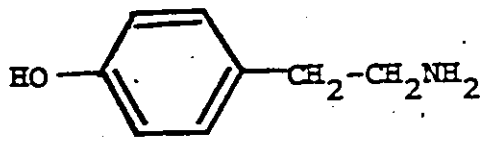
Monoamine oxidase exhibits a fairly broad substrate specificity depending on the source of the enzyme (57-61). The differences in substrate specificity among the enzymes from different organs and species, make any classification of the different substrates difficult. However, some general rules could be established.

Primary and secondary amines are readily oxidized by MAO, provided the substituent in the secondary amine is a methyl group (62). Blaschko (62) has suggested that an attack on N-methylated amines appears to be a safe criterion for the presence of a "true" MAO. The rate of oxidation of methylamino compounds is high, sometimes even higher than that of the corresponding primary amines. The tertiary amines are oxidized more slowly.

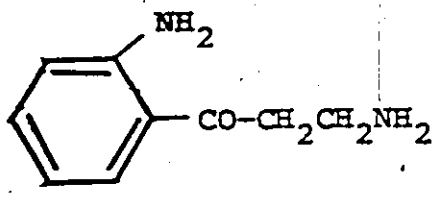
The rate of oxidation and affinity of the aliphatic amines depend on the number of carbon atoms. Methylamine is not attacked and ethylamine is oxidized slowly in some species, for example in cat and cattle (59). With increasing length of the chain, the rate of oxidation increases and maximal rates are reached with amylamine and hexylamine after which the rate falls off (1). Short chain diamines are not deaminated by MAO (14, 37, 59). However, long chain diamines

from heptamethylene diamine onward are oxidized by the enzyme. The diamine most rapidly oxidized by MAO is the compound with 13 methylene groups; beyond 13 methylene groups the rate of oxidation decreases with increasing chain length (1). Some derivatives of ethylendiamine containing a substituted amino group serve as MAO substrates (63).

Substitution of a hydrogen on the α-carbon atom of an aliphatic or aromatic amine prevents attack by the enzyme (11, 14, 59, 64, 65). Belleau and Moran (66) used tyramine (a) and kynuramine (b) together with their α-deuterium isotopes as substrates. They found that the removal of an α-hydrogen is the rate-determining step in the oxidation and that this is independent of the nature of the substrate.



(a)



(b)

The α-deuterium substrate had a much lower affinity for the enzyme. The substrates of more recognized physiological importance are β-phenylethylamine and tryptamine derivatives e.g. dopamine, noradrenaline, adrenaline, metanephrine, normetanephrine and serotonin. Though histamine is not a MAO substrate 1-4-methylhistamine is attacked by MAO. An increasing

number of methoxy substituents in the benzene ring produces a decrease in the affinity of MAO for such compounds. Thus, mescaline, β-(3,4,5-trimethoxyphenyl)ethylamine, is not a MAO substrate (19, 20, 37, 59, 65). Substitution of hydrogen on the β-carbon causes a decrease in the reactivity (14, 59, 67). If both β-hydrogens are substitued the resulting compounds do not serve as MAO substrates (68, 69). The introduction of an o-hydroxyl into catecholamines and benzylamine markedly reduced their rate of degradation (70).

Systematic study on the optical specificity of the enzyme has received relatively little attention. Blaschko et al. (13, 14) found that l-epinephrine was oxidized at a higher rate than the corresponding d-isomer by extracts of guinea pig liver or rat kidney. Pratesi and Blaschko (71) later reported that a preparation of guinea pig liver, but not of rabbit liver, showed optical specificity in deaminating β-hydroxyphenylethylamine. The guinea pig liver extracts oxidized the D-form more rapidly than the L-form. Leeper et al. (72) found that in a soluble guinea pig liver MAO preparation L-epinephrine was oxidized at a slightly higher rate (10/8) than the D-isomer, but in the case of norepinephrine the reverse occurred (12/16). Giachetti and Shore (73) found that the l-isomer of octopamine, norepinephrine and epinephrine were prefentially destroyed in different organs and preparations of rabbit and rat.

Kohn (12) and Philpot (74) found the rate of the MAO reaction depended on the partial pressure of oxygen. Aebi et al (48) confirmed this finding and reported the inhibition of MAO activity in the presence of pyruvate, which they explained on the basis of competition for oxygen. Novick (75) described increase of the reaction rate with increased oxygen tension which was dependent upon the tissue, species and substrates. The Km values for oxygen in different preparations are relatively high (53, 76, 77). These Km values are all close to the value of the oxygen concentration of air saturated water at physiological temperature (76). On the other hand the response of MAO to local fluctuations in oxygen concentration depends upon the operative concentration of the amine. The relative efficiency with which the enzyme will oxidize low concentrations (< Km) of amines will increase as the oxygen concentration falls (76). Tipton (76), using purified pig brain MAO observed that when the concentration of tyramine was 10 μM or less the activity of the enzyme was relatively insensitive to quite large fluctuations in the concentrations of oxygen. This author remarked that the enzyme activity would depend on the nature of the substrate involved. These factors are important when the partial pressure of oxygen in the tissues is considered as a regulation of MAO activity.

The literature on the kinetic data is difficult to

compare due to differences in experimental conditions and variations between different preparations as mentioned above. Alles and Heegaard (59) and Oswald and Strittmatter (61) ascribed these variations between different preparations to the possible existence of different enzymes.

The optimum pH is different for different substrates, Werle (58) gives values ranging from 6.9 to 8.4. McEwen et al. (78) found that in a partially purified MAO from human liver mitochondria, the apparent Km values for N-methylbenzylamine, and veratrylamine depended upon pH. In an analysis of their data they reported no evidence of ionizations, other than those of the free substrates, that might affect the formation of the substrate-enzyme complexes. The marked changes in the apparent Km values with changes in pH were followed by little or no change in the maximal velocities. These authors concluded that their data indicated that the non-protonated species of these substrates were bound by the enzyme. Yasunobu and Oi (54) using a purified preparation of bovine hepatic MAO found a pH effect on the formal mechanism of the enzyme. They reported constant values of Km and Vm for oxygen and benzylamine from about pH 7.3 to 10.3. Below pH 7.3, both Km and V values change with pH. Above pH 10.3 only the Km for the amine changes with pH. Combining chemical and kinetic data they proposed several formal mechanisms and derived rate expressions for the different pH

ranges which are in agreement with a "ping-pong" mechanism for the MAO reactions.

Gorkin and collaborators (79) some time ago reported alterations in the properties of MAO by the effects of oxidizing agents such as peroxides of higher saturated fatty acids, cupric ions, H_2O_2, ergosterol peroxide and o-iodoso-benzoate. These alterations consisted in an inhibition of the enzyme towards its natural substrate and appearance of a new ability to deaminate oxidatively new substrates (histamine, cadaverine, lysine, spermidine, adenosine 5'-phosphate). The enzymatic activity also became resistant to MAO inhibitors it was sensitive to carbonyl reagents. The MAO inhibition was followed by a reduction in the content of SH groups in the preparation used. Incubation of rat liver mitochondria with oleic acid, in the presence of harmine or α-methyl-tryptamine, at concentrations which inhibited the deamination of serotonin, caused almost complete inhibition of tyramine deamination but deamination of serotonin was only slightly decreased. The new ability of mitochondrial MAO to deaminate new substrates could be prevented by preincubation with MAO inhibitors. The properties of the original enzyme, as in the case of Sarcinea lutea tyramine oxidase, could partially be restored by treatment with reducing agents.

Exposure of diamine oxidase to reducing agents

decreased the rate of putrescine deamination. At the same time the ability to deaminate tyramine appeared which was inhibited by MAO inhibitors (79). A similar phenomenon of the disappearance of MAO properties, was reported by Gorkin et al. (80) to occur during the development of radiation disease in rat liver mitochondria in vivo. They suggested that these changes were due to the peroxides of higher unsaturated fatty acids released during the development of radiation disease. Diaz Borges and Drujan (81) reported an increase in MAO activity, using kynuramine as substrate, in several organs of the fish Eugerres plumieri immediately after exposure to γ-irradiation. This increase was possibly caused by factors outside of the organs where MAO was measured, since this activation could not be produced in vitro. Forty-eight hours after the exposure to γ-irradiation the MAO activity started to diminish but reached values below the control only after 72 hours. In mice, changes in MAO activity were noticed only in liver and spleen. The enzyme activity in kidney, brain and heart remained constant. In spleen, though the specific activity for MAO increased, there was actually a decrease in the total enzyme content of the whole organ following a drastic reduction in the organ weight. In liver there was a decrease in MAO activity, as noticed by Gorkin et al, in the rat liver mitochondria during the development of the radiation disease.

CHAPTER IV: INHIBITORS OF MAO

A wide variety of drugs which inhibit MAO has been introduced following the demonstration by Zeller et al. (82-84) that iproniazid, a compound which was developed as an antitubercular drug, was a potent inhibitor of this enzyme. As stated by Ho (85) MAO is perhaps the enzyme with the largest number and widest variety of inhibitors. Several reviews have been devoted to this subject (49, 85-97).

There is no representative MAO inhibitor as such. The degree of inhibition, rapidity of action, organ specificity, toxicity and pharmacological action are peculiar to each compound or group of compounds (94, 96). Marked species differences in response to MAO inhibitors also occur (94, 96). It is difficult to make generalizations as to the specific structural requirements for MAO inhibitory action, notwithstanding the prolific research carried out in this field. The inhibitory activity varies with the substrate and some inhibitors either selectively inhibit only a limited fraction of the total enzyme activity or exert selectivity toward a particular tissue or organ (85). Some of the MAO inhibitor effects could be due to either direct action of the drugs themselves or to changes in the binding of amines in the tissues rather than due to MAO inhibition (94). Among the MAO inhibitors we find derivatives of hydrazines, propargylamines, cyclopropylamines,

indolealkylamines, α-,β- and γ-carbolines, pyridines and a heterogeneous group of substances. Werle (58) distinguished two main groups of MAO inhibitors:

I. Competitive inhibitor.

1. Hydrazine derivatives.
2. α-alkylated arylalkylamines.
3. Choline-p-tolyl ether.
4. Harmala alkaloids.
5. Alkaloids as piperidine, nicotine and chlortetracycline.

II. Non competitive inhibitors.

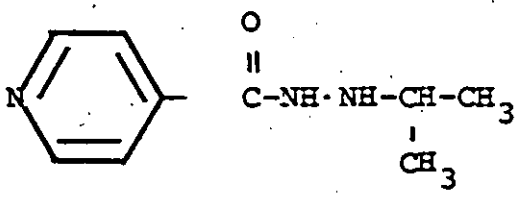
1. Amidines.
2. Antihistamines.

As this last author pointed out the in vitro effect of some of these MAO inhibitors often does not parallel their in vivo action (98, 99).

Hydrazides.

The prototype of this group is iproniazid. (1-isonicotinyl -2-isopropylhydrazine). It has been proposed

that iproniazid is converted into an active principle before



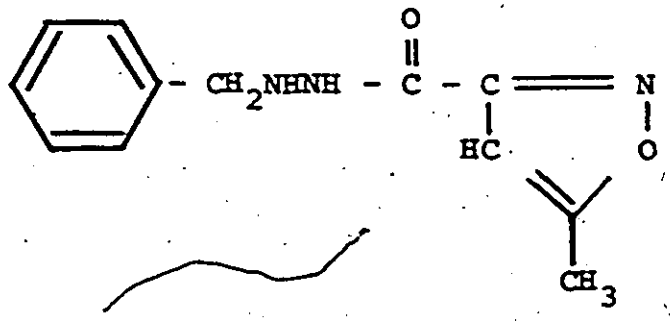
IPRONIAZID

it inactives the enzyme (85). The formation of isopropyl hydrazine, a more potent MAO inhibitor than iproniazid, was proposed (100). Another alternative possibility was the formation of 1-isopropylidene-2-isonicotinoylhydrazine by dehydrogenation of the iproniazid molecule after its attachment to the active site (100).

Iproniazid is a competitive inhibitor of the enzyme from rat liver mitochondria as tested in presence of the substrate, tyramine (100). In contrast the same MAO inhibitor acted as a noncompetitive inhibitor with respect to the substrate, veratrylamine in a purified enzyme preparation from human liver mitochondria (101).

Substitution of a chlorine atom on the para-position of the benzene ring of benzoylisopropyl hydrazides results in

a nearly three fold increase in the in vivo inhibitor activity on the oxidation of tyramine by rat liver mitochondria (102).



ISOCARBOXAZID

Isocarboxazid (5-methyl-3-isoxazolecarboxylic acid 2-benzylhydrazine) is both in vitro and in vivo a much stronger inhibitor than iproniazid for the deamination of tyramine and is less toxic (49, 103). Introduction of a dimethyl-amino group on the para-position of the benzene ring of isocarboxazid yielded a short acting MAO inhibitor [1-(p-dimethylaminobenzyl)-2-(5-methyl-3-isoxazolylcarbonyl)hydrazine] (104-106).

Hydrazines.

Vetracin (3,4-dimethoxybenzylhydrazine) had the same effect on the deamination of tyramine when the incubation was carried out in an atmosphere of oxygen or hydrogen as it had in air, and the replacement of air by nitrogen did not decrease the inhibition (107). So vetracin is different from

iproniazid which requires oxygen for the inhibition reaction (100). Kinetic studies with rat liver mitochondria showed that the inhibition by vetracin was noncompetitive with respect to tyramine, serotonin and benzylamine (85).

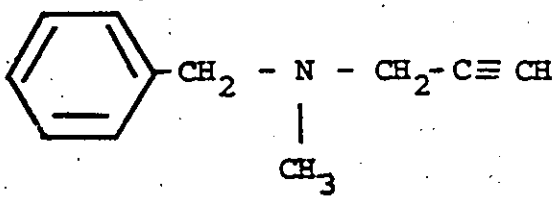
Horita (108) observed that the route of administration could be important for some hydrazines in relation to their organ specificity. Phenelzine (phenethylhydrazine) and pheniprazine (β-phenylisopropylhydrazine) showed much greater inhibition of brain MAO than that of liver MAO when it was administered subcutaneosly to rats and the reverse occurred when the compounds were given orally. This organ selectivity was not noticed with iproniazid and nialamide.

The inhibitory effect of phenylalkylhydrazine decreases with an increase in the methylene chain length up to four carbons, compounds with a five-carbon chain producing the same degree of inhibition as those with a three-carbon chain. The α-methylated analog exhibited no striking difference in inhibition (109).

The *in vivo* activity of a hydrazide may depend upon the readiness of hydrolysis of the acyl group to free alkyl- or aralkyl-hydrazines (110). The transport of the inhibitor into tissues and its tissue selectivity may be provided by the acyl portion of the hydrazide molecule (85).

Propargylines.

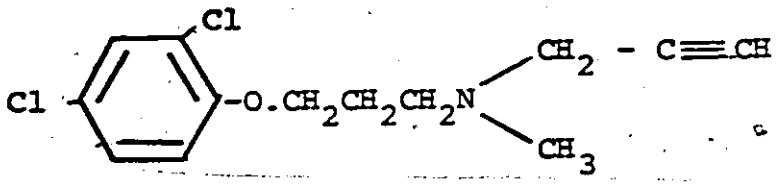
Pargyline (N-methyl-N-2-propynyl benzylamine) was found to produce inhibition, both competitive and noncompetitive, with respect to veratrylamine in purified human liver mitochondrial MAO preparation and it was



PARGYLINE

similar to that observed with tranylcypromine (101). Hellerman and Erwin (111) reported results indicating that pargyline was covalently bound to the active site of the enzyme. Hellerman et al. (56) suggested that the covalent linkage was most probably to the flavonucleotide component of the enzyme (Cofactors and sulphydryl groups. This thesis). It has been proposed that a requirement for the irreversible inhibition of tyramine deamination is the attachment of pargyline to metal ions (112). Vina et al. (113) observed that pargyline inhibited the deamination of tyramine at substantially lower concentrations than dopamine or serotonin. An inhibitor of propargylamine-type compounds containing an indane nucleus, N-methyl-N-2-propynyl-1-indanamine, was found to be characterized

by a very high potency *in vitro* but a relative lack of action on liver MAO *in vivo*. It was 20 times more potent than pargyline in inhibiting serotonin and tyramine deamination in rat brain (114). Maître (114) reported from his studies on Su-11,739(N-methyl-N-2-propynyl-1-indanamine) with various tissue preparations, that the differences in substrate specificities and inhibitor sensitivities provided additional evidence that various tissues of the same animal species contained more than one single MAO and that the proportions of the different MAO might vary considerably from one organ to another. Johnston (115) studied the effects of clorgyline [N-methyl-N-propargyl-3-(2,4-dichlorophenoxy)--propylamine hydrochloride]. He found a pair of sigmoid

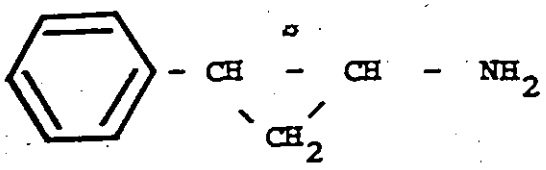


CLORGYLINE

curves joined by a horizontal section when he plotted the percentage of inhibition in rat brain MAO against the concentration of inhibitor. This author concluded that MAO is a binary system of enzymes each of which has detectably different sensitivity to clorgyline. Hall *et al.*

(116) extended this observation to a variety of species and organs and deduced that two enzymes are present in the brain of rat, man, rabbit, ox, dog and cat and also in the liver of rat and man, whereas the MAO from the brain of pig and liver of all species examined, other than rat and man, seem to be homogeneous. Hall and Logan (117) demonstrated that unlike iproniazid, the inhibition of clorgyline neither requires pre-incubation, nor was it affected by cyanide and copper. Phenylisopropylmethylproninylamine (Deprenil), a strong MAO inhibitor of this group is a potent inhibitor of the oxidative deamination of m-iodobenzylamine and benzylamine but a poor inhibitor of serotonin deamination (118).

Cyclopropylamines.



TRANYLCYPROMINE

Tranylcypromine (d-trans-2-phenylcyclopropylamine) is a better in vivo and in vitro inhibitor of MAO than iproniazid and its mode of action is different from other irreversible MAO inhibitors such as pargyline and iproniazid (85). In contrast to other irreversible inhibitors of MAO

tranylcypromine did not require either pre-incubation or the presence of oxygen. Maass and Nimmo (119) reported that rat brain MAO was inhibited noncompetitively by tranylcypromine when the substrate was serotonin. Other authors found that the inhibition was irreversible and competitive in the liver enzyme from various species for the substrate tyramine (120-123). Tranylcypromine inhibited the deamination of veratrylamine competitively and noncompetitively (mixed type) when human liver mitochondrial MAO was used.

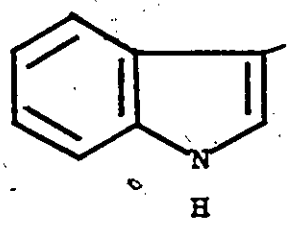
The inhibition of rat brain mitochondrial MAO by [2-(o-chlorophenoxy)ethyl]cyclopropylamine was found to be irreversible and noncompetitive with respect to serotonin or phenethylamine (124). The deamination of indolealkylamines was preferential as compared to phenethylamines when this drug was used. A comparison of this compound with pargyline was done by Fuller in a later report (125). He used phenethylamine and tryptamine as substrates and found that pargyline showed greater potency when phenylethylamine was the substrate and caused greater inhibition in tissues in which phenylethylamine oxidative capacity was high with respect to tryptamine. The opposite was the case when [2-(o-chlorophenoxy)ethyl]cyclopropylamine was the inhibitor.

Huszti et al. (126) showed that 1-(m-aminophenyl)-2-cyclopropylaminoethanol dihydrochloride preferentially

inhibited the deamination of MAO substrates such as norepinephrine and serotonin, but not tryptamine in rat brain homogenates.

Indolealkylamines.

The 3-substituted indoles are, in general, inhibitors of irreversible nature and of shorter duration (85). Huszti and Borsy (127) observed marked differences



N,N-DIETHYLTRYPTAMINE

in the inhibitory properties of N,N-diethyltryptamine and its 2-substituted derivatives dependent on whether the substrate used was tryptamine or 5-hydroxytryptamine. These differences are interesting in view of the close structural similarity between the two compounds. Barlow (128) suggested that the dependence of the inhibitory action of 2-methyl-dimethyltryptamine on the type of substrate could be due to the existence of at least two types of enzymes. Huszti and Borsy (127) felt that the theory of Zeller of eutopic and dystopic complexes (129) might be a more suitable explanation. Gorkin

et al.(130) reported similar observations with some α-substituted tryptamine derivatives. They found that α-methyltryptamine (indopan) selectively inhibited the oxidative deamination of serotonin by rat liver mitochondria in vitro as compared to tyramine or tryptamine. This selective inhibition was demonstrated in liver and brain tissues in vivo. Species differences in sensitivity of MAO preparations towards indopan in vitro were also observed by these authors.

Other heterocyclic compounds.

The harmala alkaloids, β-carboline derivatives, are MAO inhibitors with reversible effects in vitro and faster action in vivo (85). Harmaline is the most potent inhibitor of this series (131). Applied in vivo this drug protects an animal against the actions of some long-acting MAO inhibitors (132). Squires (133) has divided the mitochondrial MAO capable of deaminating kynuramine into two groups: one of these groups is inhibited by harmine but is relatively resistant to pargyline, while the other is resistant to harmine but is inhibited by low concentrations of pargyline. Gorkin et al.(134), after testing 30 carboline derivatives, found that harmine inhibited the oxidative deamination of serotonin by rat liver mitochondrial MAO at the lowest concentrations and with the greatest selectivity. They

observed 100 to 1,000 fold differences in the sensitivity of deamination of serotonin or tyramine to inhibition by harmine in different organs of animals of the same species, and also in the same organ of animals of different species. Yasuhara et al. (135) showed that harmine inhibited the oxidation of serotonin more specifically than that of tyramine in the beef brain. α- and β-carbolines are weaker inhibitors than the harmala alkaloids (85). 2-Methyl-3-piperidinopyrazine (modaline) is a rapidly acting MAO inhibitor whose effects are irreversible and of long duration (136, 137). This compound as well as 3-(5-nitrofurfurylidene) amino-2-oxazolidinone (Furazolidone), 3-[2-(4-methyl-1-piperazinyl)-5-nitrobenzylidene]amino-2-oxazolidinone and 5-oxo-(D-trans-2-phenylcyclopropyl)-1-2-pyrrolidone carboxamide require biotransformation before exerting its effect as an MAO inhibitor (85). 3-Amino-2-oxazolidinone is yet another compound with similar properties (138).

Miscellaneous.

Amphetamine and ephedrine inhibit MAO competitively and reversibly (11, 14, 59, 62, 139). MAO can be inhibited by compounds that react with sulphydryl groups such as p-chloromercuribenzoate, iodoacetate, mercury salts, arsenicals, salts of cadmium and silver as well as by an excess of certain sulphydryl compounds, methylene blue and tetrazolium

salts (88). Another group consist of the peripheral MAO inhibitors (85). This group of inhibitors generally has a charged moiety in the molecule which makes its penetration through the blood-brain barrier difficult. Compounds of diverse structure are found in this group such as benzylguanidines, guanylhydrazones of aromatic carbonyl compounds, styrylquinoliniums and β-carbolines.

Chemical substances as diverse as 2-bromo-2-phenylacetaldehyde (140, 141), nitroglycerin (142), 2-(diethylamino)ethyl 2,2-diphenyl-valerate hydrochloride (proadifen) (143), choline-p-tolyl ether (144, 145), octanol (1), local anaesthetics (146), urea (65), amidines (58, 146, 147), s-n-alkyl-isothioureas (148), sodium pentobarbital (149), hydroxyquinolines (153), thyroxine derivatives (150), estradiol (151) and various anions (152) also produce MAO inhibition.

CHAPTER V: DISTRIBUTION

MAO is widely distributed in the tissues of different animal species (1, 14, 62, 154). The amount of enzyme varies with the species (155). It is found particularly in glands, smooth muscle and nervous tissue (13, 65, 156). The highest activity of the enzyme is found in the liver and kidney of most animals. However, rat kidney is a relatively poor source of the enzyme (11, 14). The brain of Bufo marinus shows higher MAO activity towards kynuramine than the liver (157). Preparations with high MAO activity can also be obtained from pancreas, intestine, aorta and stomach (14, 65). In man the parotid and submaxillary glands represent the richest source of the enzyme (158). In the uterus the MAO activity is high. Changes in the human endometrial MAO has been reported during the menstrual cycle (159, 160) as well as in various tissues of the rat and guinea pig during the estrus cycle (160, 161). Visceral organs other than liver and kidney usually display less MAO activity (10, 11, 14). Relatively high activity of MAO is found in tissues richly endowed with sympathetic nerves as well as in the nerves themselves (162). The superior cervical ganglia of the rat is rich in MAO and the ganglionic MAO activity is greater than in other investigated, except liver (164). In the brain, MAO shows highest activity in regions with active monoamine metabolism, but also occurs in other parts

of the brain. It has been suggested that this enzyme could be a "safety factor" against the uncontrolled entrance and diffusion of monoamines into brain tissue (164). Several biochemical and histochemical studies on the distribution of this enzyme in brain are available for diverse animal species (164). This distribution is not homogeneous. In general, high activities can be found in midbrain, hypothalamus, thalamus, basal ganglia and hippocampus, though the localization varies as a function of both species and substrates (162, 164-169). By means of chemical (6-hydroxydopamine) and surgical sympathectomy the occurrence of intra and extraneuronal MAO has been studied in several tissues (170-172). The existence of differences in inhibitor sensitivity, substrate specificity and thermal inactivation of MAO in normal and denervated tissues suggested different properties for the neuronal and extraneuronal MAO (171, 173). MAO has been found in the human placenta as well as in that of other mammals (174). The human blood serum (175) and blood plasma (176) also contain MAO. Blood platelets are a relatively rich source of the enzyme (177, 178). Due to the accessibility of the blood tissue, some investigators have attempted to test the degree of MAO inhibition by means of MAO assay in the components of this tissue. Recently, Collins and Sandler (179) have critized this approach because of the multiplicity of MAO, the inhibition of MAO in platelet could not correspond to the inhibition in other tissues.

Cotzias and Dole (180) observed that the major part of MAO in rat liver was in the mitochondria. Hawkins (181) found that about two-thirds of the enzyme in liver was localized in the mitochondria and the rest of the activity resided in the microsomes. These results were confirmed more recently by other authors (182-187). De Champlain et al. (187) observed similar localization in the rat liver but in other organs the subcellular distribution was different. In the heart 50% of the activity was associated with the microsomal fraction and 20% was found in the supernatant. In the salivary glands equal amounts were found in microsomal and mitochondrial fractions. In the rat vas deferens (186, 187) and bovine thyroid (188) also exist a higher proportion of MAO in the microsomal fraction which is again at variance with the liver distribution. In the bovine adrenal medulla MAO activity seems to be found exclusively in the mitochondria (189). In brain the enzyme is also localized mainly in the mitochondrial fraction (167, 190-193). It has been shown that the enzyme occurs in synaptosomes (193) as well as in glial cells (194). Weissbach et al. (195) described a soluble MAO in guinea pig liver. Oswald and Strittmatter (61) suggested that this soluble enzyme could arise from lyzed mitochondria since in the liver of this animal MAO is easily liberated upon lysis of mitochondria. Furthermore Jarrot and Iversen (186) believe that the most likely explanation for the origin of the microsomal MAO is that this arises as an

artifact during homogenization in which the enzyme is recovered as fragments of mitochondrial outer membranes. As Tipton (196) indicated, if this is the case, the differences in properties between MAO of mitochondria and microsomes would then be modifications of the properties of the enzyme by disruption of the mitochondrial membranes.

MAO is associated with the outer membrane of mitochondria (197). This idea was originated from the studies of Schnaitman et al. (198) and it was confirmed subsequently by other reports (199-205), although Green et al. (206) reported contradicting results. Later on these last authors found a great enrichment of MAO activity in purified outer membrane fractions of beef heart mitochondria (207). MAO has been used as a "marker" of the outer mitochondrial membrane by several authors (208-215). Gorkin (208) has questioned the use of this enzyme as marker of the outer mitochondrial membrane because he found higher MAO specific activities in cytoplasmic membranes and, especially, in the nuclear membranes. Racker and Proctor (216) observed that purified monoamine oxidase combined specifically with preparations of resolved outer membrane. Oreland and Olivecrona (217-219) also recombined a solubilized MAO from pig liver mitochondria with lipid-depleted mitochondria from the same source in the presence of added phospholipids. A similar phenomenon with the same enzyme was observed using delipidated

red cell membranes and delipidated milk fat globule membranes. They found a correlation between the yield of soluble enzyme and the extraction of anionic phospholipids, cardiolipin being the major component. The soluble enzyme could interact with acidic phospholipids either in solution or bound to membrane residues. Oreland and Ekstedt (220) found differences in thermostability and resistance to digestion by trypsin between soluble and in situ bound mitochondrial MAO. The soluble MAO was more labile. When the soluble enzyme was rebound to relipidated membranes its ability to resist heat denaturation was partially restored, but the rebound enzyme had greater sensitivity to the trypsin digestion, indicating that a larger area of the rebound enzyme was exposed in comparison to the original bound enzyme. Recently Greenawalt (221) has reviewed the localization of MAO and reported preliminary results where kynuramine MAO activity showed a different distribution, in subcellular fractions of rat liver mitochondria, from those of benzylamine, serotonin and tyramine MAO activities. Kynuramine activity was almost equally distributed in the outer membrane, inner membrane and matrix fractions, while the activity for all the other substrates was largely found in the outer membrane. Kroon and Veldstra (222) separated, by means of sucrose gradient centrifugation of a crude mitochondrial fraction of rat cerebral hemisphere, the MAO activities for several substrates. Their results indicated

that dopamine and serotonin activity have the same localization in the gradient and this was different from those of noradrenaline and kynuramine activities. These authors concluded that in rat brain homogenate existed synaptosomes which preferentially deaminated dopamine and serotonin and they were different from the noradrenaline deaminating synaptosomes. The free mitochondria differed from the mitochondria enclosed in synaptosomes by their relatively high affinity for kynuramine.

CHAPTER VI: COFACTORS AND SULPHYDRYL GROUPS

In 1952, Hawkins (223) found a decrease of liver MAO activity in riboflavin deficient rats. This work was confirmed and extended by Sourkes and collaborators (224-227). Wiseman-Distler and Sourkes (226) reported that the riboflavin deficiency renders the hepatic MAO of rat more susceptible to the action of inhibitors both *in vivo* and *in vitro*. After iproniazid inhibition the restoration of dopamine oxidation was slower thatn that of serotonin oxidation (226). These studies did not distinguish whether riboflavin was an integral part of the enzyme (prosthetic group) or if it was necessary in the biosynthesis of MAO. Furthermore riboflavin antagonists could inhibit the enzyme (225, 227-229). All these findings suggested the possibility that MAO might have a flavin cofactor. Nara *et al.* (230) showed evidence for the presence of flavin in beef mitochondrial MAO. Their study was based on changes in the absorption spectrum of the enzyme as well as on identification of the flavin by a microbiological assay and by its fluorescence properties. The flavin was apparently covalently bound to the enzyme. These results were extended in other reports (231-233). Erwin and Hellerman (234) on similar basis also postulated the presence of flavin in beef kidney mitochondrial MAO. They, as well as Yasunobu and collaborators (230-233), failed to remove the flavin moiety from the apoenzyme. Tipton (235) was able to extract a

fluorescent material from a purified pig brain MAO which was identified as flavin-adenine dinucleotide (FAD). Pyridoxal phosphate was found to be absent in the preparation and the inactive FAD-free apoenzyme could be reactivated by FAD. Walker et al. (236, 237) have isolated a pentapeptide from beef liver mitochondrial MAO in which a thioether linkage of cysteine to the 8 α group of flavin was found. Furthermore Ghisla and Hemmerich (238) found that the properties of synthetic cysteinyl-8α-riboflavin are similar to those of the compound isolated by Walker et al. FAD has also been identified in the beef and human brain MAO by means of fluorescence spectra and thin layer chromatography (239-242). Similarly FAD has been demonstrated to occur in purified rat liver mitochondria MAO (243, 244). Though the flavin extracted from purified MAO preparations of pig liver behaves like FAD in a chromatrographic system, the fluorescent properties and optical spectra were found to differ from those of authentic FAD (245). The difference of the MAO flavin from FAD could be attributed, according to Oreland (245), to an amino acid still attached to the flavin, or to a difference in the flavin molecular structure.

The strength of attachment of the flavin to the enzyme is variable in different preparations. In beef kidney (234), beef-liver (231) and pig-liver (245) purified MAOs the flavin is tightly bound. In purified preparation of pig brain (235) the flavin appears to be more loosely bound

hile in preparations from rat-liver about one half of the AD is loosely bound (acid-extractable flavin) and the other alf is tightly bound (non-acid extractable flavin). The ignificance of the strength of the flavin attachment to he enzyme is unknown. This could be either due to the ifferent techniques of purifications as mentioned by ipton (196) or due to species variations.

The possible requirement of MAO for a metal cofactor has been suggested by several authors (243, 246-256). Nara et al.(252) reported purifications of beef-liver MAO where there was a direct correlation between the copper content and the specific activity of enzyme preparations. On this basis as well as on the MAO inhibition by copper chelating agents and other properties, they thought that copper could be a cofactor of the enzyme. Other metals such as iron, molybdenum, cobalt and manganese were not present in detectable quantities. Coq and Baron (254) described the reactivation of MAO by copper after prolonged dialysis. The bovine thyroid mitochondrial and microsomal MAO were reported to be inhibited by copper chelating agents and on this basis a possible requirement of copper for the enzyme activity as suggested (257, 258). Reports of other investigators with purified preparation of other sources failed to corroborate the results of Nara et al.(234, 235, 242, 243, 245). Further purification of beef liver MAO by

Yasunobu et al. (232) showed results quite different from those of Nara et al. (252). Correlation between the copper content and the specific activity of MAO in the preparation was not found during different stages of purification. According to Yasunobu and Oi (54) MAO is not considered a copper enzyme.

A possible role of iron in the action of MAO has been suggested by Symes et al. (255, 256). This metal, according to these authors, could have some role as a prosthetic group of the enzyme or as a functional part of an enzyme catalyzing the incorporation of riboflavin into the apoprotein of MAO. Futher studies are necessary for clarification of the significance of this metal.

The presence of SH groups essential for the activity of MAO was first suggested by Friedenwald and Herrmann (259). The enzyme was inhibited by organic mercurial compounds which reacted with SH groups and the inhibition was reversed by glutathione or cysteine in the presence of cyanide. Later it was demonstrated that cyanide reacted with p-chloromercuribenzoate and this was the cause of the reactivation. Since glutathione still had a reactivating effect in the absence of cyanide, it was concluded that SH groups were necessary for enzyme activity (260). Lagnado and Sourkes (246) studied the inhibition of the enzyme by metal ions and sulphydryl compounds and interpreted

their data as indicating the presence of SH groups, although they pointed out that other mechanisms for metal inhibition must also be considered. The inhibition of MAO activity by mercaptide-forming reagents was confirmed in experiments with partially purified preparations (123, 249).

Nara et al. (251, 252) reported the presence of SH groups in a purified beef-liver mitochondrial MAO preparation which did not seem to be vicinal. The enzyme preparation was very sensitive to sulphydryl reagents. Gabay and Valcourt (253) found that their purified rabbit-liver mitochondrial MAO was more sensitive to the action of p-chloromercuribenzoate than that of Nara et al. This difference may be due to the fact that this rabbit-liver preparation was subjected to repeated column chromatography (249, 253). Other preparations from different sources with different degree of purification such as beef (240, 242, 261) and human (241, 242) brain mitochondria, pig brain mitochondria (262), bovine thyroid mitochondria (257) and microsomes (258), bovine kidney mitochondria (234) and rat liver mitochondria (249, 263, 264) have been found to be affected by sulphydryl reagents. Klyashtorin and Gridneva (264) showed in rat liver mitochondrial preparations that for the inhibition of the deamination of serotonin it was necessary to block less SH groups with mercaptide-forming reagents than for the inhibition of tyramine, benzylamine and p-nitrophenylethylamine. Erwin and

Hellerman (234) found in bovine kidney MAO a decrease of the enzyme activity which kept a linear function with the amount of p-chloromercuribenzoate added. This would suggest the possible direct involvement of the SH groups in catalysis. From kinetic studies with beef liver MAO which disclosed that both p-chloromercuribenzoate and heavy metal ions are noncompetitive inhibitors, Gomes et al. (265) concluded that the SH groups are not involved in catalysis. These authors suggested that the SH groups are required for conformational stability. In a recent report Yasunobu and Oi (54) proposed a model where two SH groups were localized in the active center and five others were on the surface of the enzyme. These last five SH groups would be responsible for the conformational stability as suggested by Gomes et al. (265). Gorkin and collaborators (79, 266, 267) observed changes in the content of SH groups during the "transformation" of MAO to a diamine-oxidase-like enzyme by the influence of oxidizing agents. These authors suggested the existence of a relationship between the SH group content of MAO and the phenomenon of qualitative alterations of its catalytic properties.

CHAPTER VII: DETACHMENT OF MAO FROM BIOLOGICAL MEMBRANES. PURIFICATION

MAO is tightly bound to insoluble structures of mitochondrial membranes (62, 249) rendering the purification of the enzyme very difficult, notwithstanding the great amount of effort put to this end, since its initial discovery by Hare (8). In the initial attempt to purify this enzyme from homogenates, the soluble material was separated from the insoluble and discarded, but the degree of purification obtained was poor (8, 12, 59). Subsequently several groups of workers tried to liberate the enzyme from the biological membranes as a first step in its purification by means of different techniques such as extraction with either detergents (51, 123, 268-273) or by sonication (262, 274-276) or with both detergent and sonication (84, 261, 277, 278) or with organic solvents (279, 280). Weissbach et al. (195) obtained a soluble MAO from guinea pig liver after homogenizing the tissue with distilled water. As already mentioned, Oswald and Strittmatter (61) thought that this soluble MAO was a product of mitochondrial lysis.

The term solubilization adopted by several authors could be misleading since different criteria were used to determine whether MAO was solubilized (281) e.g. centrifugation at arbitrary time and centrifugal force, stability in the

aqueous phase after several purification steps, precipitation by salts and resuspension or dissolution in aqueous medium. This problem increases in view of the tendency of the enzyme to aggregate (196) or to form complexes with other subparticles (217, 218). Akopyan et al (282) used the term solubilization to denote a phenomenon of apparent conversion of polyionic macromolecular aggregates (e.g. mitochondria) to an optically clear, homogeneous, single aqueous phase. On the other hand in many of these attempts of solubilization the purpose was further purification for which a true solubilization was not essential (281).

The liberation of the enzyme from the mitochondria has made it possible to use standard techniques such as salt fractionation, gel filtration and ion exchange chromatography for the purification of MAO. With these and other techniques purification of this enzyme to various degrees has been achieved in beef liver (123, 232, 233, 282, 283), kidney (234), brain (240,242, 282), adrenal medulla (284) and thyroid (257, 258), pig brain (262, 285) and liver (245, 270, 280), rabbit liver (253), monkey intestine (286), human liver (78), brain (241), blood platelets (179) and placenta (287). Kapeller-Adler (300) has made a detailed survey of some of the above mentioned purification procedures.

Different methods for the purification of rat liver

mitochondrial MAO have been reported. The first significant purification of MAO from this source was achieved by Guha and Krishna Murti in 1965 (276). They subjected a sonicated preparation, with 90% of the activity of the original mitochondria, to two successive steps of chromatography on DEAE cellulose. A 350-fold purification over the homogenate was reported. When tyramine, serotonin, tryptamine and adrenaline were used as substrates, no significant difference was observed in the substrate specificity of the preparation after the chromatographic steps, as compared to the sonicated preparation. A year later, Klyashtorin et al. (263) obtained an overall 26 fold purification over the rat liver homogenate with a yield of 4%. The purification procedure involved the use of the non-ionic detergent OP-10 and chromatography on calcium phosphate gel (brushite). In a similar fashion the purified preparation retained the ability of the mitochondria to deaminate several amines as tyramine, serotonin, isoamylamine, tryptamine and dopamine as well as its sensitivity to MAO inhibitors. In the same year, Youdim and Sourkes (278) reported a purification of rat liver MAO up to 208-fold over the homogenate with a yield of 29%. The MAO activity was measured using kynuramine as substrate. The enzyme was solubilized from the mitochondria by sonication and cholic acid and purified in several steps involving ammonium sulfate, Sephadex G-200, DEAE-Sephadex A-50, ammonium sulfate and calcium phosphate gel. Ultracentrifugation

of the purified preparation showed a single peak. The molecular weight as measured by gel filtration was 290,000 and the content of iron and copper were 0.12% and 0.034%, respectively. The sedimentation coefficients yielded an estimated molecular weight of about 150,000 which was interpreted as suggesting the presence of a monomeric enzyme, and that a dimer is formed during the course of gel filtration (243). This procedure of purification was applied to monkey and rabbit liver, and similar purifications as with the rat liver mitochondria were obtained (243). Youdim et al. (160, 288, 289) applied an analogous procedure to rat liver mitochondria and to different organs of distinct species. The purified preparations obtained by these authors were subjected to acrylamide gel electrophoresis and several bands were demonstrated by means of tetrazolium staining using the method of Glenner et al. (290). These bands were recognized by this group of workers as MAO isoenzymes. Akopyan et al. (282) described another procedure for the purification of rat liver mitochondrial MAO. They subjected the mitochondria to an alkaline medium containing 1 mM benzylamine in distilled water after which the preparation was treated with a non-ionic detergent (OP-10). Following this, the sample was carried through different steps of centrifugation, treatment with alumina Cγ gel, fractionation with ammonium sulfate, dialysis and chromatography on hydroxyapatite. The purification obtained was about 180 fold over the original homogenate

(substrate kynuramine). The yield was 2%. Polyacrylamide gel electrophoresis of the preparation in presence of the detergent OP-10 resulted in three bands which could be demostrated by either protein staining (amido black) or tetrazolium staining (290).

The purified preparations mentioned above are possibly not pure proteins as mentioned by Tipton (196). The molecular weight in the different preparations of distinct species varies from 100,000 to over one million. The purified MAO from beef liver, (233), pig brain (262) and pig liver (245) has been found to aggregate. According to the content of flavin in beef liver and kidney, pig liver and brain, rat liver and human brain, the minimum molecular weight of the enzyme has been found to be about 100,000 (196).

One question arising from studies on these purified preparations is how close are their properties to those of the enzyme in its natural state, since the enzyme originally is not free from cellular membranes. Another question is related to the existence of multiplicity of the enzyme. If the purification procedure is followed using only one substrate and there is loss of the activity for other substrates, it is difficult to explain how and why this occurred. The loss in activity could be due to modifications in the catalytic properties or due to undetected loss of some

of the multiple forms, which could be specific towards one of the substrates not tested during the purification.

CHAPTER VIII: METHODS FOR THE ESTIMATION OF MAO ACTIVITY

For the assay of MAO activity numerous methods are available. The disappearance of substrates, the consumption of oxygen, or the formation of products, namely aldehyde, ammonia and hydrogen peroxide, have been measured.

The rate of OXYGEN CONSUMPTION has been widely utilized. Earlier workers measured oxygen with manometric techniques (8-11, 14, 18, 46, 59). Creasey (291) conducted a thorough study of the factors which interfere with the manometric assay of MAO. He included semicarbazide and cyanide in the reaction mixtures to block other respiratory reactions, different from those catalyzed by MAO and catalase (catalatic), which could alter the oxygen consumption. The use of the manometric technique has been criticized because it requires long periods of incubation and also a period of equilibration (292). Yet another problem is their low sensitivity (293, 294). Zeller et al.(293) found that 40 to 50 times as much tissue homogenate was required for the manometric assay as compared to the spectrophotometric method described by them. The addition of cyanide and semicarbazide produce a complex and rigid system (295). Furthermore it has been suggested that the presence of cyanide ions changes the

susceptibility of MAO to certain inhibitors (100, 293, 296). In spite of the objections mentioned above, the manometric technique as standarized by Creasey (291) has been a very useful tool. This technique is particularly convenient for the comparison of the activity of the same sample towards different substrates.

Many of the difficulties in measuring oxygen consumption with manometric methods have been overcome by the use of polarographic methods. Lessler and Brierley (297) have reviewed the application of the oxygen electrodes to biochemical analysis. The Clark-type electrode (298) has been used to assay of MAO activity instead of the Warburg apparatus (149, 255, 272, 299, 300). This type of electrode can operate well in media with different substrates, different gas content and different pH. It can give continuous information of instantaneous reaction rates and requires relatively short periods of time for equilibration and monitoring processes. In comparison to manometric techniques its sensitivity is highly increased.

The estimation of liberated AMMONIA during the enzymic reaction is a reliable and accurate method of assay for MAO (296). This method has been used for many years (8, 10-12,46, 74) and permits the comparison of activity different substrates. The determination of ammonia has been

made with several procedures involving one of the following operations: a) vacuum distillation (8,11, 74) b) the Conway micro-diffusion (46, 247, 301) c) diffusion in the Warburg manometric apparatus (276, 302) d) ammonia assay directly in the incubation mixtures after deproteinization(165, 303). The liberated ammonia was estimated by nesslerization (8, 10-12, 74, 301, 302, 304) or by other techniques (165, 303, 305-308). The measurement of the rate of ammonia production in the MAO reaction by following the oxidation of NADH in presence of glutamate dehydrogenase has been suggested by Lorenz et al. (306). The determination of MAO by measuring ammonia formation has several shortcomings. The methods are generally tedious, insensitive and a continuous monitoring of the enzymic reaction is not possible (309). There is also the problem of eliminating the ammonia from external sources and also the elimination of endogenous ammonia from reactions other than those catalysed by MAO.

MAO activity has also been measured COLORIMETRICALLY using tyramine and serotonin as substrates (310, 311). Green and Haughton (294) suggested another technique in which the enzyme was incubated in the presence of tyramine and semicarbazide. At the end of the incubation period the semicarbazone formed was converted into the corresponding

2:4-dinitrophenylhydrazone the absorbance of which was measured at 450 mμ. p-Nitrophenylethylamine has been used as a substrate and its colored oxidation product has been estimated for the assay of MAO activity (263, 269, 312). Zeller et al. (313) used o-aminophenylethylamine which was converted to indole in the presence of MAO. The indole formed was measured by the procedure of Turner (314). The production of hydrogen peroxide has been measured by coupling MAO with peroxidase in the presence of o-dianisidine which produces a stable chromophore (315, 316). Sjoerdsma et al. tested the monoamine oxidase inhibition in man by measuring the urinary levels of 5-hydroxyindolacetic acid after an oral load of serotonin (317, 318).

SPECTROPHOTOMETRIC methods have been frequently employed for the determination of monoamine oxidase. While simple to perform these methods have certain limitations. Many of them are not useful for the comparison of the activity between different substrates. Not all the MAO preparations are suitable for spectrophotometric assay since they must be free from materials which would interfere with the assay measurement. Several workers have used benzylamine and its derivatives as substrates, for this assay. Benzylamine was first used by Tabor et al. (23) for the assay of beef plasma amine oxidase, but it is also applicable to MAO (319). With this substrate increase in absorbance at 250 mμ, the absorbance peak of

benzaldehyde, is recorded. A drawback of this method is that proteins also have a strong absorbance at this wavelength. Other derivatives of benzylamine which produce aldehydes with peaks of absorption in other regions of the ultraviolet spectrum, such as m-nitro-p-hydroxybenzylamine (309) and p-dimethylaminobenzylamine (320, 321) have also been used to measure MAO. Zeller et al. (293) assayed MAO activity by measuring the formation of m-iodobenzaldehyde, at 253nm, after incubation of MAO preparations with m-iodobenzylamine. They found that this was a better substrate than benzylamine for MAO assay. Weissbach et al. (322) described a method based on the rate of disappearance of kynuramine which has been widely used. It has been critized because the initial concentration of kynuramine is relatively low and the initial optical readings are high (293, 319). These authors have also proposed to measure the rate of formation of 4-hydroxyquinoline. Obata et al. (323) measured the residual amines after their incubation with MAO in presence of 2,4,6-trinitrobenzene-1-sulfonic acid. Popov et al. (324) assayed MAO activity measuring aldehyde semicarbazone formed from semicarbazide and aldehydes primarily arising in oxidative deamination of amines. With the last two methods it is possible to use several amines, but they do not give information about instantaneous rates.

The FLUOROMETRIC methods are very sensitive. Several criteria have been followed in the development of these methods. The consumption of serotonin (325, 326) and norepinephrine (325) have been used to estimate MAO activity. The methods based on the consumption of substrate are not advisable because of their lack of accuracy and specificity (327). Lovenberg et al. (328) and Udenfriend (329) used tryptamine as a substrate and measured the rate of formation of indoleacetic acid, in presence of MAO and aldehyde dehydrogenase. Udenfriend (329), for large amount of enzyme, suggested to separate the indoleacetic acid from tryptamine by catien exchange resin. A similar method was described by Hidaka et al. (330) using serotonin as substrate and separating the product of serotonin oxidation using column chromatography on Dowex-50. Rosengren (331) estimated brain MAO activity by measuring the 3,4-dihydroxyphenylacetic acid formed when incubating brain homogenates with dopamine. Kraml (332) developed a sensitive fluorometric procedure based on the spectrophotometric method of Weissbach et al. (322). In the method of Kraml, 4-hydroxyquinoline, the product from the spontaneous cyclization of the intermediate aldehyde formed by the oxidation of kynuramine, was measured fluorometrically. In this method, trichloroacetic acid, which produced a strong quenching was used for deproteinizing the samples. The use of TCA limited the sensitivity of the method. Several modifications of this method have been

reported. Drujan and Diaz Borges (157) suggested the use of ethanol instead of trichloroacetic acid. Century and Rupp (333) replaced the trichloroacetic acid with perchloric acid and obtained more stable readings. Squires and Lassen (334) used NaOH to stop the reaction instead of the protein precipitant mentioned above. Takahashi and Takahara (335) suggested a method where 5-hydroxykynuramine was used as substrate. The product of the oxidative deamination of the substrate, 4,6-quinolinediol, was separated by paper chromatography and measured by fluorometry. Diaz Borges and Drujan (81) successfully applied the Kraml principle to the assay of MAO *in vivo*. Sjoerdsma *et al.*(336, 337) used the measurement of tryptamine in urine as an index to monoamine oxidase inhibition in man. The velocity of formation of H_2O_2 has also been assayed fluorometrically. The methods using this principle are based on the formation of a highly fluorescent compound when H_2O_2 reacts with either homovanillic acid (338, 339) or p-hydroxyphenylacetic acid (340), in presence of peroxidase. The estimation of MAO activity by measuring the rate of H_2O_2 formation, makes it possible to use several substrates, though the formation of fluorophors is interfered with compounds such as catecholamines and serotonin.

The methods which use RADIOISOTOPES are by far the most sensitive. They are based, in general on the incubation

of the sample with the radioactive substrate and separation of the radioactive products either by extraction with organic solvents (138, 169, 176, 177, 272, 341-346) or column chromatography (177, 346, 347). These methods, though very specific have two principal difficulties. One is the impossibility of getting continuous information about instantaneous rate. The other one is related to the quantitative recovery of the products (345-347). Several substrates such as tryptamine (177, 341, 345-347), tyramine (169, 176, 177, 344, 348), serotonin (169, 177, 272, 342, 344, 349, 350), butylamine (343) phenethylamine (138), benzylamine (177, 272), norepinephrine (346) and dopamine (169, 346) have been used . The metabolites of the radioactive substrates have also been separated by thin layer chromatography after extraction with organic solvents (349, 350). Fellman et al.(348) measured the tritium released by oxidation of tyramine-1,2-^{3}H by monoamine oxidase. Comparative studies on the different procedures for the separation of the radioactive products has been reported (346, 347). Symes et al.(256, 351) estimated MAO activity in vivo by measuring the $^{14}CO_2$ after administering n-pentylamine-1-^{14}C intraperitonially.

For the localization of MAO in different tissues several HISTOCHEMICAL techniques have been described. The first of these methods was reported by Oster and Schlossman (352) in 1942. The aldehyde produced upon deamination of

tyramine was reacted with a appropriate reagent and the Schiff's base formed was localized histochemically. The formation of a dark brown pigment when the tissues are incubated with tryptamine (353) or serotonin (354) has also been used to localize MAO. All latter methods lack accuracy and specificity (355, 356). Koelle and Valk (355) used 2-hydroxy-3-naphthoic acid hydrazine to trap the aldehyde of tryptamine as its hydrazone. The condensation product is then converted to a bluish-purple pigment by coupling with tetrazolium o-dianisidine. The validity of the method was assessed by the absence of reaction either in presence of MAO inhibitor or without substrates. It is not possible to use this method in the central nervous system or in tissues containing much oxidizable lipid. Francis (357) introduced neotetrazolium for the detection of MAO. This method was unsatisfactory in the hands of most workers. Glenner et al. (290) substituted tryptamine for tyramine and neotetrazolium for a more reducible tetrazolium salt. This method of Glenner et al. has been the most widely applied in light microscopic histochemical studies (358). Tetrazolium salts have also been widely applied to the localization of MAO after electrophoresis (76, 160, 271, 288, 359). Several authors have reviewed the histochemical techniques mentioned above (356, 358, 360, 361). Graham and Karnovsky (362) coupled the MAO reaction to peroxidase activity with the formation

of a colored insoluble precipitate. The same authors concluded that it was more difficult to visualize and photograph the colored precipitate in their method than in the method of Glenner et al.(290): Boadle and Bloom (363) have adapted the light microscopic histochemical method of Glenner et al.(290) to electron microscopy using osmiophilic ditetrazolium salt, thiocarbamyl nitroblue tetrazolium. This technique gives no reaction in tissues such as ganglia or vas deferens. Recently, Bloom et al.(358), looking for a more sensitive and accurate electron microscopic technique, used a reaction in which the final product is a precipitate of cupric ferrocyanide. This product was formed when the aldehyde of the MAO reaction reduced ferricyanide to ferrocyanide. Then the transition metal precipitate catalysed an osmiophilic polymer generation. This new technique is presently being studied and some technical details remain unresolved.

Other principles distinct to those already mentioned above have been used for measuring MAO. Manukhi (365) has measured the comsumption of norepinephrine in a bioassay using the pressor reaction of spinal rabbits and cats. The tetrazolium salts have also been used for biochemical determination of MAO in vitro (271, 364). Mason and Olson (366) have measured the MAO activity on the basis of differential amperometric measurement at tubular carbon

electrodes. They coupled the H_2O_2 produced in the MAO reaction enzymatically to another redox couple, $K_4Fe(CN)_6$-$K_3Fe(CN)_6$, by means of a peroxidase reaction. The $K_3Fe(CN)_6$ formation is assayed amperometrically by reduction to $K_4Fe(CN)_6$ at the tubular carbon through which the reaction mixture flows. The $K_3Fe(CN)_6$ formation is proportional to the rate of the MAO catalyzed reaction.

CHAPTER IX: MULTIPLICITY

Enzymes capable of oxidizing monoamines were first reported by Hare (8), Pughand Quastel (10) and Blaschko et al. (13). Several authors suggested (11, 12, 14) that the observed activity was due to MAO which has since been considered as one enzyme capable of oxidizing all monoamines. On the other hand, as we can notice from the preceding chapters, several questions about this last statement arise when considering the properties of the enzyme such as substrate specificity, inhibition and distribution. In fact evidence that MAO is more than one enzyme has gradually accumulated (160, 288, 289, 367). This evidence has come either from studies on the interaction of MAO with its substrates and inhibitors or from separation of enzyme fractions with different properties using chromatographic and electrophoretic techniques.

Alles and Heegaard (59) studied the substrate specificity of liver MAO of different species. They concluded, "the way that the maximum of activity varies in a particular homologous series (substrates) with enzyme of different sources is very marked, and may be taken as good evidence of there being different enzymes present". Similar conclusions were reached by Hagen and Weiner (57) when they discussed the differences in substrate specificity between

various species and organs. Hope and Smith (60) suggested that each organ might contain a distinct enzyme after studying the substrate specificity of MAO in different mouse organs. Kobayashi and Schayer (274) measured the oxidation of labelled tyramine and tryptamine in a mixture of both substrates using a solubilized preparation of rat liver mitochondria. These authors also measured the tyramine-^{14}C consumption in a mixture of nonlabelled phenylethylamine and i-amylamine. They concluded that tyramine and tryptamine are oxidized at the same active center, though it was possible to determine whether or not the oxidation of the four amines was carried out by one enzyme alone.

Hardegg and Heilbronn (368) using rat liver mitochondria found that iproniazid inhibited the enzymatic activity toward tyramine. At the same time, the inactivaction of tyramine activity proceeded at two different rates. They suggested that the two substrates probably were oxidized by two enzymes; serotonin was oxidized by one enzyme, whereas tyramine was oxidized by two enzymes. According to Barlow (128), it appeared possible to distinguish two types of guinea pig liver and rat fundus MAOs: one of which has a higher affinity for serotonin than the other and is more susceptible to inhibition by 2-methyldimethyltryptamine. Barbato and Abood (123) found in a purified beef liver MAO preparation that when the effect of pH on the activity was measured with

benzylamine as substrate, the enzyme alone had an optimum pH of 8.0, while in presence of phenylcyclopropylamine there was a sharp pH optimum of 7.0. When the concentration of phenylcyclopropylamine was increased a secondary optimum at pH 8.0 disappeared. With iproniazid the pH-activity curve was found to be similar to that of the enzyme alone. These results, according to Barbato and Abood, suggested the presence of two MAOs not equally sensitive to the inhibitors mentioned above.

Oswald and Strittmatter (61) found quite different relative rates of activity with different substrates in various tissues of rat and guinea pig as well as different Km values in different tissues. These authors mentioned several possibilities for explaining these results. Tissues might (a) contain a different single MAO, (b) contain a different group of MAOs, or (c) contain different proportions of several monoamine oxidases common to several tissues. On the other hand, they observed apparent competition for enzyme in multiple substrate experiments and this suggested that each tissue contained a single enzyme active on the four substrates examined (tyramine, serotonin, benzylamine and m-xylylenediamine). But as Severina and Gorkin (369) pointed out "competitive interactions between amines observed by the method of mixed substrates cannot be considered as simple evidence for the presence in a

preparation of one enzyme, since it is known that there are cases of changes in enzyme activity under the influence of different compounds which do not react directly with active centers (so -called allosteric or conformation cofactors)". These two authors, using rat liver mitochondria and two substrates, benzylamine and tyramine, studied the influence of heat, pH, chelating agents and sodium oleate on MAO activity. They showed that with each of these experimental conditions the MAO activity with a given substrate varied differently in comparison with the other substrate. These results could be explained, according to the authors, by assuming the presence of two enzymes or two active centers on the surface of one macromolecule. Later Gorkin and co-workers found other examples of selective inhibition such as the influence of hydroxyquinolines on the oxidation of tyramine and benzylamine (153), the inhibitory action of proflavine on the deamination of tyramine, serotonin, tryptamine, isoamylamine, dopamine and benzylamine (229), the major sensitivity of the deamination of tyramine to inhibition by pargyline as compared to that of dopamine or serotonin (113), the action of harmine on the deamination of serotonin and tyramine (140) and the inhibition of serotonin MAO activity by α-methyltryptamine (130). Severina and Sheremet'evskaya (370) found selective inhibition of tyramine and phenylethylamine as well as tyramine and benzylamine in rat

liver mitochondrial MAO subjected to controlled heating and treatment with urea. p-Methoxy-β-phenylethylamine had pH-dependent changes analogous to the deamination of tyramine. Gorkin and Romanova (371) studied the effects in vivo of pargyline (N-methyl-N-benzylpropynilamine HCl), phenelzine (β-phenylethylhydrazine HCl), nialamide [1-isonicotinoyl-2-(benzylcarboxamidoethyl) hydrazine], modaline (2-methyl-3-piperidinopyrazine sulfate) and Ro 4-2308(N^1-d-hydroxypropionyl-N^1, N^2-di-isopropyl-hydrazine) on the deamination of tyramine and serotonin in several tissues. With the exception of nialamide all the inhibitors caused selective inhibition with the substrates studied, but this selective inhibition was dependent on the organ. Correlation between the results of selective inhibition in vitro with those obtained in vivo was observed only in experiments with some inhibitors and in certain tissues.

Huszti and Borsy (127), as mentioned above (inhibitors, p. 29), considered the theory of Zeller of eutopic and dystopic complexes (129) a more suitable explanation for the selective inhibition of serotonin MAO activity than the multiplicity of MAO proposed by Barlow (128) since they did not find a compound which would inhibit the oxidation of serotonin only, or tryptamine only. Furthermore, they felt that the low inhibitory effect of 4-methoxyphenyl-diethyltryptamine and 3,4-dimethoxyphenyl-diethyltryptamine

was also better explained by the theory of Zeller. Fuller and Walters (372) observed in rat liver mitochondria that there was no relation between the degree of inhibition of kynuramine oxidation and efficacy of an amine as substrate. This might be considered in terms of Zeller's theory (129), where the formation of dystopic complexes would influence the degree of inhibition, whereas the formation of eutopic complexes would determine its ability as substrate. Fuller and Walters reasoned that their data were compatible with a single MAO that acted on all the amines studied or that a family of MAO enzymes existed with broad or overlapping substrate specificities.

Youdim and Sourkes (227) studied the effects of heat and inhibitors on the MAO activity at different pH. The pH-activity curve of the enzyme with kynuramine as substrate had a shoulder around pH 6.5 and a peak at pH 7.4 in phosphate buffer or at pH 8.1 in borate buffer. The activity at higher pH was more sensitive to heat and tranylcypromine whereas the inhibition by pargyline was more marked at pH 6.5. Chelating agents such as 8-hydroxyquinoline and thenoyltrifluoroacetone also inhibited the enzyme more strongly at the lower pH. The authors indicated several possibilities to suitably explain this phenomenon, such as (a) two distinct enzymes with different pH maxima (b) formation of active enzyme-substrate complexes (c) presence

of an ampholyte inhibitor (d) presence of more than one active site in the same enzyme. Attempts to separate different enzymes by sucrose density gradient centrifugation were unsuccessful.

Van Woert and Cotzias (152) found that the degree of inhibition by various anions depended on whether serotonin or tyramine was used to measure the MAO activity. Similarly, the inhibition was affected differently by temperature and pH depending on the substrate used. According to these authors, the differential inhibition of MAO by anions suggested the existence of more than one active site or more than one enzyme subunit in rat liver MAO. Maître (114), testing the effects of a non-hydrazine MAO inhibitor, N-methyl-N-2-propynyl-1-indanamine, found that this substance inhibited brain MAO more than liver MAO when injected, and, as with other inhibitors, the degree of inhibition was dependent on the substrate used, i.e., tyramine or serotonin, in measuring MAO activity. Maître considered his data as further evidence that various tissues of the same animal contained more than a single MAO. Squires and Lassen (334) suggested that the incomplete inhibition of MAO by γ-morpholino-butyrophenone reflects the existence of two or more forms of the enzyme. Fuller (373) studied the action of six inhibitors on the deamination of phenethylamine, tyramine, tryptamine and serotonin. N-(2-chlorophenoxyethyl)-

cyclopropylamine and harmaline inhibited the enzyme maximally when tryptamine or serotonin was used as the substrate. Trancylpromine and pargyline were most active as inhibitors of the oxidation of phenethylamine or tyramine. According to Fuller these results could be indicative of the existence of separate enzymes for oxidizing phenyl and indole-alkylamines. But he pointed out that this work, at the same time, should probably not be used as strong evidence for MAO multiplicity, since an inhibitor might act on a single enzyme and influence at varying degrees its combination with different substrates. The variations observed with different MAO inhibitors and different pH depending on the substrate used led Coq and Baron (374) to accept the presence of at least two enzymes in rat liver mitochondria.

Johnston (115) concluded that the pair of sigmoid curves joined by a horizontal section, observed when the percentage of inhibition (tyramine as substrate) of rat brain MAO was plotted against the concentration of clorgyline, were due to the existence of a binary system of enzymes. One sigmoid curve would represent a relatively clorgyline sensitive MAO, enzyme A, while the second would represent a relatively clorgyline resistant MAO, enzyme B. Hall et al. (116) extended the observation of Johnston to other species. They found that in rat liver and brain the component A was

active upon tyramine and serotonin, while the component B acted on tyramine and benzylamine. Both enzymes deaminated dopamine and tryptamine. They concluded that the brain of man, rabbit, ox, dog and cat as well as the liver of man also contained a binary system of enzymes, while the MAO from the brain of the pig and the livers of the other species studied other than rat and man appeared to be homogeneous. Goridis and Neff in similar experiments to those of Johnston studied the "enzymes A and B" in rat superior cervical ganglia, cerebral hemispheres, brain and pineal gland (375-377) as well as in human lumbar sympathetic trunk and pineal gland (378). The possibility that the pair of sigmoid curves, observed with clorgyline as inhibitor, was related to the permeability of the mitochondrial membranes was excluded by them since they observed the same behavior with a solubilized rat brain MAO preparation. Since each one of the MAOs of pineal gland and superior cervical ganglia preparations was inhibited to about the same percentage by clorgyline, when compared before and after freezing followed by dialysis, it was also ruled out that endogenous amines or synaptosomes in these preparations differentially protected MAO against inhibition by clorgyline (344). The proportion of these enzymes in some organs according to these authors, was as follows (375-377): pineal gland 15% of A and 85% of B, superior cervical ganglia 90% of A and 10% of B and rat brain 60% of A and 40% of B. Because the ratio of

activities between tyramine and serotonin varies from tissue to tissue, they (375, 377) suggested that "enzymes A and B" represent classes of MAO rather than single enzymes. Norepinephrine and metanephrine seem to be deaminated by enzyme type A (376). The same group (344) studied some properties of MAO from pineal gland (type B) and sympathetic ganglia (type A). They found the sympathetic ganglia enzyme had lower Km for tyramine than the enzyme of the pineal, was heat stable and was readily inactivated by trypsin, while pineal MAO was not readily inactivated by trypsin and was heat-labile.

Jarrott (171) found, after surgical sympathectomy in rabbit submaxillary gland and iris, a decrease in MAO activity for tyramine, serotonin and benzylamine. The decrease in serotonin MAO activity was greater than that for tyramine although there was no difference between the percentage decrease of benzylamine and tyramine MAO activity. In vas deferens after sympathectomy the percentage decrease of activity seen with benzylamine in rabbit and guinea pig was significantly less than the decrease seen with tyramine or serotonin. The lost and remaining MAO activities did not seem to correspond to the enzyme A and B described by Johnston, since it was shown by experiments with clorgyline that there was no complete loss of either component. On the other hand, there is no correspondence between the curves of vas deferens, normal and denervated, after plotting the percentage of

uninhibited homogenate vs $-\log_{10}$ M concentration of the inhibitors clorgyline and M&B 9303 (May & Baker) In view of this Jarrott concluded that, in rat vas deferens, MAO was not a binary mixture but consists of a group of three or more enzymes. The same author showed that extraneuronal MAO of vas deferens was inactivated by incubation at 58°C at a faster rate than the neuronal MAO. He pointed out that although the extraneuronal and neuronal MAO are different in properties such as inhibitor sensitivity, substrate preference and heat stability, this did not conclusively demonstrate that the two forms of the enzyme were isoenzymes. Tipton (76) had earlier suggested that the differing substrate specificity of neuronal and extraneuronal MAO could be interpreted as being due to a single enzyme existing in different lipid environments. The same author (141) found in rat liver mitochondria that the time course of the inhibition with 2-bromo-2-phenylacetaldehyde, an analogue of the aldehyde products from substrates such as benzylamine and phenethylamine, when shown as semilogarithmic plots produced a straight line for the inhibition of serotonin MAO activity while with tyramine and benzylamine as substrate the graphs are more complicated. According to him the graphs with each one of the last two substrates could be interpreted as being composed of two first order curves. Moreover, combined experiments with 2-bromo-2-phenylacetaldehyde and clorgyline suggested the presence of two components in the clorgyline-insensitive enzyme (B), which differed in their

reactivities towards 2-bromo-2-phenylacetaldehyde, although he did not discard the possibility of two reactive groups on the same enzyme. On the other hand, pig brain mitochondrial MAO appeared as homogeneous in experiments in which the homogeneity of this enzyme was investigated using mixed substrates, partial temperature denaturation and the inhibitors harmine and 2-bromo-2-phenylacetaldehyde with five different substrates (tyramine, tryptamine, L-adrenaline, serotonin and D-L-m-O-methylnoradrenaline) (140).

Huszti et al. (126) found that the inhibition with l-m-aminophenyl-2-cyclo-propylamine-ethanol dihydrochloride (AB-15) was dependent on the substrates and the enzyme preparation used. These authors pointed out that it was not known whether the difference in MAOs was due to different subfragments of the enzyme or if different single enzymes were responsible for the deamination of the different amines. Fuller and Roush (125) studied the inhibition of MAO, in rats and mice, by N-cyclopropyl-2-chlorophenoxyethylamine (Lilly 51641) or pargyline. The enzyme activity was measured with phenylethylamine or tryptamine. Their results showed that these inhibitors were selective on the two substrates, and also revealed differences in their action on different organs. The authors interpreted these results as an indication of the existence of multiple enzymes, although they could not distinguish between different explanations such as separate

enzyme proteins, isoenzymes or the same protein in different molecular environments. The selective inhibition of MAO has been extensively reviewed by Fuller (379). According to him the most compelling evidence that selective inhibition is due to effects on multiple MAO forms comes from experiments with irreversible inhibitors. The theory for these experiments is briefly as follows: the rate constant k was determined in the equation

$$E + I \longrightarrow EI$$

where E= enzyme
I= inhibitor
EI= undissociable enzyme inhibitor complex.

If a single enzyme oxidized two substrates S_1 and S_2, then, $k_1 = k_2$, but if two separate enzymes exists k_1 will be different from k_2. Various examples of the last case, with MAO, were shown. The multiple forms of MAO were interpreted in a similar form by Fuller and Roush (125).

In the mouse, evidence for the existence of several forms of mitochondrial MAO in different tissues was shown on the basis of inhibitor and thermal inactivation experiments (133). According to Squires (133) the multiple forms of MAO in the mouse could be divided into two groups; one group

which is inhibited by harmine but relatively resistant to pargyline and another group resistant to harmine but inhibited by low concentrations of pargyline. The harmine-sensitive group was further separated into two other groups by heat inactivation experiments. Recently, these experiments were extended to eight mammalian species (380) and to other inhibitors. Squires (380) correlated his results with those of Johnston (115) and other authors and concluded: (a) In intact mitochondria MAO may, usually but not invariably, be divided into two fractions on the basis of selective inhibition. (b) In most preparations clorgyline, harmine and Lilly 51641 are selective inhibitors of fraction A while B is usually inhibited by low concentrations of pargyline or deprenyl. (c) The A and/or B fraction in several mitochondrial preparations seem to be heterogeneous. (d) There does not appear to be any correlation between the presence of an amine and forms of MAO which deaminate it in different cell types. (e) Pig brain, liver and kidney seem to lack the A fraction almost completely. The B fraction contains different proportions of thermostable and thermolabile forms in these organs. In guinea pig the B fraction is thermostable and homogeneous while the A fraction is thermolabile and heterogeneous. (f) In general, the ratio of fraction A to B (A/B) is high in the small intestine. In mouse, rabbit, cat, dog, pig and ox the A form is almost absent from liver while in the same organ of rat, guinea pig and human the A

activity is quite high.

Egashira et al.(381) found that sodium nitrite inhibited rat liver MAO as measured with tyramine or serotonin as substrate. However with benzylamine, butylamine, amylamine, hexylamine and β-phenylethylamine there was an increase in the enzyme activity. In rat brain, an increase in MAO activity was noticed with this compound when tyramine or butylamine was the substrate. No significant effect in this last organ was observed when serotonin, benzylamine or tryptamine was used as the substrate. They suggested that their results might imply the existence of multiple forms of MAO. Sierens and D'Iorio (272, 281) reported evidence for the multiplicity of rat liver MAO based on the kinetics of the enzyme with serotonin and benzylamine. Three kinds of experiments were performed (a) the apparent Km and Ki of each substrate were determined using a mixture of radioactive and nonradioactive serotonin and benzylamine. The Km of serotonin was significantly different from the Ki whereas for benzylamine the Km and Ki were not significantly different. (b) They tested the influence of several concentrations of nonradioactive serotonin on the slopes of the reciprocal plot of reaction velocity against substrate concentrations when the MAO activity was measured with benzylamine-^{14}C. A hyperbolic inhibition by nonradioactive serotonin on the slope (Km/V) of the benzylamine reaction was found. (c) The

enzymatic reaction velocity was measured in the presence of several concentrations of serotonin and the Lineweaver-Bürk plot was found to coincide with an hyperbola. As these authors mentioned, all experimental results could be explained on the basis of a two-enzyme model, although each experiment could be explained by alternative models such as either allosteric site of activation or inhibition or differences in reaction with a second substrate (oxygen). These authors separated two fractions of MAO by subjecting deoxycholate solubilized rat liver mitochondria to polyacrylamide gel electrophoresis. One fraction acted on benzylamine and the other fraction acted on both benzylamine and serotonin.

Along with the indirect evidence mentioned above, the separation of multiple forms of MAO has been attempted using various physicochemical methods. As early as 1952, Werle and Röewer (382) identified enzymes from different animal sources capable of oxidizing either aliphatic or aromatic monoamines. In 1963, Gorkin (269) reported the separation of two distinct MAO activities from rat liver mitochondria using a non-ionic detergent and chromatography on a calcium phosphate gel column. One MAO fraction deaminated p-nitrophenylethylamine and m-nitro-p-hydroxybenzylamine. However, only a small percentage (6%) of m-nitro-p-hydroxybenzylamine activity was recovered. Later on he

confirmed his finding but the recovery for the later activity was not more than 25% (383). Kim and D'Iorio (384) and Youdim and Sandler (287) independently reported the first electrophoretic separations of different fractions of MAO identified with tetrazolium salts. Since then, several reports on the separation of distinct MAO fractions from different species and organs such as rat liver (271, 272, 282, 385-391), brain (222, 392-394), uterus (161) and heart (385), chick brain (393), embryos of South African clawed toad Xenopus laevis (395), rabbit liver (386), pig liver (245), beef liver (233, 386, 396), brain (240, 397-399) and adrenal medulla (76, 284), and human placenta (388), endometrium (161), brain (400) and liver (389, 390) have accumulated. The significance of the above studies dealing with the separation of different MAO fractions will be discussed in the experimental part of this thesis.

EXPERIMENTAL

SECTION I: LIBERATION OF MONOAMINE OXIDASE FROM RAT LIVER MITOCHONDRIA

The term solubilization is used here as adopted by Akopyan (282) to denote a phenomenon of apparent conversion of polyionic macromolecular aggregates, in this case rat liver mitochondrial fraction, to an optically clear, homogeneous, single aqueous phase.

The purpose of solubilization is to obtain a preparation in which the MAO substrate specificity pattern is similar to that of the original mitochondrial preparation in question. The solubilization process should also facilitate the utilization of the preparation for separation of the different forms of MAO.

As discussed elsewhere various procedures have been employed to solubilize the mitochondrial MAO. The degree of success has been variable. However, in most cases the effect of solubilization on the activity of MAO with different substrates has not been studied. Although a particular method evaluated with one substrate may be promising, the same procedure may not be adequate when other substrates are used.

A solubilization technique making use of a non-ionic detergent and sonication will be described in this section together with some aspects of the influence of an ionic detergent, sodium deoxycholate, upon the MAO activity of rat liver mitochondria.

METHODS

All the chemicals used throughout this work were of reagent grade or of the highest purity available and were generally purchased from Fisher Scientific Co. Limited or Canadian Laboratory Supplies Limited. In the case of some special chemicals the source is indicated in parenthesis in the text.

Preparation of rat liver mitochondria: Male Sprague-Dawley rats weighing between 200-250g and starved overnight were decapitated and the livers were dissected out. The liver tissue was homogenized immediately with cold 0.25 M sucrose in 0.05M phosphate buffer, pH 7.4. The mitochondrial fraction was obtained by differential centrifugation according to the method of Schneider and Hogeboom (401).

MAO assay by oxygen consumption: The activity of MAO with different substrates was determined polarographically, using an oxygen monitor (Yellow Spring

Instruments, Co. Model 53.Biological oxygen monitor) with a Clark electrode (see p. 52, chapter VIII). The output of this apparatus was connected to the input of a recorder, Photovolt Varicord, model 43. The linear scale was used and the sensitivity was adjusted to 100mV full scale. When higher sensitivity was required it was increased up to 10 times. In order to restore the reading to a range which could be registered on the recorder chart, a variable offset voltage source* (fig. 1) was introduced between the output signal of the oxygen monitor and the input signal of the recorder (fig. 2).

The mixture for the enzyme assay was similar to that described for the manometric method of Creasey (291). The incubation mixture contained 200 µmoles of phosphate buffer pH 7.4, 2 µmoles of neutralized KCN, 20 µmoles of neutralized semicarbazide, the substrate to a final concentration of 3 mM, with the exception of tyramine (6 mM), and the enzyme preparation. The total volume of the incubation medium was 3 ml. In order to increase the sensitivity the volume in some cases was also reduced to 1.2 ml the final concentration of the components of the assay remaining the same and the micro accessory kit of the YSI oxygen monitor was used.

After proper testing of the oxygen electrode the

* The variable voltage offset source was designed by Mr. Arthur Lysionok for our experiments.

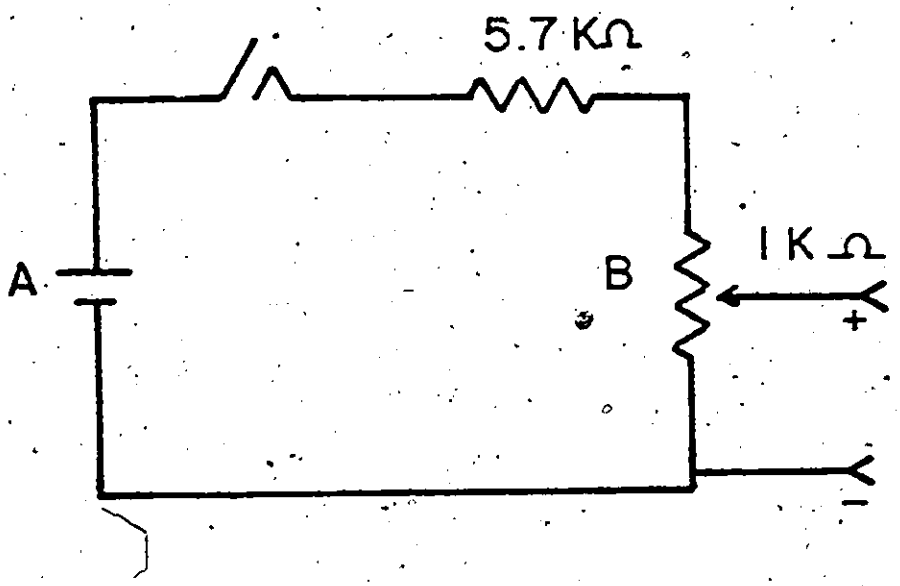

5.7 KΩ
A
B
1 KΩ
+
−

Figure I.

Variable offset voltage source. A. Mercury cell. B. 10 turns potentiometer with turn counting dial.

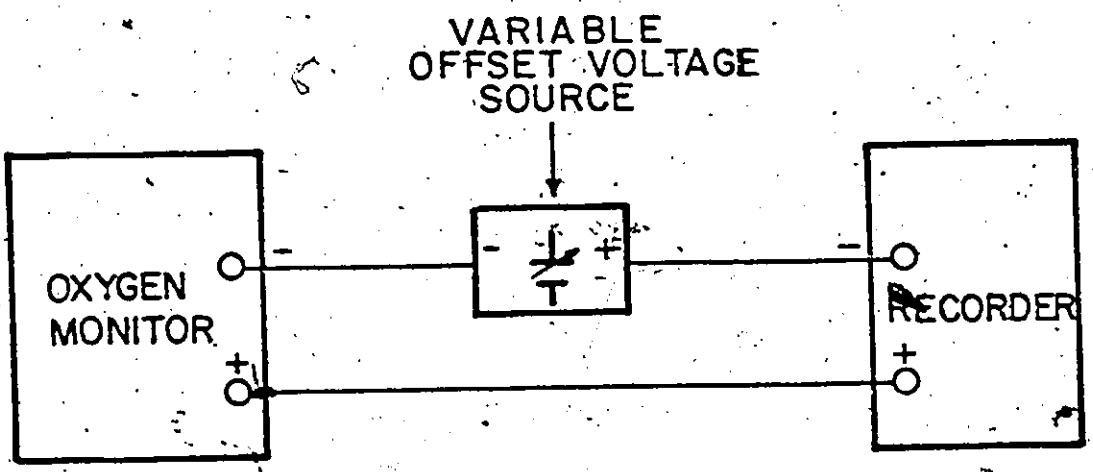

VARIABLE
OFFSET VOLTAGE
SOURCE
OXYGEN
MONITOR
RECORDER
-
+
-
+
-
+
-
+

Figure 2.

Arrangement of the oxygen monitor, variable offset voltage source and recorder for assay of MAO by oxygen consumption.

apparatus was standardized. The scale of the oxygen monitor (0-100) was adjusted to 90, in the presence of air saturated distilled water. All the procedures were carried out at 37°. The assay mixture without the enzyme was equilibrate with air for 5 minutes by constant stirring. The concentration of O_2 was measured for 3 to 5 minutes (substrate blank). The enzyme reaction was started immediately and measured up to about 6-8 minutes. The blanks consisted of a similar mixture but without substrate. The initial velocity oxygen consumption was estimated by drawing a tangent to the recorded line through its origin and was expressed as percentage of oxygen consumed in 5 minutes. In general the reaction was linear during the time of the measurement.

The readings expressed in percentage can be converted to µmoles of oxygen, using the appropiate conversion factors. It was assumed that at 37° and 1 atmosphere of air pressure, 1 ml of water dissolves 0.217 µmoles of oxygen (402). The conversion factor for total volumes of 3 ml and 1.2 ml was 0.0072 µmoles and 0.0029 µmoles, respectively.

Since the Clark electrode measures the activity and not the concentration of oxygen present (403) and the substances dissolved in the reaction mixture can change the oxygen solubility, it was necessary to estimate the extent to which solubility was changed. Therefore, true oxygen

content of the assay medium was estimated by the procedure described by Dixon and Kleppe (404). This procedure is based on deriving a correction factor from the recorder deflections produced when a known amount of oxygen is introduced into air-saturated water and air-saturated reaction mixture. In this assay the correction factor was estimated as follows. The reading of the oxygen monitor was adjusted to 70 in presence of 3 ml of air-saturated distilled water containing 133 Sigma units of catalase (Sigma Chemical Co.) and then 20 µl of 0.1% H_2O_2 were added and an increase in the reading was observed. The same steps were carried out with 3 ml of the reaction mixture. It was found that in this case the increased reading in the reaction mixture was 15% more than that detected in distilled water. This difference denoted the change in the activity of oxygen caused by the dissolved substances in the reaction mixture. Therefore this percentage was used to correcting the conversion factor. The values of the conversion factor after this correction are 0.0061 and 0.0025 µmoles for the reaction mixture with volumes of 3 ml and 1.2 ml, respectively. The conversion factor was the same with different substrates such as benzylamine (Eastman Kodak Co. or Sigma Chemical Co.), serotonin (Sigma Chemical Co.), tryptamine (Sigma Chemical Co.), kynuramine (Sigma Chemical Co.) to a final concentration of 3 mM or tyramine (Sigma Chemical Co.) to a final concentration of 6 mM.

Solubilization of rat liver mitochondrial fraction by a non-ionic detergent and sonication: The mitochondrial fraction was treated with 12.5% (w/v) Lubrol W (a cetyl-alcohol-polyoxyethylene condensate, n: average 16, Canadian Industries Ltd.) for 16 to 20 hours, in presence of 0.01M Tris-HCl buffer, pH 8.8, benzylamine and serotonin (3 mM) and 30% (w/v) sucrose. The final volume was 8.2 ml. The suspension with a protein concentration of about 10 mg/ml (10.4 mg ± 0.4 S.E. / ml) was kept under an atmosphere of hydrogen for 3-4 hours, in a 50 ml Elenmeyer flask in order to displace the oxygen present in the solution. The hydrogen was previously passed through an alkaline solution of pyrogallol as described by Verevkina et al.(275). After this treatment the sample was subjected to intermittent sonication using a Biosonic III (Bronwill Scientific, Inc.) ultrasound device with a 4 mm diameter probe. It was utilized to an intensity of 20% of the maximal output (maximal permissible output with this probe 35%). The sample was sonicated for 1 minute and after an interval of 3 minutes it was sonicated again. The cycles were repeated until the extent of sonication required was complete. These operations were performed at 0 - 4°.

Other techniques: A Beckman Acta™ III UV-Visible spectrophotometer was employed at room temperature for absorbance measurements.

Protein was estimated by the method of Lowry et al. (405). Bovine serum albumin (Sigma Chemical Co.), in which the degree of hydration was determined by lyophilization, was used as standard. In samples containing Lubrol W a fine precipitate appeared on the addition of the Folin-Ciocalteau reagent. This precipitate was eliminated by centrifugation at 1000 g for 5 minutes before taking readings (406). Table 1 illustrates the influence of various concentrations of this nonionic detergent on the estimation of protein by the method of Lowry et al. after centrifugation of the samples as mentioned above. It was found that the presence of Lubrol W did not change the absorbance of the standard protein up to certain concentration. Care was taken not to exceed this concentration by proper dilution of the sample. The interference of sucrose with the assay (407) in some samples, was overcome either by dialysis or dilution. Tris buffer also interferes with the assay (408) but it was present in low concentrations and was usually further diluted.

The enzyme preparations were centrifuged in a Sorvall RC2-B refrigerated centrifuge with an SS-34 rotor for centrifugal force less than 46000 g. For greater centrifugal force an ultracentrifuge Spinco model L with a rotor # 40 was used.

RESULTS

TABLE 1

Influence of Lubrol W on the estimation of protein by the method of Lowry et al (405)*.

µg of protein (Bovine serum albumin)	Concentration of Lubrol W**					
	0%	1%	2%	4%	6 %	8%
	Optical density***					
10	0.052	0.057	0.059	0.054	0.009	0.012
25	0.112	0.122	0.122	0.130	0.026	0.025
50	0.212	0.221	0.236	0.218	0.062	0.046
75	0.288	0.318	0.308	0.295	0.068	0.049
100	0.383	0.386	0.405	0.385	0.090	0.063

* For details see text.
** Concentration of Lubrol in 200 µl of solution which was added to different amounts of protein. Final volume of the assay mixture was 4.8 ml.
*** Blank subtracted.

Table 2 gives a comparison of the MAO activities of mitochondria solubilized by sodium deoxycholate alone and by the same detergent in the presence of the MAO substrates, benzylamine and serotonin, and subjected to dialysis against 0.01 M phosphate buffer pH 7.4 for 36 hours. After this time the assays of all the samples were carried out. The values are expressed as percentage of the MAO activity of mitochondria suspended in buffer. These percentages were calculated from the specific activities of the samples. The MAO activity solubilized with deoxycholate without the substrates was found to be unstable. The loss of activity for serotonin was much higher than that for benzylamine. On the other hand when the sample was solubilized in presence of MAO substrates and dialyzed for 36 hours, the recovery of both the activities were better, but the loss of activity for serotonin was always greater than that for benzylamine. These experiments were carried out, in a preliminary effort, to test the possibility if mitochondria, solubilized by this ionic detergent could be used to separate the multiple forms of MAO by sucrose gradient electrophoresis. Changing the 0.01 M phosphate buffer, pH 7.4 for 0.01 Tris-HCl buffer, pH 8.8 or phosphate buffer 0.2 M, pH 7.4 produced similar effects.

Table 3 illustrates the influence of the solubilization of rat liver mitochondria by Lubrol W and

TABLE 2

Effect of deoxycholate on MAO activity in the rat liver mitochondria.

	Benzylamine	Serotonin
Deoxycholate (1%)	18.6 (4.3 34.5)	1.7 (0.0 7.2)
Deoxycholate (1%), benzylamine (3 mM), serotonin (3 mM) and dialysis (36 hours)	61.0 (42.1 85.9)	22.9 (5.2 44.2)

The values are expressed as percentage of MAO activity of the original mitochondria kept in a hypoosmotic medium. MAO activity was measured by oxygen consumption (see text). All procedures before the assay were carried out at pH 7.4 (0.01 M phosphate buffer) and at 4° during 36 hours. Each value is the mean of 6 experiments. Values in brackets represent the range.

TABLE 3

Effect of Lubrol W and sonication on benzylamine, serotonin and kynuramine MAO activities in the rat liver mitochondria.

	Benzylamine	Serotonin	Kynuramine
Lubrol W (12.5%)	88.2 (80.0 105.2)	86.0 (71.4 108.9)	80.0 (69.9 86.8)
Lubrol W (12.5%) and sonication.	83.2 (74.6 102.2)	78.6 (62.1 93.0)	76.1 (54.8 90.6)

The values are expressed as percentage of MAO activity of the original mitochondria kept in a hypoosmotic medium. MAO activity was measured by oxygen consumption (see text). Benzylamine and serotonin to a final concentration of 3 mM were added to the samples with the Lubrol. The samples were dialyzed before the MAO assay for for 36 hours. All procedures before the assay were carried out at pH 8.8 and 4°. Each value is the mean of 5 experiments. Values in brackets represent the range.

sonication on MAO activity as assayed with three substrates: benzylamine, serotonin and kynuramine. The values are expressed as percentage of activity in mitochondria suspended in buffer and, as in table 2, the percentages are calculated from the specific activities of the samples. With this procedure it was observed that the MAO activity decreased by approximately the same extent, about 20%, for all the substrates studied. This procedure was designed after repeated trial and error. In a preliminary experiment different concentrations of Lubrol W were added to the mitochondria and the O.D. measured at 550 nm (fig. 3). Even though Lubrol had very little absorption in this region of the spectrum (maximal absorption at 236 nm according to our data), the samples were read against reference samples containing the same buffer and the same concentration of Lubrol W. It was found that at a Lubrol concentration of about 12.5% a maximal decrease in the optical density was obtained and after increasing the detergent concentration, no further decrease in O.D. was observed. In view of this the concentration of 12.5% was selected for our standard procedure. Similarly it was found that the decrease in O.D. was even greater (up to 20%) if the Lubrol W was allowed to dissolve slowly in the preparation instead of adding a solution of the detergent. In view of this the former procedure was adopted.

The amount of sonication was determined by a similar

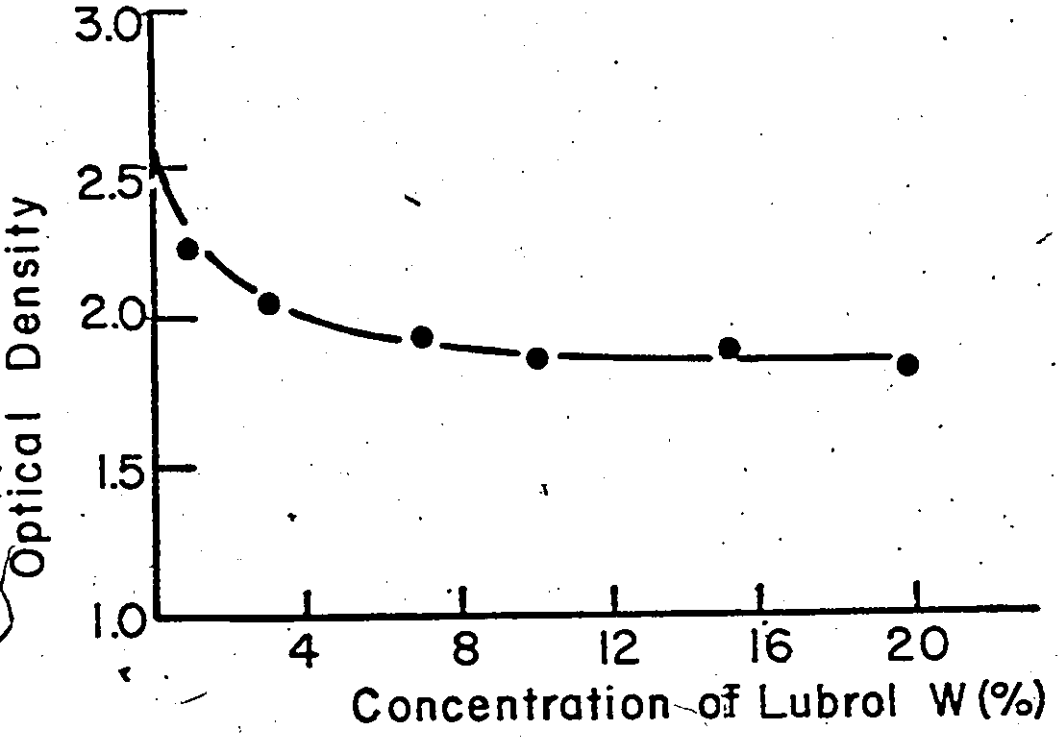

3.0
2.5
2.0
1.5
1.0
Optical Density
4
8
12
16
20
Concentration of Lubrol W (%)

Figure 3.

Influence of Lubrol W on the absorbance of a suspension of rat liver mitochondria in Tris-HCl buffer 0.01 M, pH 8.8. The O.D. was measured at 550 nm. Lubrol W concentration is expressed in gr/100 ml.

procedure, as described by Verevkina et al. (275). A sample treated with 12.5% Lubrol was subjected to sonication for different periods of time (fig. 4) and after 15 minutes no decrease in O.D. was observed and therefore the period of sonication was standardized to 15 minutes. The concentration of Lubrol and the extent of sonication were further teste for their effect on the activity of MAO as described above, by ultracentrifugation and by subsequent experiments to be described in the succeeding sections.

About 85% of the original MAO activity was found in the supernatant obtained on centrifugation of the mitochondria solubilized as above, at 105,000 g for 2 hours; though there was some variation depending on the experiment and the substrate used (see fig. 5). After increasing the centrifugal force to 145,000 g and the period of centrifugation to 3 hours, the amount of MAO activity remaining in the supernatant was 86.2% and 83.6% for benzylamine and serotonin, respectively. When the same procedure of solubilization was followed up with 3% Lubrol W, only 30% of the MAO activities for serotonin and benzylamine were recovered in the supernatant after centrifugation to 105,000 g for 2 hours.

In these experiments the values of MAO activity for the mitochondria resuspended in a hypo-osmotic medium and

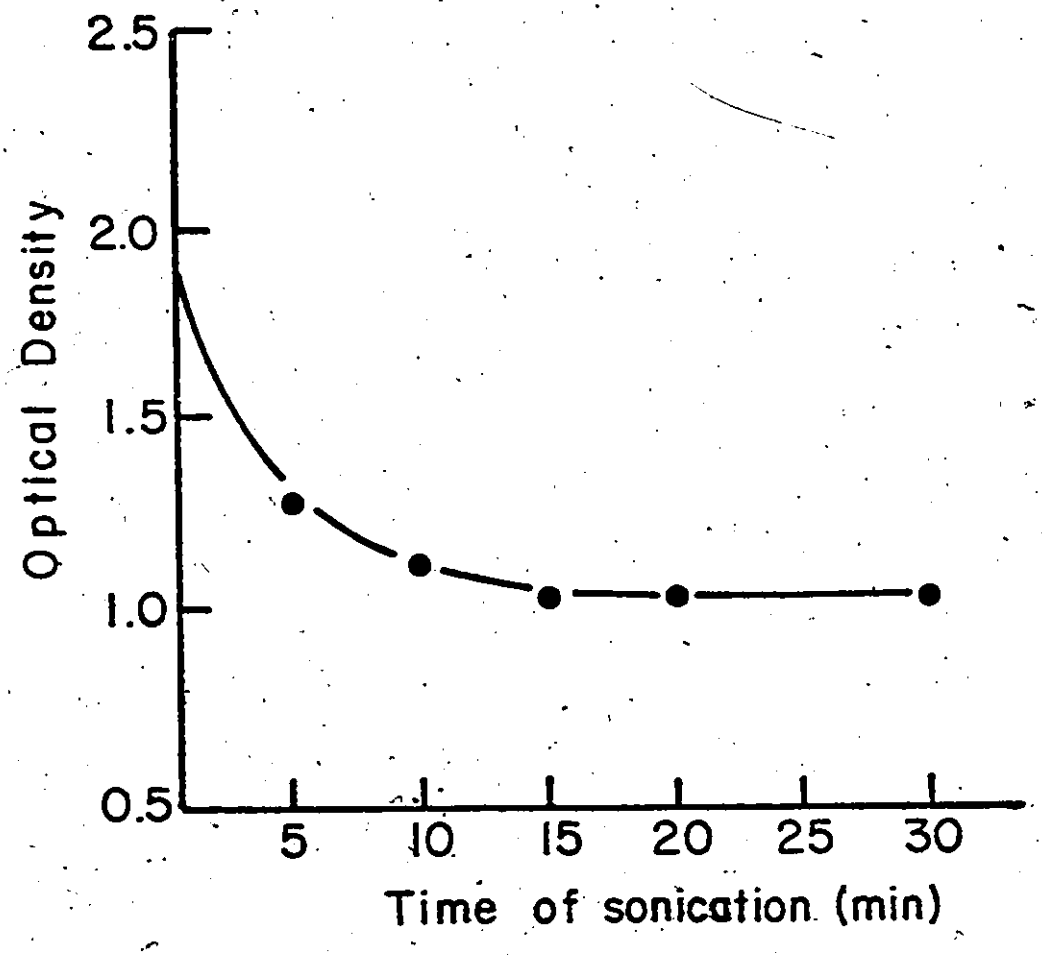

2.5
2.0
1.5
1.0
0.5
Optical Density
5
10
15
20
25
30
Time of sonication (min)

Figure 4.

Influence of the time of sonication on the absorbance of a suspension of rat liver mitochondria in Tris-HCl buffer 0.01 M, pH 8.8 treated with 12.5% Lubrol W. The sonication was carried out in a Biosonic III ultrasound device under the same conditions described in the text (see p. 88).

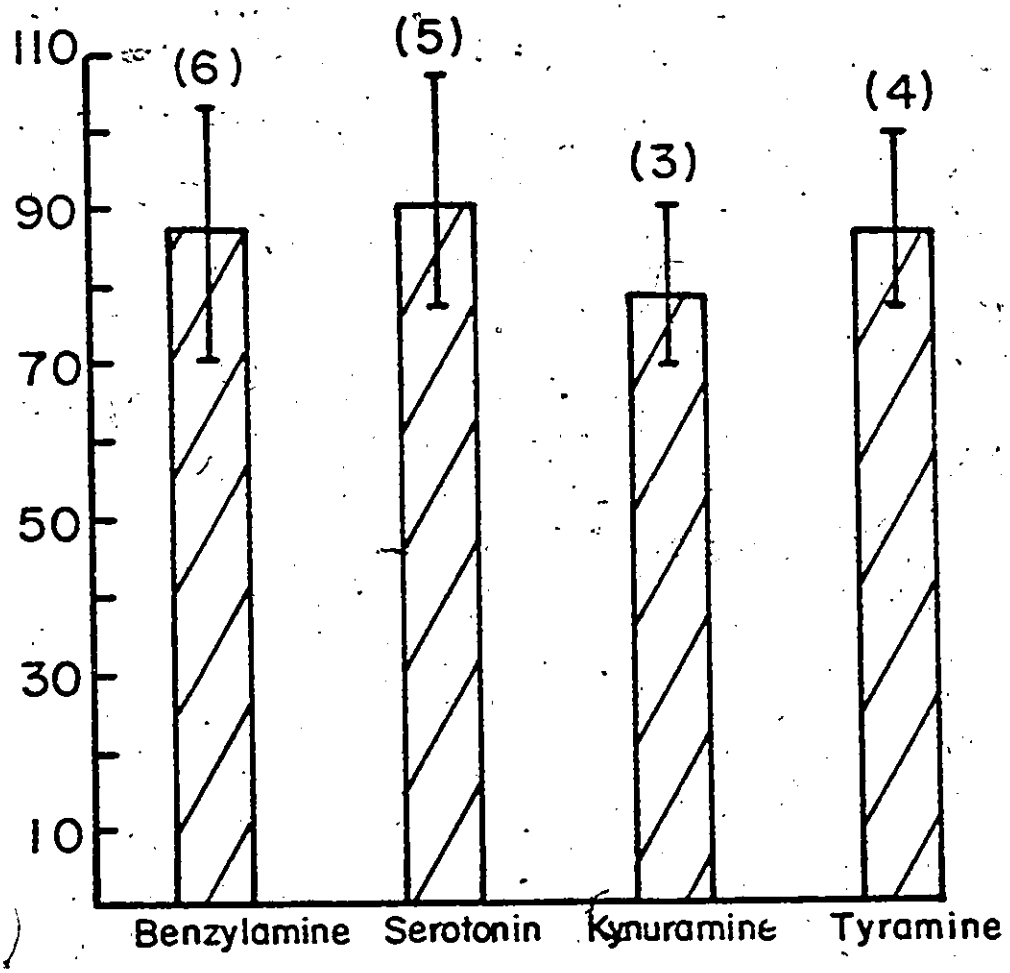

110
90
70
50
30
10
(6)
(5)
(3)
(4)
Benzylamine
Serotonin
Kynuramine
Tyramine

Figure 5.

Activity of MAO remaining in the supernatant after centrifugation at 105,000 g x 2 hours. The heights of the columns represent the mean of the values of MAO activity which is expressed as percentage of the original solubilized sample. The bars indicate the range of these values and the figures in brackets the number of samples.

expressed as µatoms of oxygen (mean ± S.E.) /hour/mg protein with different substrates were:

Benzylamine	0.576 ± 0.024
Serotonin	0.512 ± 0.028
Kynuramine	0.485 ± 0.031
Tyramine	0.784 ± 0.038
Tryptamine	0.310 ± 0.024

DISCUSSION

From a survey of various papers dealing with the liberation of MAO from mitochondrial membranes it is apparent that the term solubilization cannot be used in a general sense as different authors have chosen different criteria for considering their enzymes as soluble. As mentioned elsewhere MAO has been extracted from the cellular membranes principally by treatment with detergents (51, 123, 268, 273) or by sonication (262, 274, 276) or by the use of both (84, 261, 277, 278). Though extraction with organic solvents were unsuccessful at first (123, 268), Hollunger and Oreland (279, 280) have recently reported a successful solubilization of pig liver mitochondrial MAO employing 2-butanone. The yield of soluble enzyme was about 25% with a total recovery of about 65% of activity. In general the degree of solubilization has been determined estimating the MAO activity in the supernatant fraction obtained on submitting the mitochondrial fraction treated by different methods to high-speed centrifugation. However, it has to be borne in mind that treatments such as sonic irradiation, mechanical disruption, or extraction at alkaline pH may result in the dispersion of lipoprotein or formation of small membranes fragments that do not sediment readily and remain in the soluble fraction of the cell (409). On the other hand, for preparations solubilized with detergents it is difficult

to distinguish between true solution and formation of surfactant-protein or lipoprotein complexes of low density which will not sediment at high centrifugal force. The dispersing agent must be removed before assessing the degree of solubilization, but this is not always possible, especially in MAO preparation solubilized with high concentration of high molecular weight detergents. The detergents are usually precipitated along with the protein in many of the standard protein fractionation techniques (262). Erwin and Hellerman (234) have reported the presence of digitonin, the surfactant used for solubilizing the enzyme, in purified MAO preparations of bovine kidney. In other preparations in which other detergents have been used to solubilize the enzyme, these are present even after extensive purification (76, 219, 242, 410). We did not test if our MAO preparations were in "true" solution, because the non-ionic detergent used, Lubrol W, was present in high concentration and it was not diffusible. Notwithstanding this, after treatment with this detergent and sonication an appreciable amount of the enzyme, as measured by different substrates remained in the supernatant after ultracentrifugation (fig. 5). This compares favorably with different MAO preparation solubilized by various methods (81, 268, 270, 273-275, 277, 278, 396). In view of these facts we adopted the term solubilization as defined by Akopyan et al. (282) (see p. 64 and p.79, this thesis) which does not necessarily mean that the enzyme is in "true" solution.

Since MAO is firmly bound to the mitochondrial membranes (249), several attempts were made before this enzyme could be successfully detached from this organelle without considerable loss in enzyme activity. Furthermore a method reported successful with a particular MAO preparation is not necessarily applicable to another preparation, even when is it used with another organ of the same species (279). All the procedures toward this end have been tested on the basis of trial and error for each source of the enzyme.

Different detergents have been utilized for the solubilization of MAO. Cotzias et al.(268) reported an increase in MAO activity for tyramine in homogenates of rat and rabbit liver treated with the detergents isooctyl-phenoxypolyethoxyethanol (an active constituent of Cutscum), polyoxyethylenelauryl alcohol and Alconox^{R}. The maximal increase was obtained with isooctylphenoxypolyethoxyethanol. The greater part of the MAO activity was in the supernatant after centrifugation at 140,000 g for 180 minutes. This would seem to be a good criterion commonly employed to indicate that a high solubilization rate was achieved. However, it was found that when the sample was frozen without changing the centrifugal force the fractions obtained from the top were inactive while the bottom fractions contained the highest activity. Gorkin et al.(319) studied the factors affecting the solubilization of rat liver mitochondrial MAO using the

non-ionic detergents isooctylphenoxypolyethoxyethanol, Brunegal 0, OP-7 and OP-10. Substitution of OP-10 by the other detergents with the exception of OP-7 had no marked effect on the MAO activity estimated spectrophotometrically with benzylamine as substrate or on the progress of the solubilization followed by a turbidometric method. This last methodology was similar to the methods used in the present study to select the optimal concentration of Lubrol W and the amount of sonication. Gorkin et al. (319) found that after a certain concentration of the detergent an inhibition of the enzyme activity was observed. On the other hand the amount of protein solubilized increased proportionally to the concentration of detergent up to a certain limit, after which the proportionality was lost. They also described an increase in the rate and intensity of the solubilization with an increase of pH from 6.75 up to 8.65. Due consideration was given to all these factors in devising the present procedure.

Mushahwar et al. (258) solubilized bovine microsomal MAO with Tergital-NPX and also found a critical concentration of the detergents when the solubilization of MAO was maximal. The recovery of solubilized MAO was 80% as measured with tyramine as substrate. A study of the effects of saponine, Tween 20, sodium lauryl sulfate and digitonin on the solubilization of rat liver mitochondrial MAO was reported by Coq and Baron (273). They used serotonin as substrate.

The greater solubilization, up to 50%, was obtained with sodium lauryl sulfate. Tween 20 inactivated the enzyme while with digitonin and saponine the activity of the solubilized MAO was 20% as evaluated after centrifugation at 100,000 g for 30 minutes. This low recovery in the supernatant could be ascribed to the substrate used and perhaps with other substrates the conditions could have been better. We have already shown a selective inhibition of serotonin MAO activity by sodium deoxycholate. Van Woert and Cotzias (152) found that the detergent Igepal Co-630 (a polyethyleneoxy derivative) also primarily affected the deamination of serotonin by rat liver mitochondria as compared to that of tyramine.

Beef liver mitochondrial MAO has been solubilized with detergents using several procedures. Barbato and Abood (123) treated this preparation with Triton X-100 several times and increase in the pH to 8.5 in different steps. They obtained 42% of the original activity in the supernatant after centrifugation at 100,000 g for 30 minutes. Gomes et al. (233) who used the same detergent designated their preparation as "soluble" only after several steps of purification. They considered the enzyme as soluble when it remained stabilized in the aqueous phase without adding detergent. But it is possible that the detergent could have been attached to the enzyme even during the last step of

purification. More recently Shih and Eiduson (394), after trying 10 different procedures, found that the procedure of Gomes et al (233) was the most adequate one for solubilizing rat brain mitochondrial MAO. They tested the solubilization by measuring the MAO remaining in the supernatant after centrifugation. The conditions of centrifugation and the substrates used were not reported. Kinemuchi (396) using sodium cholate obtained 60% of the MAO activity, as measured with tyramine as substrate, in the supernatant after centrifugation at 50,000 g for 30 minutes. This shows how difficult it is to compare different procedures, even in the same preparation. Another procedure used by Harada et al.(240) with beef brain mitochondria was heating the preparation at 40° for 10 minutes and adding Nonion NS-210 to the preparation after cooling. Using kynuramine as substrate they detected 33% of the MAO activity in the supernatant after centrifugation at 107,000 g for one hour. They also found a 3-fold increase in specific activity in their preparation. They consider this procedure better than other procedures of solubilization making use of Cutscum and Triton X-100.

Sourkes (243) discarded the use of a non-ionic detergent for solubilizing rat liver mitochondria, because of its nondiffusibility through dialysis membranes and its tendency to bind strongly to the proteins. He suggested the

use of sonication under standardized conditions. The optimal conditions for sonication such as volume of the sample, intensity of vibration, concentration of protein, pH, viscosity and the presence of other substances must be chosen according to the material to be sonicated and the apparatus at hand (409, 411). The ultrasonic waves have inactivating effect on the enzymes which are usually revealed when enzymes are irradiated in the presence of oxygen, the enzyme activity being more resistant when the irradiation is carried out in a aqueous medium saturated with hydrogen. Detachment of low-molecular weight peptides from protein molecules has been observed as a result of irradiation with sonic waves. Depending on the nature of the gas present form[illegible] fragments of proteins are transformed to stable molecules or interact one with each other, causing an increase in the molecular weight of the protein. These changes in structure do not necessarily lead to a loss in the original enzymatic properties of the proteins. All these findings have been reviewed by El'piner (412).

As early as 1955 Kobayashi and Schayer (274) used sonication to solubilize rat liver mitochondrial MAO. They sonicated the preparation for 1 hour and obtained the same specific activity (tyramine as substrate), as in the original mitochondria, in the supernatant after centrifugation at 21,000 g for 2 hours. Verevkina et al. (275) sonicated the

same organelle after equilibrating the medium with an atmosphere of hydrogen. Sonication for a period of 60 minutes was without any effect on MAO benzylamine activity. They found that the enzyme was not in the free state. After centrifugation at 105,000 g for 1 hour all the enzyme precipitate and in the electron microscope 50-200 Å particles were observed. Guha and Krishna Murti (276) exposed a suspension of rat liver mitochondria in 0.01 M phosphate buffer pH 7.6 to ultrasonic waves for 25 minutes. After centrifugation at 4,050,000 g-minutes they obtained 90% of the activity in the supernatant, with an 11 fold purification as compared to the original homogenate. Tyramine was used as substrate for the assay of MAO activity. Ganrot and Rosengren (270) sonicated a pig liver mitochondrial preparation at pH 9. MAO remained in the supernatant after centrifugation at 40,000 g for 2 hours. However, they commented that the preparation could not be used as a starting material for purification, though they did not mention the reasons. Perhaps, the most successful solubilization with sonication was obtained by Tipton (262) using pig brain mitochondrial MAO. By successive freezing, thawing and sonication he could liberate up to 60-80% of the enzyme which was purified by subsequent steps. His procedure was followed with tyramine as a substrate. Murali and Radhakrishnan (286) applied a similar procedure to MAO from monkey intestine.

The combination of sonication with detergent has also been used by several authors with variable degrees of success. Zeller et al.(84) reported the liberation of MAO from hog liver mitochondria by means of deoxycholic acid and sonic oscillations. No further particulars of this procedure are found in the literature. Sakamoto et al.(277) subjected beef liver mitochondria to sonication and cholic acid, but the recovery in the supernatant after centrifugation at 105,000 g for 2 hours was only 4%. Nagatsu (261) studied several procedures for liberating MAO from beef brain mitochondria. The solubilization was measured determining the MAO with kynuramine as substrate in the supernatant after centrifugation at 105,000 g for 1 hour. He found that with Cutscum (1.2%, final concentration) the higher the pH the higher was the activity in the supernatant. But the enzyme was unstable in alkaline pH. He found the combination of Cutscum with sonication at pH 7.1-7.2, to be the most effective medium for solubilizing the enzyme, achieving a solubilization of 70%. Gabay and Valcourt (253) used a similar procedure for solubilizing the rabbit liver mitochondrial MAO. These authors noticed that too large a volume of sample and too long a period of sonication resulted in greater loss of the enzyme. They reported as unsatisfactory the solubilization with Triton X-100. Youdim and Sourkes (278) treated rat liver mitochondria with sonication in presence of benzylamine after which cholic

acid was added. They obtained up to 85% (kynuramine as substrate) of the original mitochondrial activity in the supernatant after centrifugation at 160,000 g for 90 minutes. Benzylamine was used on the basis of an earlier report by the same authors (227). In this work they showed that substrates of the enzyme, such as benzylamine, dopamine, epinine and isoamylamine, were able to protect the enzyme against denaturation by heat. Later on, Youdim and Sandler (287) modified the procedure of Youdim and Sourkes substituting cholic acid with Triton X-100.

It is well known that an increase in pH facilitates the solubilization of several membrane systems (409). In the case of MAO Nagatsu (261), as mentioned above, observed that Cutscum was more effective for solubilizing the enzyme in an alkaline medium but the enzyme was unstable. Akopyan et al. (267, 282) obtained solubilization of MAO from mitochondria of beef liver and brain and rat liver based on two facts: the protective effects of the MAO substrates on the enzyme and the increase in the solubilizing action of non-ionic detergents in alkaline medium. They treated the mitochondria with non-ionic detergents (OP-10 or Triton X-100, depending on the species) in the presence of benzylamine which was used to increase the pH and to protect the enzyme against inactivation.

It is difficult to compare and select from the technique of solubilization which would be most ideal for an organ of a determined species since the source of the enzyme and the methods to estimate its activity are quite different. The majority of these procedures have been designed to be followed by specific methodology for further purification or electrophoretic separation. Some authors have reported difficulties in applying solubilization procedures which have been found suitable for other MAO preparations (240, 257, 261, 281, 394). As some authors specify, problems arising from the use of detergents could be overcome by resorting to sonication (243, 262) or extraction with organic solvents (245). The detergents, besides the difficulties mentioned above, can produce alterations in the conformation of proteins (413). But the ultrasonic waves have a marked influence on the structure of the protein. When the pig liver mitochondria was treated with an organic solvent (279) only a relatively small proportion of MAO was solubilized. On the other hand, with or without detergents, the question always remains if the enzyme isolated by any means is identical to the original protein in the mitochondria. Certainly, the most probable answer will be that this "soluble" protein is somewhat different and could perhaps be related to the original physiological conditions when the change occurring in the MAO preparations are fully understood.

Sierens (281) studied exhaustively several procedures for solubilizing rat liver mitochondrial MAO. From deoxycholate (1%), Tween 20 (1%), Tween 40 (1%), Tween 81 (1%), Lubrol W (5%) and sonication, only the treatments with Lubrol W and deoxycholate liberated 20% or more of the original activity. The liberation of MAO was estimated by measuring the enzyme activity with benzylamine in the supernatant obtained after centrifugation at 100,000 g for 2 hours and 45 minutes. This author also found, using density gradient centrifugation and electron microscopy that sodium deoxycholate produced a more fine dispersion of the mitochondria. But he reported that MAO in presence of this detergent was unstable as estimated with benzylamine as substrate. This shortcoming was partly overcome by using the deoxycholate in presence of benzylamine. Furthermore the separation of two MAO fractions of rat liver mitochondria solubilized with this detergent with polyacrylamide gel electrophoresis was also reported (see p. 77 , this thesis). One fraction was active on benzylamine and the other was active on benzylamine and serotonin. We already showed that deoxycholate affected the serotonin MAO activity (Table 3). This effect of the detergent gives rise to two explanations other than MAO multiplicity. The first possibility could be that one fraction would be made up of the enzyme with the detergent attached to it, thus inhibiting the serotonin activity, and the other fraction would be free of

deoxycholate and possess both serotonin and benzylamine activity. This possibility was tested by us (367) under the same conditions described by Sierens and D'Iorio (272, 281) using deoxycholate-^{14}C. It was found that the detergent migrated faster than the fractions with MAO. Furthermore the fraction with only benzylamine activity was the one that migrated the least and consequently was further away from bulk of the detergent, thereby excluding the possibility of a fraction attached to the detergent and another free of the detergent. The conditions of the sample after being subjected to polyacrylamide gel electrophoresis could be comparable to those in table 3 where the enzyme was solubilized with deoxycholate in the presence of substrates and immediately subjected to dialysis. The second possibility could be that the detergent produced irreversible damage to the serotonin activity in one fraction but not in the other. The likelihood of this suggestion is strengthened by the fact that the serotonin activity is greatly diminished in the presence of deoxycholate and this activity even following dialysis is poorer than that of benzylamine.

In view of our interest to studying if these substrates, benzylamine and serotonin, were deaminated by different enzymes, we tried to devise a procedure to detach the enzyme from the mitochondria where none of the substrates studied were selectively affected. At the same

time it was necessary to insure that there was no inactivation during or after solubilization treatment. This procedure would also have to be drastic enough to achieve the separation of the multiple forms of MAO. The combination of Lubrol W with sonication in presence of substrates in an alkaline medium equilibrated in an atmosphere of H_2 affected the activity of all these three substrates; benzylamine, serotonin and kynuramine, approximately to the same extent (about 20% of inactivation). Sucrose was originally added with the intention of using the preparation for experiments with sucrose gradient electrophoresis (Section II), but it was found that the recovery after sonication was low when it was omitted. Furthermore it has a protective effect on the proteins (414). Consequently we decided to use sucrose in the standard procedure for all these experiments. Though a systematic study was not carried out we did notice that when there was a decrease in enzymatic activity the loss in serotonin activity was always greater than that of benzylamine.

SECTION II: DENSITY GRADIENT ELECTROPHORESIS

After the liberation of MAO from the mitochondrial membranes the next step was to explore the possible occurrence of multiple forms of MAO. Sierens and D'Iorio obtained indirect evidence for the existence of different enzymes for the oxidative deamination of benzylamine and serotonin, in accordance with other authors (see chapter IX, this thesis). Even though they reported the separation on polyacrylamide gel electrophoresis, of two fractions with different substrate specificities, the selective inhibitory action on the serotonin activity by the detergent used, sodium deoxycholate, as mentioned above (section I, table 2) led to the possibility that these differences in substrate specificity could be due to effects of the detergent. The difficulties in interpreting the results when using detergents which could inhibit MAO selectively, such as deoxycholate are eliminated in the procedure of solubilization used in the present work.

For the separation of multiple forms of MAO two approaches have been made. In one the enzyme was subjected to electrophoresis after partial purification and in the other crude preparations of the enzyme were separated chromatographically or electrophoretically after solubilization with sonication and/or detergent. When the

first procedure was used the degree of purification was generally followed by measurements of the MAO activity with one substrate (245, 252, 253, 262, 278, 282). This has the disadvantage that the activity for some substrates could be lost without being detected. The second approach presents the disadvantage encountered with crude preparations, such, as low specific activity and interference with some assays of enzymatic activity. The latter approach, however, permits, with relatively few steps, a preliminary visualization of the possible multiple forms, thus helping to plan the subsequent steps in the purification of the different enzyme fractions. This approach has been used by different authors (269, 271, 272, 281, 287, 359, 384, 390, 391) but unfortunately some of these reports were preliminary and were not followed by other reports (269, 287, 390).

In order to study whether a different enzyme deaminated benzylamine and serotonin as well as kynuramine, we subjected our solubilized preparation to sucrose gradient electrophoresis. This electrophoretic method offered the advantage of permitting the use of large amounts of samples free from sorption effects thereby eliminating the necessity to elute, which is normally required with other procedures.

METHODS

For chemicals used see section I, p. 80.

Sucrose gradient electrophoresis: The equipment used was a LKB column electrophoresis apparatus model 3340C, an ISCO (Instrumentation Specialties Company, Inc.) Dialagrad programmed gradient pump model 190, a Buchler power supply model No. 3-1014A, a fraction collector ISCO model 326 and a Buchler polystaltic pump model 2-6100. The arrangement of all this equipment is illustrated in figure 6.

The LKB column electrophoresis apparatus consists of a column (fig. 6, A) which is connected by ground joints to a cathode (fg. 6, C) and to an anode (fig. 6, B) vessel. The separation of the sample was carried out in this column. Its bottom can be opened or closed by means of a funnel (D) the end of which was attached to a tube of 3.5 mm diameter. This tube was connected to a two-way valve (fig. 6, F) and through this to the programmed gradient pump and to a peristaltic pump. For some procedures the peristaltic pump was substituted by a syringe.

The procedure followed before the introduction of the sample was described in the manual supplied with the electrophoresis apparatus. The light solution consisted of 0.01 M Tris-HCl buffer and the heavier solution of 50% sucrose in 0.01 M Tris-HCl buffer. After filling the apparatus with both

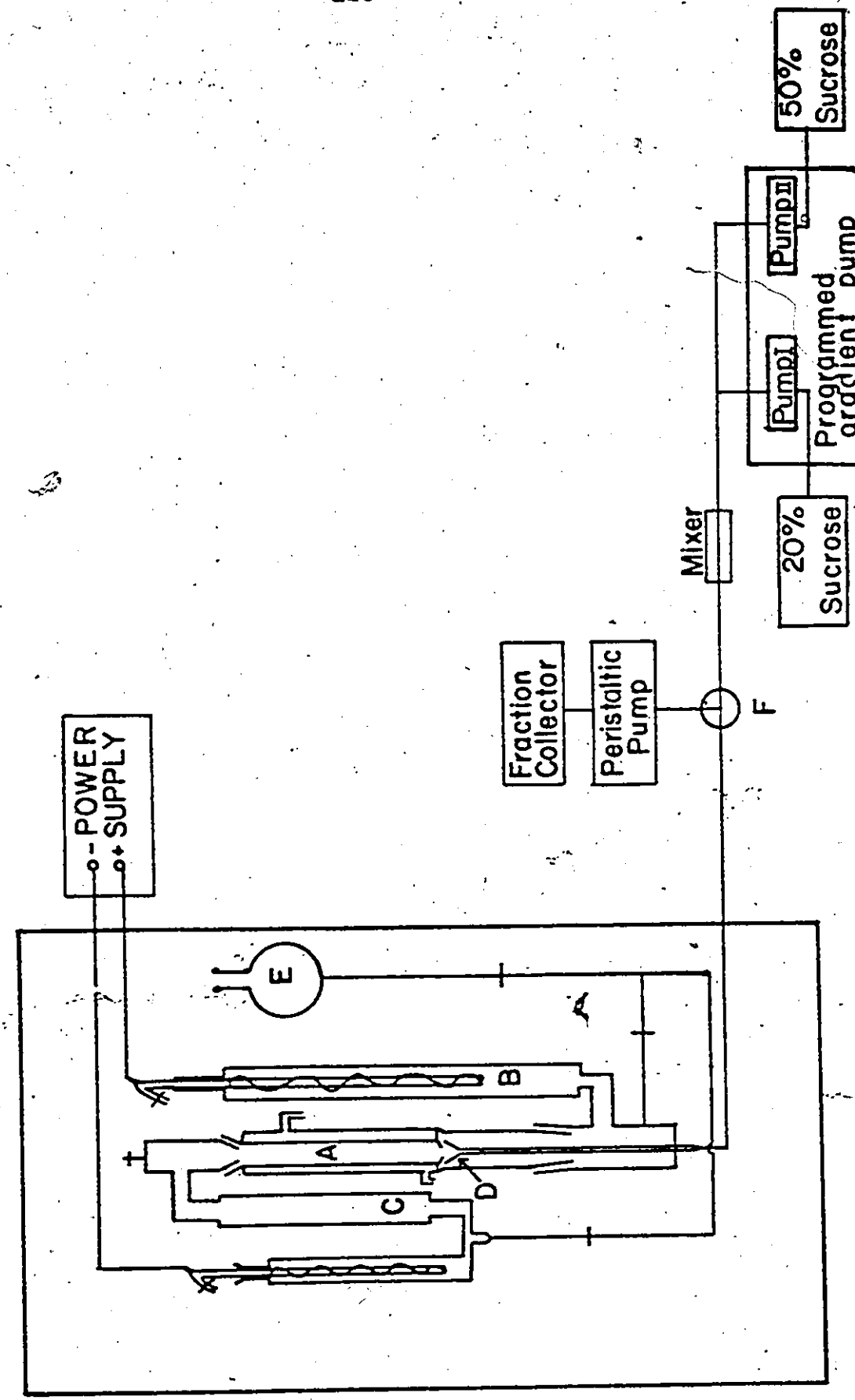

50% Sucrose
Pump II
Pump I
Programmed gradient pump
20% Sucrose
Mixer
Fraction Collector
Peristaltic Pump
F
- POWER
+ SUPPLY
E
B
A
D
C

Figure 6.

Arrangement of the equipment used for sucrose gradient electrophoresis. (A) Column for the electrophoretic separation of the sample. (B) Anode vessel. (C) Cathode vessel. (D) Funnel for closing or opening the bottom of the column A. (E) Mariotte flask. (F) Two-way valve.

solutions and closing the bottom with the funnel, all the heavier solution was extracted from the column where the actual separation was carried out. Then a linear gradient was applied through the glass tube united to the funnel by means of one programmed gradient pump apparatus, which consisted of two independent pumps. One was connected to a 20% solution of sucrose in 0.01 M Tris-HCl buffer and the other to a 50% solution of sucrose in the same buffer. The program consisted of 50 different steps. It was adjusted in such a way that in each step the flow rate of the heavier solution increased to 2% of the total flow rate (100 ml/h) while the less viscous solution had a flow rate decrement of the same amount. The program was set at 2 hours. If any adjustment was done in the total flow rate, a corresponding adjustment was made in the program time to compensate for it. The gradient pump was stopped when the gradient reached a density similar to that of the sample. The sample was rat liver mitochondrial preparation prepared by the procedure described in section I and centrifuged at 40,000 g for 15 minutes. The density of this sample was estimated by a previous run of the programmed gradient pump connected to the fraction collector. Each fraction represented a step of the program. A drop of the sample was added to each fraction and in this way its density was evaluated. Then a volume equivalent to that of the sample (4 to 6 ml), was extracted from the column. The sample was applied through the two-way valve with a syringe. The

application of the sample was not quantitative because some of it usually was lost during this step. On the other hand to make certain that no air penetrated into the column, it was necessary to evacuate some of the sample before connecting the programmed gradient pump to the column. The gradient was continued after omitting 10 steps. When the program was finished the pump was allowed to work for another 10 minutes period under the same conditions as the last step of the program. From then on the procedure followed was exactly the one described in the manual of the electrophoresis apparatus. The electrophoresis was run at 1,000 V and 3.5 mA with the Buchler power supply set at constant voltage. After the electrophoresis the funnel was lifted in order to close the bottom of the column. The different fractions were collected by means of a fraction collector and a peristaltic pump which was connected to the two-way valve.

Radioactive assay of MAO: For this assay radioactive benzylamine-^{14}C (International Chemical & Nuclear Corp.) and serotonin-^{14}C (New England Nuclear) were utilized as substrates. The method used was that described by Otsuka and Kobayashi (176) as adapted by Sierens and D'Iorio (281, 272). It was based on the incubation of the enzyme with the radioactive substrates in a buffered medium and extraction of the products with toluene after acidification with HCl.

e

The incubation and extraction of the products were carried out in polyethylene centrifuge tubes (A.H. Thomas Scientific Company) which had a capacity of 0.4 ml. The reaction mixture was identical to the one used by Sierens and D'Iorio (272, 281). It contained 20 µl of 0.2 M phosphate buffer (pH 7.4), 5 µl of radioactive substrate solution and 5 µl of the enzyme preparation. The final concentration of the substrates was 0.3 mM (2.0 mCi/mmole) and 1.5 mM (9.3 mCi/mmole) for benzylamine and serotonin, respectively. The different components of the reaction were mixed by centrifugation with a Beckman 152 microfuge for 5 seconds. The incubation was carried out at 37 °C for 30 minutes in a shaking water bath (Eberbach Corporation). At the end of the incubation the tubes were placed in ice and 10 µl of 5N HCl were immediately added. The transfer of the aqueous solutions was done with polyethylene micropipettes (B & B brand, Bio-Rad) or with microliter Eppendorf pipettes. When it was necessary to use glass micropipettes a thin film of silicone stopcock grease was applied to the tip of the pipette.

The products of the enzyme reaction were extracted with 100 µl of toluene and mixed for 2 minutes on a Vortex Genie mixer (Fisher Scientific Co.). The organic phase was separated from the aqueous phase by centrifugation in the Beckman microfuge. Fifty µl of the organic phase were taken and transferred to a counting vial containing 15 ml of

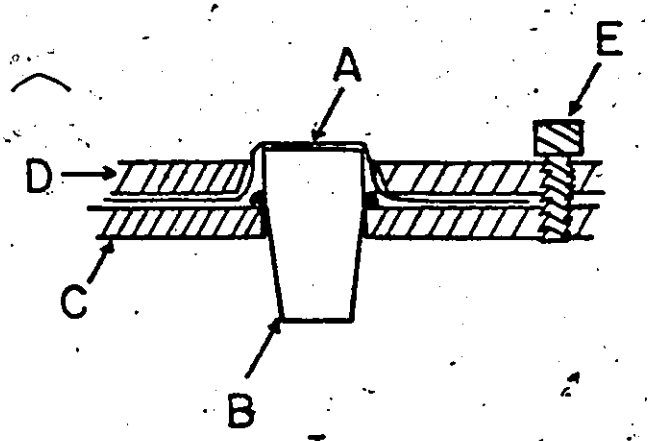
Omnifluor (4 g/l. New England Nuclear) in toluene. The counting was carried out in a Mark 1 liquid scintillation counter (Nuclear-Chicago Corporation). The quenching was estimated by the external standard method following the procedure described in the counter manual. The counting efficiency approximately the same for all the samples (about 84%). As the efficiency of the extraction was unknown the values are only comparative. MAO activity is expressed as counts per minute (c.p.m.) in 100 µl of extraction solvent. The correction for quenching was applied and the blank was subtracted. The blank consisted of the same reaction mixture but instead of 5 µl of enzyme 5 µl of the same buffer present in the enzyme reaction mixture were added.

Fluorometric assay of MAO: The procedure was that described by Drujan and Diaz Borges (157). As mentioned in chapter VIII (p. 56), it was based on the principle of the method described by Kraml (332) in which the 4-hydroxyquinoline, formed by the spontaneous cyclization of the product of the action of MAO on kynuramine, was measured fluorometrically.

The reaction mixture consisted of 1 ml of sample, diluted or undiluted according to its activity, 0.25 ml of 0.2 M Tris-HCl buffer (pH 7.4) and 0.25 ml of a kynuramine solution. The final concentration of the substrate was 0.2 mM. The samples were incubated at 37° C for 30 minutes in a

shaking water bath. At the end of the incubation 6 ml of absolute ethanol was added. If reduced volumes of the sample were utilized, the volumes of all the other reagents were reduced proportionally. After centrifugation 1 ml aliquot of the supernatant was made alkaline with 3 ml of 0.1 N NaOH. The intensity of the fluorescence was measured in an Aminco Bowman spectrophotofluorometer at a wavelength of 325 nm and 390 nm (uncorrected) for activation and emission, respectively. The concentration of 4-hydroxyquinoline was estimated by comparison with a standard curve of the same compound (Koch-Light Laboratories). The corresponding blank was made the same way as in the radioactive substrate assay. The MAO activity was expressed as nmoles of 4-hydroxyquinoline formed in 30 minutes. The wavelengths for measuring the intensity of the fluorescence were previously selected by testing the spectrophotofluorometer with a solution of 4-hydroxyquinoline.

Dialysis: Immediately after collecting the different fractions, they were subjected to dialysis overnight against 0.01 M phosphate buffer pH 7.4. For the simultaneous dialysis of all the fractions a simple device was designed (fig. 7). This device consisted of two plexiglas racks with multiple holes. In one of these racks, small plastic disposable beakers (2 ml, Fisher Scientific Co.) were placed and covered with a cellulose dialysis



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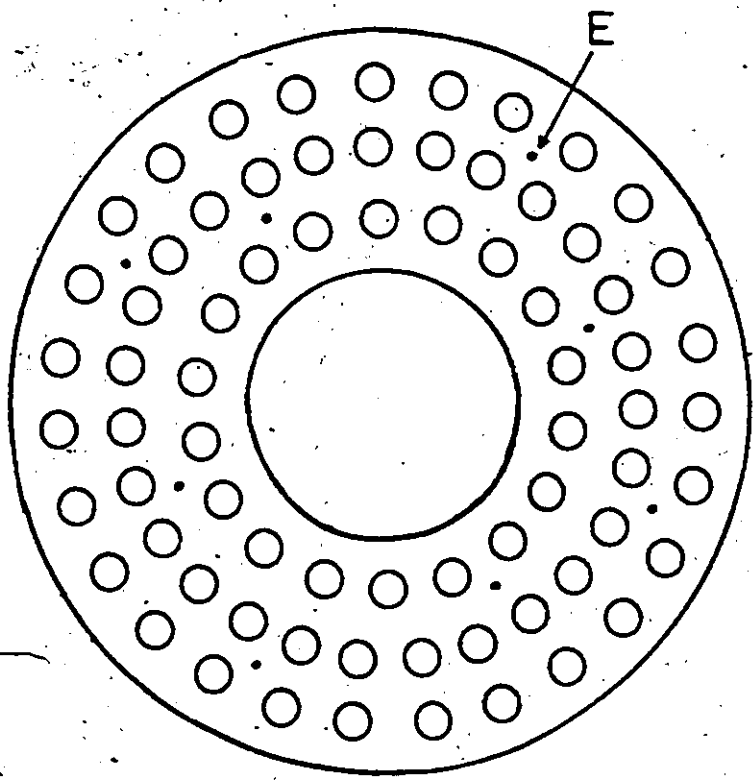


II

Figure 7.

Diagramatic representation of the dialysis device used in our experiments. (I) Front cut (II) Top view (A) Dialysis membrane. (B) Plastic beaker. (C) and (D) plexiglass racks. (E) Nylon screws.

membrane. The second rack was put on top of the first rack in such a way that the top of the beakers with the dialysis membrane fitted into the holes of the second rack. The diameter of the holes in this second rack was greater where it was first in contact with the dialysis membrane and then became slightly and progressively smaller. This was the object to avoid cutting the dialysis membranes with the edges. The pressure in order to have a good seal was obtained using nylon screws. The beakers were placed with the dialysis membrane facing down to permit a better dialysis of the sucrose. The problem of manipulation of a highly viscous solution and the interference of sucrose with the methods used for the assay of protein were thus eliminated.

Sucrose concentration: It was estimated by refractometry using an American Optical T/C Goldberg type refractometer.

Protein concentration: The protein was assayed by the method of Lowry et al.(405), taking the necessary precautions with regard to the presence of detergent as mentioned in section I.

RESULTS

Sucrose concentration of the

different fractions after electrophoresis is illustrated in figure 8. It can be observed that the gradient is approximately linear with a slight deviation in the zone of application of the sample. After the dialysis of the different fractions, the concentration of sucrose was in no case greater than 3%. A disadvantage with dialysis was the change in volume and loss of a small portion of the sample when it was removed from the dialysis device. The change in volume was, however, limited because the dialysis membrane was fixed under tension and by the rigid wall of the plastic beaker.

The results obtained with sucrose gradient electrophoresis are illustrated in figures 9, 10 and 11. Figures 9 and 10 show an example of the results obtained when the solubilized mitochondria were subjected to sucrose gradient electrophoresis for 4.5 hours. It is evident from these figures that kynuramine MAO activity followed a similar pattern of distribution within the different fractions as the benzylamine activity. The serotonin activity was distributed in several peaks and some of these were observed to have little benzylamine and kynuramine activity. In figure 11 the electrophoresis was run under the same conditions for 7 hours. Kynuramine activity, which is not shown in the graph, had a similar distribution to that of benzylamine activity. Serotonin activity appeared, as noted above, in several peaks with some of them almost devoid of the other activities.

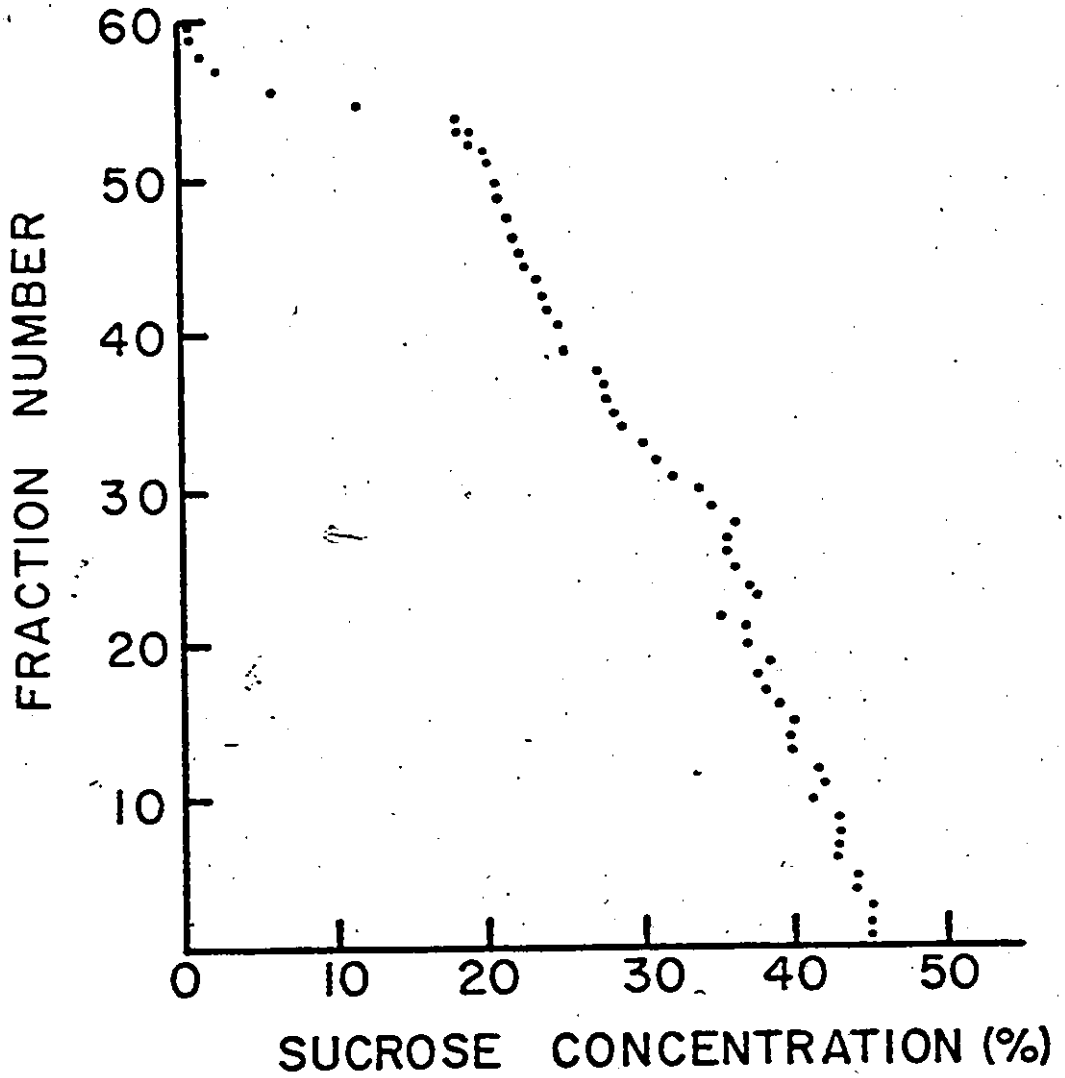

FRACTION NUMBER
60
50
40
30
20
10
0
10
20
30
40
50
SUCROSE CONCENTRATION (%)

Figure 8.

Concentration of sucrose in different fractions after sucrose gradient electrophoresis.

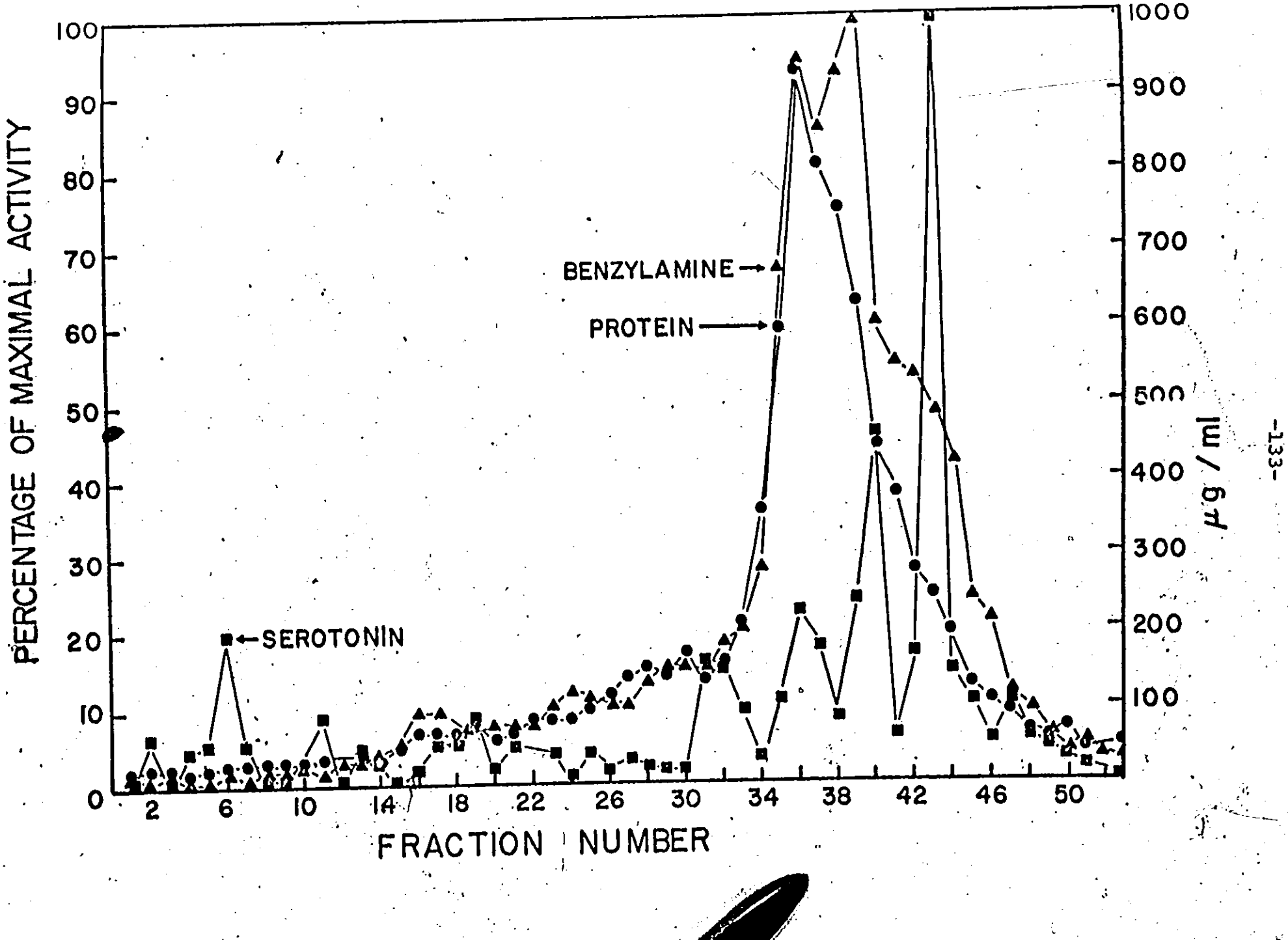

PERCENTAGE OF MAXIMAL ACTIVITY
100
90
80
70
60
50
40
30
20
10
0
2
6
10
14
18
22
26
30
34
38
42
46
50
FRACTION NUMBER
BENZYLAMINE
PROTEIN
SEROTONIN
1000
900
800
700
600
500
400
300
200
100
μg / ml

Figure 9.

Distribution of MAO activity for benzylamine and serotonin after sucrose gradient electrophoresis. The electrophoretic run lasted 4.5 hours. The MAO activities are expressed as a percentage of the fraction with maximal activity (serotonin 3986 d.p.m./5 µl/30 min; benzylamine 1713 d.p.m./5 µl/30 min). The fractions are numbered from anode to cathode. For more details see the text.

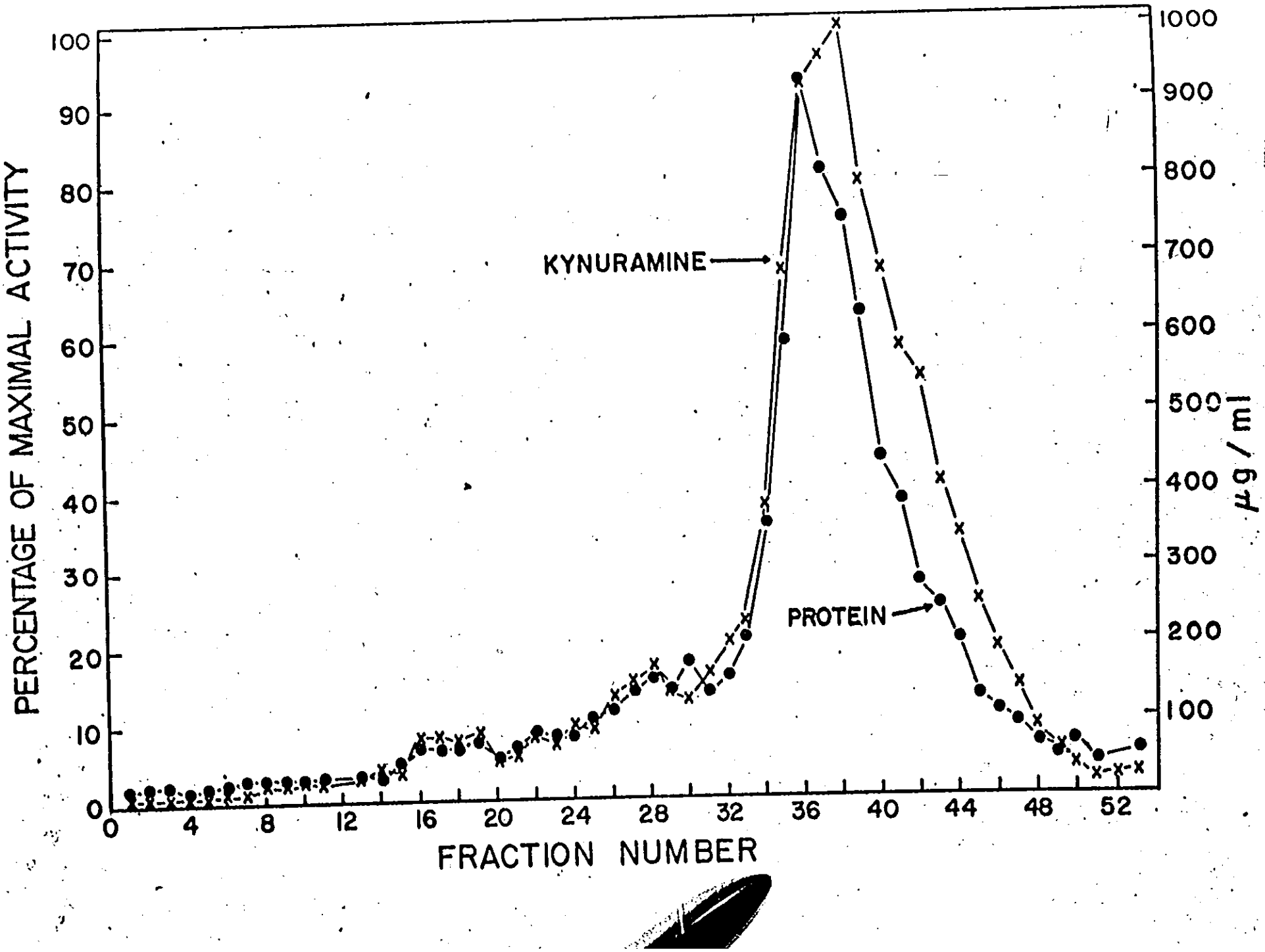

PERCENTAGE OF MAXIMAL ACTIVITY
μg / ml
FRACTION NUMBER
KYNURAMINE
PROTEIN
0
4
8
12
16
20
24
28
32
36
40
44
48
52
10
30
40
50
60
70
80
90
100
200
300
400
500
600
700
800
900
1000

Figure 10.

Distribution of MAO activity for kynuramine after sucrose gradient electrophoresis. The experiment is the same as that described in figure 9. The MAO activities are expressed as a percentage of the fraction with maximal activity (43.6 nmoles 4-hydroxyquinoline/ml/30 min.). The fractions are numbered from anode to cathode. The protein concentration is expressed as µg per ml of each fractions. For more details see the text.

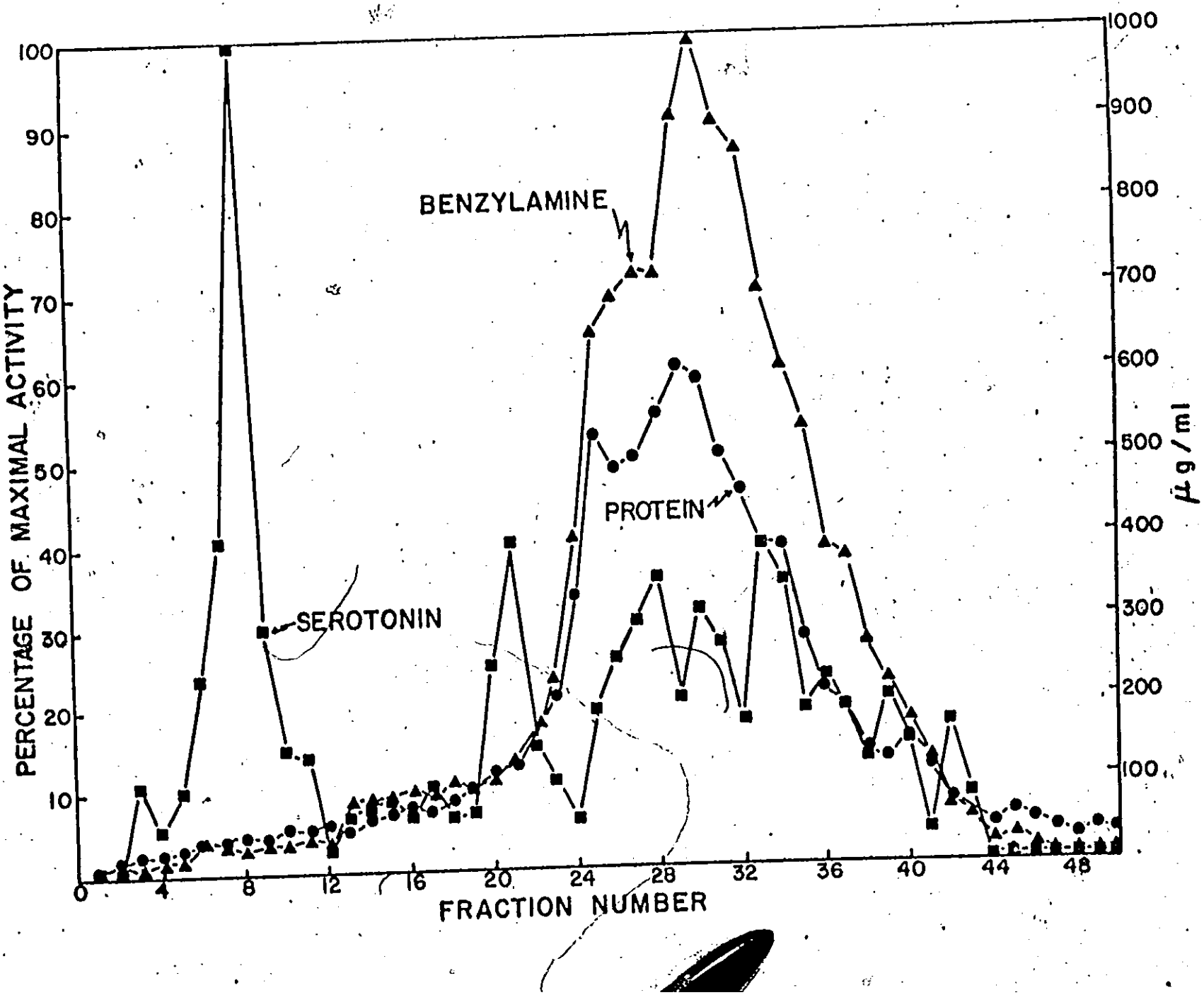

BENZYLAMINE
PROTEIN
SEROTONIN
PERCENTAGE OF MAXIMAL ACTIVITY
FRACTION NUMBER
μg/ml
100
90
80
70
60
50
40
30
20
10
0
4
8
12
16
20
24
28
32
36
40
44
48
1000
900
800
700
600
500
400
300
200
100

Figure 11.

Distribution of MAO activity for benzylamine and serotonin after sucrose gradient electrophoresis. The conditions and the legend of this figure are the same as those described in figure 9, with the exception that the electrophoretic run lasted 7 hours. The values of MAO activity for the fractions with maximal activity taken as 100% were 1337 d.p.m./5 µl/30 min. and 2051 d.p.m./5 µl/30 min. for serotonin and benzylamine, respectively.

It was difficult to get an idea of the recovery of the different activities owing to the non-quantitative application of the sample and to the unknown variation in the volume of the different fractions after dialysis. Furthermore in the case of serotonin activity there was interference by proteins in the recovery of the products of the MAO catalyzed reaction (see section III, p. 181). A rough estimation of the recovery of the different activities in four experiments where the electrophoresis was run for 4.5-5.0 hours gave the following values: 83-100% for benzylamine, 47-100% for serotonin and 75-100% for kynuramine. For the calculation of these recoveries the sonicated sample and a 100% recovery of protein were taken as references.

Though the separations shown in this section seem to be promising for the use of this procedure as a preparative method, unfortunately several shortcomings made it impractical for this purpose. The electrophoretic separation of the crude solubilized mitochondria was quite complex and it was not possible to get a similar separation consistently in spite of employing the same procedure. Hence the difficulty in isolating the different fractions of enzyme activity. Furthermore the MAO activity for serotonin was labile, specially after dialysis, which interfered with the characterization of the fractions.

DISCUSSION

The electrophoresis was carried out after taking into consideration the principles illustrated by Svensson (415) with the necessary modifications to suit the present experimental conditions. The sample was applied in the middle of the sucrose gradient between a "density shelf" which makes the isodensity condition for the application of the sample less critical. The stabilization of this zone was also reached at the time when the more dense part of the gradient was injected into the column. No droplet sedimentation was observed following the procedure described. Some broadening of the initial zone occurred during the electrophoresis by the higher conductivity of the samples, but this did not interfere with the electrophoresis. This could have contributed to the stability of the migrating zones by diluting the complex mixture applied to the column. As mentioned in chapter IX, several authors (61, 76, 116, 171, 229, 272, 281, 381) have obtained indirect evidence that the MAO substrates, benzylamine and serotonin, are deaminated by different enzymes. Moreover Sierens and D'Iorio (272, 281) using a rat liver mitochondrial preparation, were able to separate two fractions on polyacrylamide gel electrophoresis with different substrate specificities. The present result show that the migration of serotonin activity is different from that of benzylamine and

kynuramine. No difference was found between the migrations of benzylamine and kynuramine. These results also show that some serotonin activity can be isolated free from the activities of benzylamine and kynuramine. This is difficult to explain on the basis of selective inactivation of benzylamine activity, because as mentioned elsewhere in this thesis (p. 116, p. 139) when the enzyme was inactivated the activity towards serotonin was affected to a greater extent. Also the treatment of solubilization did not inactivate preferentially the activity towards a particular substrate. An alternative explanation and a more likely one in view of the difference in this pattern of migration is that we are dealing with different enzymes. Sierens and D'Iorio (272, 281) described one fraction that was active only towards benzylamine and another which was active towards benzylamine and serotonin. Assuming that the detergent did not produce irreversible damage to the fraction active towards benzylamine only those results and the results shown here strongly militate in favor of at least two different activities, one for serotonin and the other for benzylamine and kynuramine.

In 1968, Ragland (386) reported a partial separation of the MAO activity for kynuramine, tyramine and serotonin in liver mitochondria of beef, rat and rabbit.

The mitochondria, depending on the species, were solubilized in presence of Cutscum or Lauryl sulfate and Cutscum. After centrifugation at 150,000 g for 2 hours, the supernatant was subjected to gel filtration through a Sephadex G-200 column equilibrated with 2% Cutscum. The recovery of MAO activity from the column varied between 40 and 80% with different substrates and different species. Furthermore, the same author found that the enzyme was not stable in the detergent used. If Cutscum was omitted from the eluting buffer all the MAO activity was eluted immediately after the void volume. Later Gorkin (390) reported a partial separation of the MAO activities for serotonin, tyramine and p-nitrophenylethylamine and of the activities for m-nitro-p-hydroxybenzylamine and kynuramine by means of sucrose gradient electrophoresis. He used rat liver mitochondria dispersed by sonication or sonication and urea. When sonication alone was used the separation was obtained after 63 hours and up to 85 hours, but separation was not achieved with shorter run (24-48 hours). After treatment of the sonicated preparation with urea the separation of the MAO activities was obtained even within 43 hour runs. These results which confirm his earlier finding (269) are consistent with those of Ragland (386) and others (367, 391). Therefore the results shown in the present work corroborated the findings of other authors in rat liver mitochondria although different procedures were used .

Unfortunately the reports of Gorkin (269, 390) and Ragland (386) were of a preliminary nature and were not followed up.

Tipton (262) separated two active fractions of purified pig brain MAO preparation on Sephadex G-200 chromatography. The fraction eluted first, according to Tipton, might represent a lipid complex or a tetrameric form of the enzyme. Gomes et al. (233) after several steps of purification and chromatography on hydroxylapatite obtained three fractions of beef liver mitochondrial MAO. The first fraction which was not adsorbed to hydroxylapatite was unstable. A physicochemical study of the properties of the other two fractions indicated that one fraction could be a trimer of the other one. Hence the sulphydryl groups, the flavin, ribose and adenine content per 10^5 g were about the same and the molecular weights were 405,000 and 1,280,000 respectively. There was a difference in the phospholipid content. The pH optimun and the temperature stability were not different and the substrate specificity of the three fractions were similar. The higher activity was for benzylamine, the substrate used to following the purification. The activity for serotonin was rather low. The enzyme was observed to aggregate easily, specially the component with higher molecular weight. Gomes et al. concluded that the multiple components of the enzyme were not isoenzymes but

fragments in which the enzyme existed in different polymeric states. The same preparation, beef liver mitochondria, was studied by Kinemuchi (396). He solubilized it with sodium cholate and after centrifugation separated two fractions, MAO-1 and MAO-2, by means of fractionation with ammonium sulfate. MAO-1 precipitated at a saturation of 30 to 40% while MAO-2 precipitated between 40 and 50%. MAO-2 was more active than MAO-1 with all the substrates studied. The Km values for tyramine and serotonin were different. Furthermore, there was a difference in the temperature-inactivation curves of the two fractions as well as slight differences in the pH optima of MAO-1 and MAO-2 when β-phenylethylamine or serotonin was used as substrate. In view of these differences in properties and based on the evidence of MAO multiplicity obtained by other authors using different preparations, the author concluded that MAO-1 and MAO-2 seemed to be isoenzymes. No further evidence was presented in order to discard the possibility of changes in the properties of the enzyme at different states of aggregation. Hartman et al. (416, 417) reported the antigenic identity of the three MAO fractions separated by Gomes et al. (233). They suggested that the difference in properties of the enzymes separated by Gomes et al. could be due to different degrees of aggregation, binding to detergent or other physical alterations of the same protein. If we assume that the difference in properties between MAO-1 and MAO-2 are not due to these causes but to

the existence of different enzymes, it is possible that Gomes et al. isolated only one component of the multiple MAO forms and missed others during the procedures of purification. As we mentioned elsewhere if the procedure of purification is not followed up with different substrates and if there are changes in the substrate specificity it is possible that same MAO components, specific towards some substrates, could be lost undetected.

Oreland (219, 245) after solubilizing and purifying pig liver mitochondrial MAO subjected the preparation to gel filtration on Sephadex G-200 and found two peaks, one retarded and the other excluded. If the retarded peak was refiltrated on the same column it appeared as a single excluded peak. He repeated these experiments including several concentrations of sodium cholate in the elution buffer. A third retarded peak appeared when the concentration of the detergent was increased while the excluded peak disappeared progressively. When the included peaks were chromatographed again but eluted without the detergent an excluded peak appeared and only traces of the included peaks existed. In consequence Oreland concluded that the various peaks seem to be the result of a strong tendency of the enzyme to aggregate. As the purified preparation had similar substrate specificity as the original mitochondria, he discarded the possibility of the selective purification

of only one multiple MAO form. Furthermore, after a comparative study of several properties of the soluble enzyme and the enzyme rebound to lipids (220), such as thermostabilities, sensitivity to trypsin and influence to inhibitors, as mentioned elsewhere in this thesis, it was suggested that these results could be in agreement with the existence of only one enzyme in pig liver mitochondria.

Harada et al. (240) separated two components from partially purified beef brain MAO by continuous flow electrophoresis. These two components, enzyme 1 and 2, apparently did not have different molecular weights as judged by Sepharose 6B gel filtration. Other properties, such as pH optimum, Km value and the effects of inhibitors when kynuramine was used as substrate are similar. However, the substrate specificities were different. With kynuramine, benzylamine, tyramine, normetanephrine and tryptamine enzyme 1 had higher activity towards tyramine while enzyme 2 had higher activity towards normetanephrine. The authors suggested the possibility that both components belong to the same enzyme protein and were derived by the ability of the detergent to alter the structure of the protein. However, they concluded that the importance of these components in vivo remains to be investigated further. Hidaka et al. (418) found that it was possible to precipitate immunologically approximately 80% of the MAO activity from

partially purified brain and liver extracts using anti-liver MAO. With further addition of an excess of antiglobulin the rest of the liver MAO precipitated, but this did not happen in the case of brain MAO. Hartman (397) reported the isolation of beef brain MAO that did not cross-react with the anti-liver MAO antibody using immunoprecipitation and chromatography (Sephadex G-200) procedures. He called it "brain-specific MAO" and found that properties such as the rate of deamination of kynuramine the inhibition of kynuramine by iproniazid and pargyline, and the relative rate of deamination of kynuramine, benzylamine and tyramine were different from those of liver MAO, purified by the method of Gomes et al.(233), total brain MAO and serum amine oxidase. Recently, McCauley and Racker (399) also separated two MAO fractions from a partially purified bovine brain mitochondrial preparation by immunoprecipitation. MAO A did not cross-react with an anti-bovine liver MAO antibody. It deaminated tyramine, tryptamine norepinephrine, serotonin and dopamine. There was little activity towards benzylamine. Harmaline was a good inhibitor of MAO A but not pargyline. MAO B was precipitated by the antibody against bovine liver MAO and the precipitate was enzymatically active. This fraction was active on benzylamine, tyramine, tryptamine and dopamine, while the activity towards norepinephrine or serotonin was little or none. MAO B was resistant to harmaline but it was sensitive to inhibition by pargyline

MAO A was more heat labile than MAO B. According to McCauley and Racker these enzymes would correspond to the enzymes A and B described by Johnston (115) on the basis of experiments with the MAO inhibitor clorgyline. At the same time they rule out that Hidaka et al. (418) and Hartman (397) were dealing with enzyme A, because their non-precipitable fraction was active on benzylamine and furthermore, in their procedure of purification and immunoprecipitation they included heating steps (37^{0} for 30 minutes) which inactivated MAO A.

Shih and Eiduson (394) reported the separation of three MAO fractions from rat brain mitochondria by agarose columns and Sephadex electrophoresis. Though one fraction seemed to be more active towards benzylamine than towards serotonin as compared to the other fractions, the data are not conclusive about the significance of these fractions. The molecular weights seem to be different and the possible existence of aggregates was considered. Studies with the inhibitor pargyline (^{14}C-labelled) showed that this inhibitor seemed to be associated with the fractions possessing MAO activity. On the other hand they found that the ratio of the distribution of MAO activity to benzylamine, serotonin and kynuramine, between the soluble and the particulate fraction, was different for each substrate. The distribution of the radioactive pargyline was also different

for the different substrates. By assuming that there was little binding of pargyline to anything but MAO, they concluded that their data would suggest a possible change in the conformation of MAO in such a way that the specific activity would decrease; but they also pointed out the insufficiency of their data to distinguish from other possible explanations. Severina and Zhivotova (419) found during the purification of rat liver mitochondria a selective inhibition of the deamination of various amines depending on their chemical structure. The deamination of tyramine and 4-NH_2-β-phenylethylamine decreases 50% while the deamination of β-phenylethylamine, its 4Cl-derivative and benzylamine was not affected. The purification steps were followed using p-nitrophenylethylamine as substrate. Though these results could explain the existence of two enzymes, they suggested that these modifications in the activity were due to spatial changes arising in the enzyme molecule during solubilization and treatment with Triton X-100. It is also possible that both these factors lead to the alterations in the substrate specificity.

The fractions obtained by sucrose gradient electrophoresis with selective specificity towards serotonin seem to correspond to enzyme A described in brain by McCauley and Racker (399). This enzyme A, as mentioned above, seems to correspond to enzyme A postulated by Johnston (115) and

studied in different organs of distinct species by other authors (see chapter IX, this thesis). The results obtained by Kroon and Veldstra (222) with a crude mitochondrial fraction of rat cerebral hemisphere and those reported in a preliminary form by Greenawalt (221) about the subcellular distribution of the MAO activity for several substrates (see chapter V, this thesis) are consistent with our results. Both authors found different localization for the kynuramine and serotonin activities, though Greenawalt also described differences between kynuramine and benzylamine, which are not confirmed in this report. These results along with others already described in this thesis constitute strong evidence for the existence of different enzymes although there was only the partial separation of the MAO activities and the identification of serotonin specific fractions was rendered difficult due to their instability.

SECTION III: POLYACRYLAMIDE GEL ELECTROPHORESIS

There are several reports in the literature on the separation of multiple bands of MAO means of electrophoresis (76, 160, 161, 233, 240, 271, 272, 281, 282, 284, 287-289, 359, 384, 385, 387-393, 395, 400, 420-423). Polyacrylamide gel electrophoresis has been commonly employed. In the majority of cases MAO has been localized by the method of Glenner et al. (290) which utilizes tetrazolium salts. This method has been questioned in relation to its specificity towards MAO and will be discussed in this section. On the other hand the difficulty in identifying the fractions separated by sucrose gradient electrophoresis render interesting the possibility of corroborating these results using other methods of separation. Polyacrylamide gel electrophoresis gives finer separation. The MAO activity was localized directly on the gel with radioactive substrates as suggested by Sierens and D'Iorio (272, 281). In addition to serotonin and benzylamine, two other substrates, tyramine and tryptamine, were selected because they also have been involved with studies of MAO multiplicity (see chapter IX, this thesis). Some problems found during our experiments and related to the use of radioactive methods in the assay of MAO are also studied.

METHODS

For chemicals used see section I, p. 80.

Preparation of the sample: Rat liver mitochondria were solubilized in 0.1 M Tris-HCl buffer, pH 8.8, as described in section I, centrifuged at 40,000 g for 15 minutes and dialyzed against the same buffer for about 2 hours. The sample was then diluted three times with distilled water and an aliquot of about 130 µg of protein was subjected to polyacrylamide gel electrophoresis. All the operations were performed at 0-4°.

Polyacrylamide gel electrophoresis: The gels were prepared as described by Sierens and D'Iorio (272, 281) based on the disc electrophoretic technique of Ornstein (424) and Davis (425) with the following modification. Agarose was included in the separating gel as suggested by Peacock and Dingman (426). The inclusion of agarose made the gels harder which permitted the use of a lower concentration of acrylamide. In our experiments as in those of Sierens and D'Iorio (272, 281) the concentration of agarose and acrylamide in the agarose-acrylamide gel was 0.5% and 4% (w/v), respectively. Further modifications were found to be necessary to carry out these experiments. Lubrol W was included in the eluting buffers and in the acrylamide and acrylamide-agarose gels as described by Dulaney and Touster (427). This detergent was the same used for solubilization of the

sample and its final concentration was 0.4% (w/v). With further increase in the concentration of detergent there was a tendency of the gels to slip out of the tube and this made the operations difficult. Riboflavin was used as catalyst in both acrylamide and acrylamide-agarose gels as suggested by others (286, 428, 429). Ammonium persulfate which has been used to polymerise the gels has been shown to have a denaturing effect on enzymes in addition to producing electrophoretic artifacts (428-430). The gels were also prerun with reduced glutathione in order to remove any undesirable component which could be present after the polymerisation step and also to reduce any oxidants that could not be removed by the preelectrophoresis alone (341). Without the later modifications the activity in the gels for benzylamine was poor and the activity for serotonin was not observed in most cases. Furthermore the electrophoretic migration was not reproducible.

The electrophoresis was carried out in a Canalco model 12 or in a Buchler polyanalyst electrophoresis apparatus. The gels were prepared in 8 x 0.5 cm glass tubes. The solutions and the procedures for the preparation of the gels were as follows:

Solutions:

I. Eluting buffer

60 g Tris
288 g glycine
water to 2 l

II. Acrylamide-agarose gel

(a) Acrylamide monomer.

19 g acrylamide (for electrophoresis, Eastman Organic Chemicals)
1 g N,N'-methylenebisacrylamide (Eastman Organic Chemicals)
water to 100 ml.

(b) Riboflavin

2.5 mg riboflavin
water to 50 ml

(c) TEMED

0.25 ml N,N,N',N'-tetramethylethylene diamine (Eastman Organic Chemicals)
water to 100 ml

(d) Buffer.

45.48 g Tris
60 ml 1N HCl
water to 100 ml

(e) Sucrose-Lubrol W

40 g sucrose
0.8 g Lubrol W
water to 100 ml

III. Acrylamide gel

(a) Acrylamide monomer
10 g acrylamide (for electrophoresis, Eastman Organic Chemicals)
2.5 g N,N'-methylenebisacrylamide
water to 100 ml.

(b) Riboflavin

2 mg riboflavin
water to 50 ml

(c) Buffer-TEMED

48 ml 1N HCl
5.98 g Tris
0.46 ml N,N,N',N'-tetramethyl-ethylenediamine (Eastman Organic Chemicals)
water to 100 ml

(d) Sucrose-Lubrol W

The same as II, d

Procedure:

For the preparation of the acrylamide-agarose gel 125 mg agarose (Schwarz/Mann, special grade, purchased from Picker Nuclear Canada) were dissolved by boiling in 12.5 ml of sucrose-Lubrol W solution (II, e). The solution was then cooled to 40°. Simultaneously, in another flask 5.0 ml of acrylamide monomer (II, a), 2.5 ml TEMED solution (II, c) and 2.5 ml of acrylamide-agarose gel buffer (II, d) were mixed and warmed up to 40°. Before heating, both mixtures were put under vacuum for a short time. When the temperature reached 40°, the two solutions were mixed and 2.5 ml of riboflavin solution were added (II, b). Immediately, the glass tube was filled up to 1 cm from the top. After overlaying with distilled water, the polymerisation was triggered by subjecting the preparation to fluorescent light

for 30 minutes. After this time the overlaying water was carefully suctioned off and the top of the gel was washed twice with distilled water. Then, the water was replaced by acrylamide gel solution. This solution was prepared by mixing 2 parts of acrylamide monomer (III, a) with 1 part of riboflavin (III, b), 1 part of buffer-TEMED (III, c) and 4 parts of Sucrose-Lubrol W solution (III, d). Before adding the acrylamide solution to the top of the acrylamide-agarose gel, the solution was degassed by vacuum. The procedure of polymerisation was the same as adopted for the acrylamide agarose gel. When the polymerisation was completed the top of the acrylamide gel and the bottom of the acrylamide-agarose gel were washed with eluting buffer. The tubes containing the gels were placed in the electrophoresis apparatus and the reservoires filled with elution buffer (I). Care was taken to remove air bubbles from the top of the tubes. 50 µl of 4 mM glutathione in 4% sucrose were applied on top of each gel and a preelectrophoresis was run at a current of 3 mA per gel for 1 hour. The positive electrode was connected to the lower buffer reservoir.

At the end of the preelectrophoresis the enzyme preparation was subjected to electrophoresis after refilling both reservoirs with fresh eluting buffer. The preelectrophoresis and the electrophoresis were carried out at constant current and at 4°. At the end of the run the gels

were taken from the tubes and cut into thin discs (about 25 mg) with a Canalco gel slicer. The MAO activity was assayed directly on the gel fractions with radioactive substrates (272, 281, 421-423). The acrylamide gel (about 100 mg) was assayed without further divisions.

Radioactive assay for MAO: Each one of the thin gel discs was put in a 0.4 ml polyethylene centrifuge tube (section II, p. 124) and forced down by a short centrifugation. 10 µl of acrylamide-agarose gel buffer (solution II, d diluted 1:10) were added and the thin disc was fragmented with a Delrin^{R} homogenizer (Physics workshop, University of Ottawa) which fitted into the small polyethylene tube. Then, 5 µl of radioactive substrate were added to the tube and the contents were mixed by centrifugation. During this procedure the polyethylene tubes were kept in ice except for the short intervals (about 10 seconds) of centrifugation. The incubation was carried out at 37° for 60 minutes with constant shaking. At the end of this period the tubes were again placed in ice and 10 µl of 5 N HCl were added. The products of the enzymatic reaction were extracted with 200 µl of an organic solvent and 100 µl of the extract was mixed with 15 ml of a 4 g/l solution of Ommifluor (New England Nuclear) in toluene. The procedure for the extraction of the products and counting of the radioactivity was the same as described in section II (p. 124). The quenching was also

the same irrespective of the solvent used for the extraction of the enzymatic products.

Besides benzylamine-^{14}C, and serotonin-^{14}C, tyramine^{14}C (New England Nuclear) and tryptamine-^{14}C (New England Nuclear) were also utilized as substrates. The solvents used for extraction were diethyl ether for the products of tyramine and serotonin and toluene for the products of benzylamine and tryptamine.

The use of diethyl ether for the extraction of the products of serotonin deamination was suggested by Baker (342). Baker pointed out that serotonin was extracted by toluene and not by diethyl ether based on the findings of Quay (432). In a preliminary experiment we found that diethyl ether was six times more effective than toluene in extracting the products of serotonin, but the blank value was increased to the same extent. On the other hand it is not likely that large amounts of serotonin can be extracted at pH 0.45, pH which was the pH of the assay medium in our experiments (p. 161) after adding HCl. The increase in the efficiency of extraction permits the use of a lower specific activity in the radioactive serotonin solution than that used in the experiments described section II. The same solvent was employed for the extraction of tyramine products after comparison with three other solvents in preliminary experiments to be described subsequently.

Tryptamine and benzylamine were extracted with toluene as described by Wurtman and Axelrod (341) and Sierens and D'Iorio (272, 281), respectively.

Another modification was the inclusion of a crude aldehyde dehydrogenase preparation in the incubation mixture except in the assay with benzylamine. Aldehyde dehydrogenase has been employed for MAO assays by other authors (328, 330, 375). The aldehyde dehydrogenase and cofactors added were included in the 10 µl acrylamide-agarose gel buffer in the following amounts: 0.15 µmoles of nicotinamide-adenine dinucleotide (NAD), 0.85 µmoles of nicotinamide and 2 µl of crude aldehyde dehydrogenase.

The crude aldehyde dehydrogenase was prepared according to the procedure of Lovenberg et al. (328). A guinea pig weighing between 240 and 300 g was injected intraperitoneally with 12.5 mg/kg β-phenylisopropylhydrazine HCl (Catron, gift from Lakeside Laboratories, Milwaukee, Wis.). After 24 hours the animal was killed and the kidneys were removed and kept in the cold. The following procedures were carried out at 0-4°. The kidneys were homogenized in 4 volumes of a solution of 0.25 M sucrose in 0.05 M phosphate buffer pH 7.4 and the suspension was centrifuged at 78,000 g for 1 hour. The floating fatty layer was removed and the supernatant was used as the crude enzyme preparation. The enzyme preparation was kept frozen

for not more than 4 days. The activity of the enzyme was determined spectrophotometrically as described by Racker (433).

The assay mixture in a total volume of 3 ml comprised the following components:

2.4 ml distilled water
0.3 ml of 0.1 sodium pyrophosphate buffer pH 9.3
0.1 ml of 0.2% solution of neutralized NAD
0.1 ml of enzyme suspension.

The above solutions were pipetted out into a quartz cell of 1 cm light path, the reaction was initiated by adding 0.1 ml of 0.5% solution of acetaldehyde to the assay mixture and the change in absorbance at 340 nm was followed with time. The reference cell contained all the reagents except the substrate. One unit of enzyme activity is defined as an optical density increment of 0.001 per minute under the above conditions. The specific activity obtained with crude aldehyde dehydrogenase preparation was 166 ± 12 (S.E.) units/ml and the protein concentration was about 12.9 ± 0.74 (S.E.) mg/ml.

The radioactive aqueous solutions added to the assay mixture were the following: 10.8 mM (specific activity 2.5 mCi/mmole) tyramine, 6 mM (specific activity 3.0 mCi/mmole)

serotonin, 6 mM (specific activity 2.03 mCi/mmole) benzylamine and 3 mM (specific activity 3.0 mCi/mmole) tryptamine

Preliminary experiments were carried out in order to study the possibility of improving the recovery of enzymatic products when organic solvents were used. The binding of these products to acid-insoluble material (347, 434) which interferes with the radioactive assay of MAO (347) has been reported. In the present experiments, the influence of a crude aldehyde preparation on the radiometric assay of MAO was studied. Rat liver mitochondria suspended in distilled water were used as the source of the enzyme. The enzyme reaction mixture, consisting of 5 µl of substrate and 20 µl of 0.2 M phosphate buffer pH 7.4, was similar to that described under section II p. 124.

In some experiments aldehyde dehydrogenase and solubilized mitochondrial preparation devoid of MAO activity were included in 20 µl of 0.2 M phosphate buffer pH 7.4. When added to the assay mixture the concentrations of the crude aldehyde dehydrogenase, NAD and nicotinamide were one half of those present in the acrylamide-agarose gel buffer used for the assay of the gel fractions. The MAO inactive solubilized mitochondrial preparation was obtained by incubating a sample, similar to the one utilized in the electrophoresis, at 43-45° for 6 hours and leaving it up to 12 hours at room

temperature. The incubation was carried out at 37° for 30 minutes with constant shaking. The termination of the reaction, the extraction of the products and the counting of the radioactivity was done as described above except that the incubation mixture was extracted three times. After the first extraction the tubes were placed in a bucket of alcohol-dry ice and the aqueous phase was frozen. The remaining organic solvent was discarded. Two hundred µl of fresh organic solvent were added to the aqueous layer and after shaking for 2 minutes on a Vortex Genie mixer (Fisher Scientific Co.) the samples were centrifuged. One hundred µl of the organic layer was then transferred to the counting vial and the procedure was repeated twice. Further extraction did not improve the recovery of the products.

For comparative purpose some experiments were performed with the assay method of Robinson et al. (177). This method is based on the selective adsorption of the MAO substrates on the resin Amberlite CG-50. The assay mixture and the conditions of incubation were the same as those described for experiments where the products of MAO reaction were extracted with organic solvents. At the end of the incubation a 20 µl aliquot of the incubation medium was applied to an Amberlite CG-50 column and was immediately eluted with deionized water. The conditioning of the resin was done as described below following the procedure of

Pisano (435).

Amberlite CG-50(H) of 100-200 mesh (BDH Chemicals Ltd., Poole, England) was suspended in 3 volumes of distilled water and stirred for 3 minutes. After settling for 30 minutes the supernatant was discarded. The procedure was repeated until the supernatant was clear after settling for 15 minutes. The last supernatant was not discarded and 2 volumes of 10 N NaOH were added over a period of 15 minutes with continuous stirring. The stirring was continued for 2 hours. Then the suspension was allowed to settle for 15 minutes and the supernatant was discarded. The resin was washed several times with distilled water and then five volumes of 6 N HCl were added and the suspension stirred for 30 minutes. Then the resin was washed with water several times and converted back to the sodium form by 10 N NaOH and washing with water as described above. The resin was suspended in an equal volume of deionized water, stirred and brought to pH 6.0-6.5 with glacial acetic acid. The Amberlite was ready for use when the pH remained constant after 30 minutes of continuous stirring.

The Amberlite columns (0.5x3.8 cm) were prepared in Pasteur capillary pipettes which were plugged with glass wool. Deionized water was used for all procedures. When the resin settled the columns were washed with 4 ml of water.

After applying the sample the column was washed with 4 ml of deionized water. Increasing the volume of elution did not improve the recovery of products. An aliquot of the eluate was added to 14 ml of Bray solution and the radioactivity was counted in a Mark 1 liquid-scintillation counter (Nuclear Chicago Corporation) by the external standard method. For the preparation of the Bray solution naphthalene (Scintillation grade), 2,5-diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazolyl) benzene (POPOP) were purchased from ICN and p-dioxane (scintanalyzed) and methanol (spectranalyzed) from Fisher Scientific Co. Limited. The proportion of the different substances was the same as described by Bray (436).

Staining procedure for the localization of MAO in acrylamide gel: After acrylamide gel electrophoresis of solubilized rat liver mitochondria was performed as described above, some gels were stained by the method of Glenner et al. (290) as described by Youdim et al. (388). The incubation of gels was carried out in the dark at 37° for about 45 minutes with a solution of the following composition: tryptamine hydrochloride (30 mg), sodium sulfate (18mg), nitro blue tetrazolium (10 mg, 2,2-Di-p-nitrophenyl-5-5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene)-ditetrazolium chloride, Sigma Chemical Company) and 10 ml of 1.0 M phosphate buffer pH 7.4. When tryptamine was substituted by benzylamine, serotonin or tyramine the same concentration was used.

Other techniques: The assay of MAO by oxygen consumption used in this section is identical to that described in section I. In this assay catalase was not added to the assay medium (299) because all the samples assayed contained significant amounts of catalase.

The activity of catalase was measured by the Sigma method. A 30% solution of H_2O_2, in 0.05 M phosphate buffer pH 7.0 was diluted to give an O.D. between 0.550 and 0.520 at 240 nm with a quartz cell of 1 cm light path. The enzyme assay was performed in quartz cells (1 cm light path) at 25° by adding 0.1 ml of the sample to 2.9 ml of the substrate and the time required for $O.D._{240}$ to decrease from 0.450 to 0.400 was measured. This time corresponds to the decomposition of 3.45 µmoles of H_2O_2 in the 3 ml solution. One Sigma unit is the amount of enzyme which can catalyze the decomposition of one micromole of H_2O_2 per minute under these conditions.

The proteins were estimated according to Lowry et al. (405).

RESULTS

Figure 12 illustrates the distribution of MAO activity after polyacrylamide gel electrophoresis when the run was carried out from cathode to anode for 75 minutes with

a current of 2 mA per gel. Benzylamine MAO activity is localized in the acrylamide gel. Serotonin MAO activity is distributed in different peaks at the beginning and at the end of the acrylamide-agarose gel though some activity is observed in the acrylamide gel. Part of the tyramine activity remains with the activity for benzylamine in the acrylamide gel and part is localized in the middle of the acrylamide-agarose gel. With a longer duration of electrophoresis (2 hours) the activities for benzylamine, serotonin and tyramine are observed in the acrylamide gel but serotonin activity is also noticed in the acrylamide-agarose gel (fig. 13). A clear separation of the serotonin MAO activity from that of benzylamine can be observed when the electrophoresis is run for 2 hours and the current is increased to 3 mA per gel as shown in figure 14. In contrast the serotonin activity, the tyramine activity remains in the acrylamide gel although penetration of this activity into the beginning of the acrylamide-agarose gel is also observed.

When the electrophoresis is run from anode to cathode using a current of 2 mA per gel for 75 minutes (fig. 15) serotonin MAO activity appears along the gel in several bands while tyramine activity is localized in two peaks at the beginning and at the end of the acrylamide-agarose gel. However, benzylamine activity could not be detected. Similar results with benzylamine activity were obtained in

electrophoresis that lasted for 0.5 and 2 hours. When the current was increased to 3 mA per gel and the electrophoresis lasted for 2 hours (fig. 16) tyramine activity was localized in the acrylamide gel, benzylamine activity was absent and serotonin activity was localized in the acrylamide gel and in the first third of the acrylamide-agarose gel.

Figure 17 illustrates the distribution of MAO activity when tryptamine was used a substrate and the electrophoresis was run for 75 minutes with a current of 2 mA per gel. The distribution of tryptamine activity is similar to that of serotonin under identical electrophoretic conditions (fig. 12).

A series of preliminary experiments was carried out in order to standardize the conditions for the assay of MAO with radioactive substrates when organic solvents were used for the extraction of the MAO reaction products. The results obtained with these experiments are illustrated in figures 18-25 and tables 4-8.

A perusal of table 4 would show that diethyl ether is more useful for the extraction of the tyramine products than toluene, anisole and ethyl acetate. Anisole (176) is comparable to diethyl ether after four extractions, with similar proportion of radioactive counts between blank and sample,

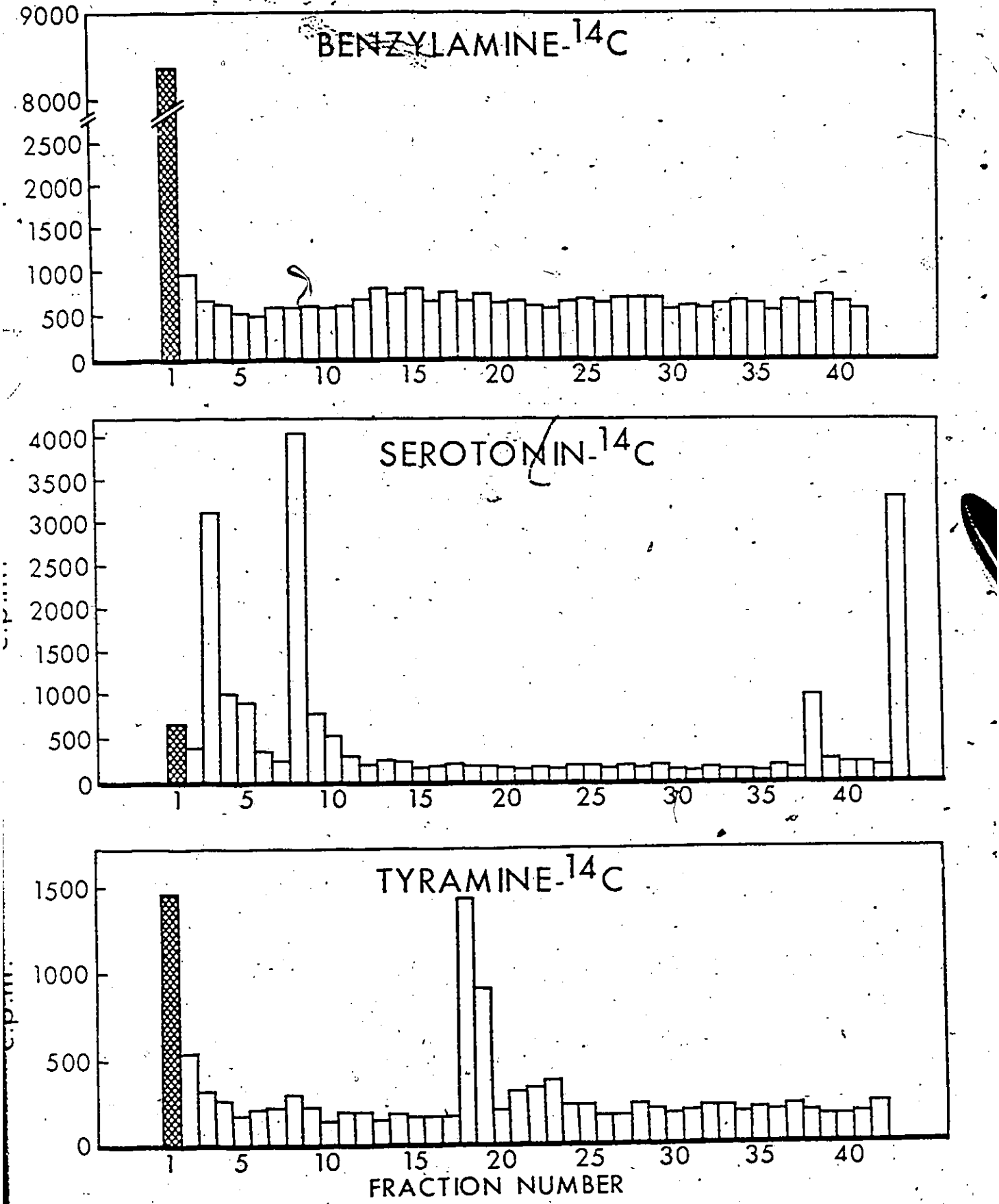

BENZYLAMINE-14C
9000
8000
2500
2000
1500
1000
500
0
1
5
10
15
20
25
30
35
40
SEROTONIN-14C
4000
3500
3000
2500
2000
1500
1000
500
0
TYRAMINE-14C
1500
1000
500
0
FRACTION NUMBER

Figure 12.

Polyacrylamide gel electrophoresis of rat liver mitochondria. The electrophoresis was carried out for 75 minutes from cathode to anode with a current of 2 mA per gel. MAO activity was assayed directly on the gel with benzylamine (top graph), serotonin (middle graph) and tyramine (bottom graph) as substrates. The activity was expressed as c.p.m. in an aliquot of 100 µl of organic solvent/fraction/hour, without blank subtraction, after extraction of the products of the MAO reaction with 200 µl of organic solvent. For more details see text.

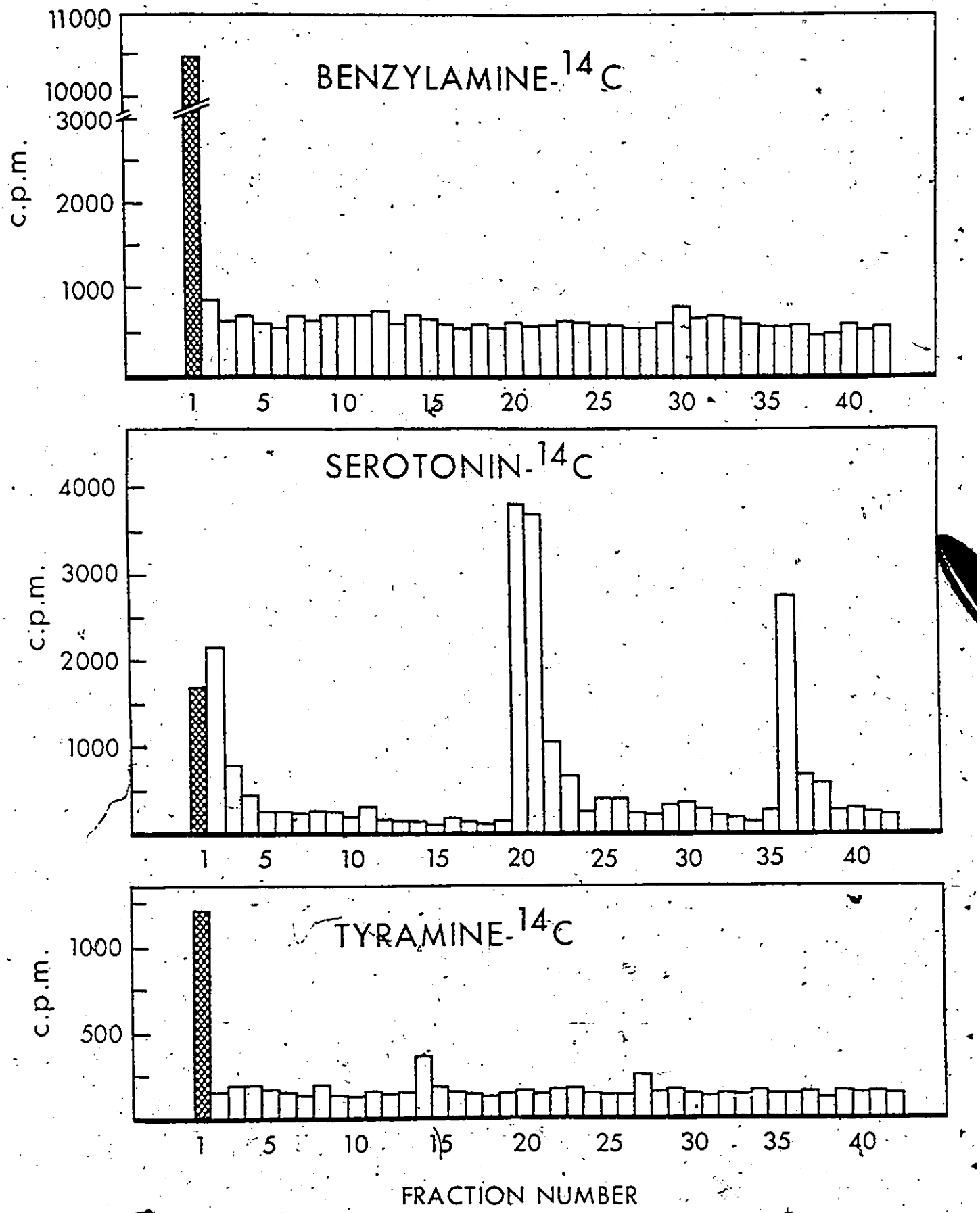

BENZYLAMINE-14C
SEROTONIN-14C
TYRAMINE-14C
c.p.m.
11000
10000
3000
2000
1000
4000
3000
2000
1000
1000
500
1
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure 13.

Polyacrylamide gel electrophoresis of rat liver mitochondria. The electrophoresis was carried out for 120 minutes from cathode to anode with a current of 2 mA per gel. MAO activity was assayed directly on the gel with benzylamine (top graph), serotonin (middle graph) and tyramine (bottom graph) as substrates. The activity was expressed as c.p.m. in an aliquot of 100 µl of organic solvent/fraction/hour, without blank subtraction, after extraction of the products of the MAO reaction with 200 µl of organic solvent. For more details see text.

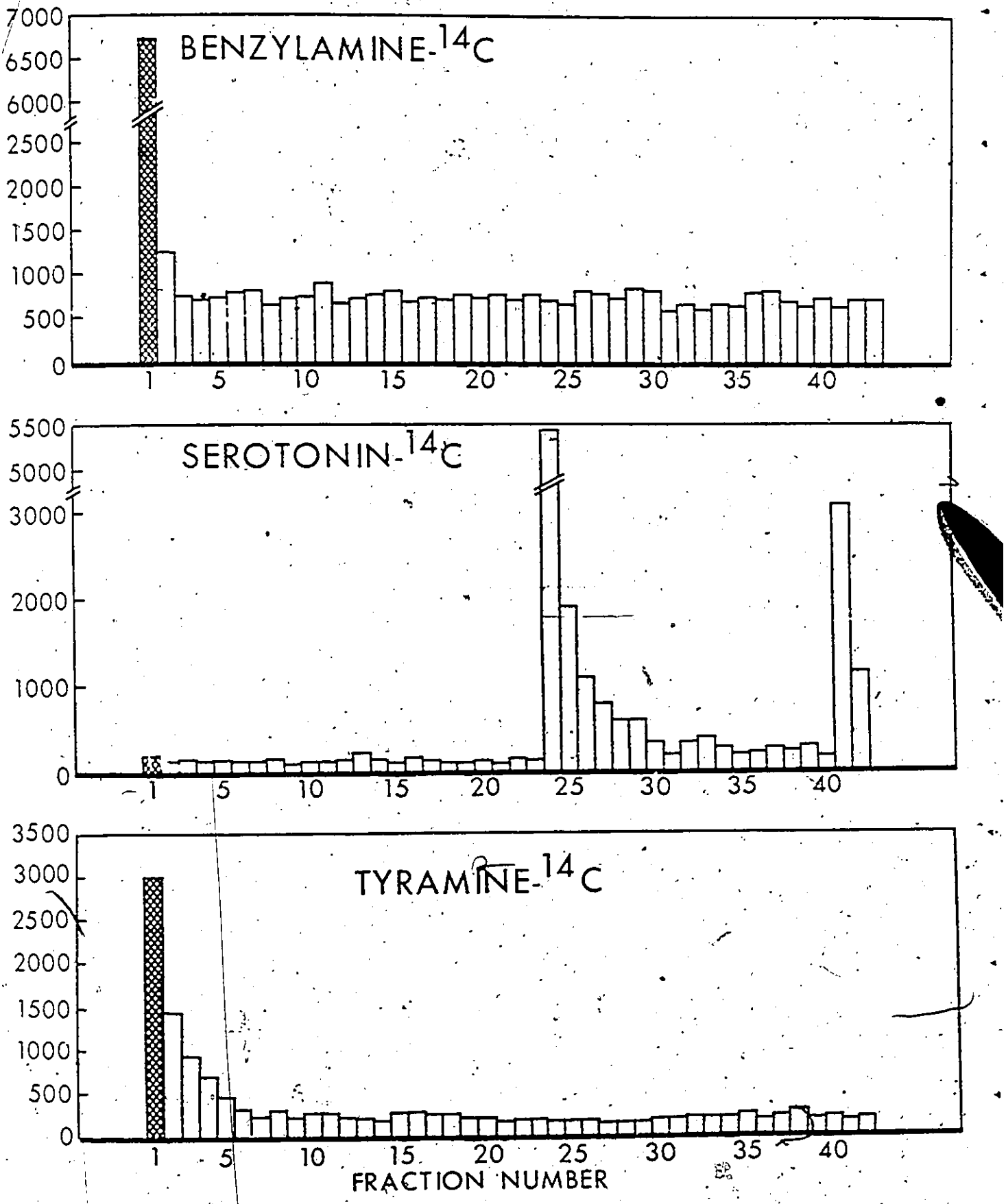

BENZYLAMINE-14C
7000
6500
6000
2500
2000
1500
1000
500
0
1
5
10
15
20
25
30
35
40
SEROTONIN-14C
5500
5000
3000
2000
1000
0
1
5
10
15
20
25
30
35
40
TYRAMINE-14C
3500
3000
2500
2000
1500
1000
500
0
1
5
10
15
20
25
30
35
40
FRACTION NUMBER

Figure 14.

Polyacrylamide gel electrophoresis of rat liver mitochondria. The electrophoresis was carried out for 120 minutes from cathode to anode with a current of 3 mA per gel. MAO activity was assayed directly on the gel with benzylamine (top graph), serotonin (middle graph) and tyramine (bottom graph) as substrates. The activity was expressed as c.p.m. in an aliquot of 100 µl of organic solvent/fraction/hour, without blank subtraction, after extraction of the products of the MAO reaction with 200 µl of organic solvent. For more details see text.

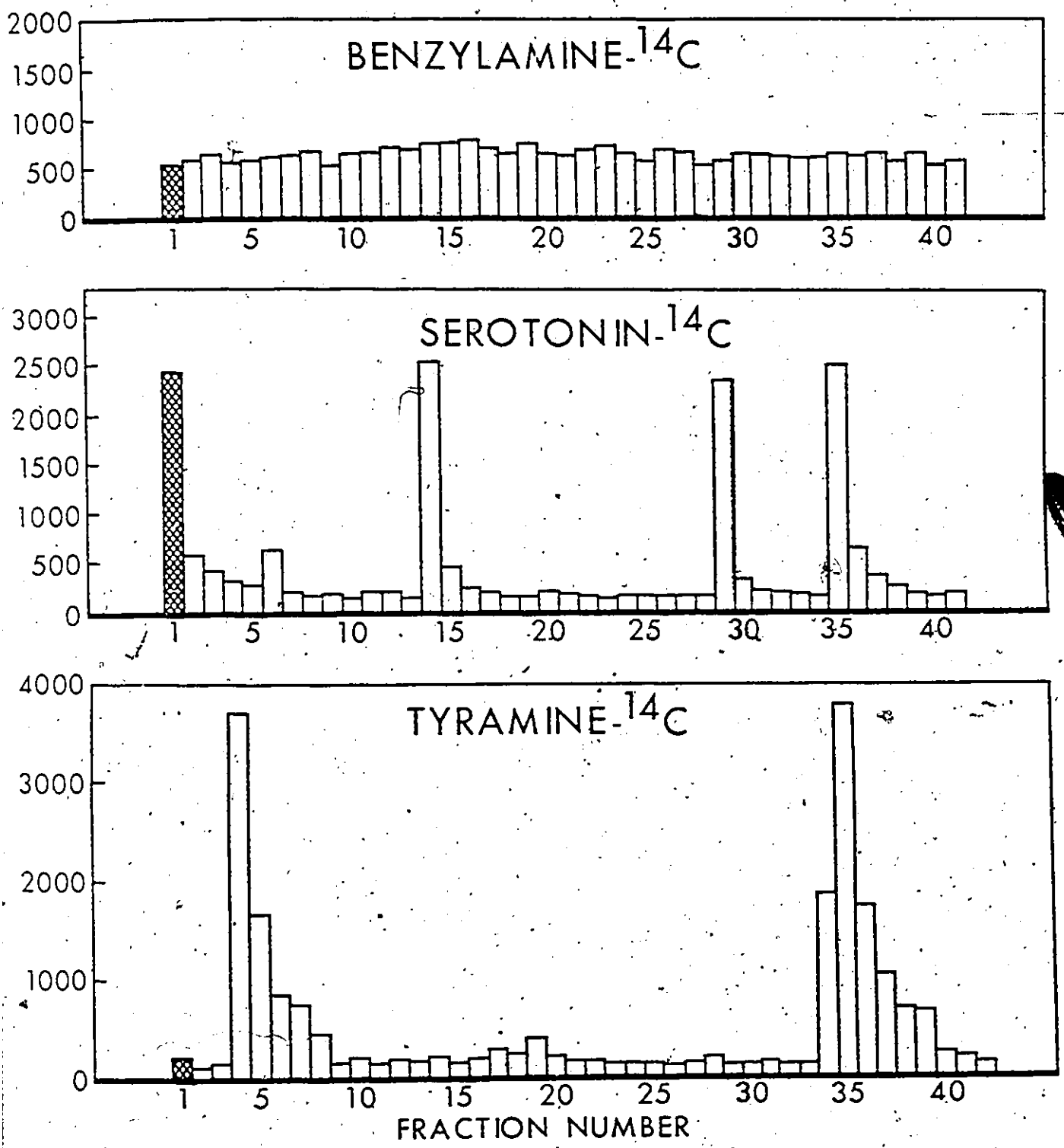

BENZYLAMINE-14C
SEROTONIN-14C
TYRAMINE-14C
FRACTION NUMBER

Figure 15.

Polyacrylamide gel electrophoresis of rat liver mitochondria. The electrophoresis was carried out for 75 minutes from anode to cathode with a current of 2 mA per gel. MAO activity was assayed directly on the gel with benzylamine (top graph), serotonin (middle graph) and tyramine (bottom graph) as substrates. The activity was expressed as c.p.m. in a aliquot of 100 µl of organic solvent/fraction/hour, without blank subtraction, after extraction of the products of the MAO reaction with 200 µl of organic solvent. For more details see text.

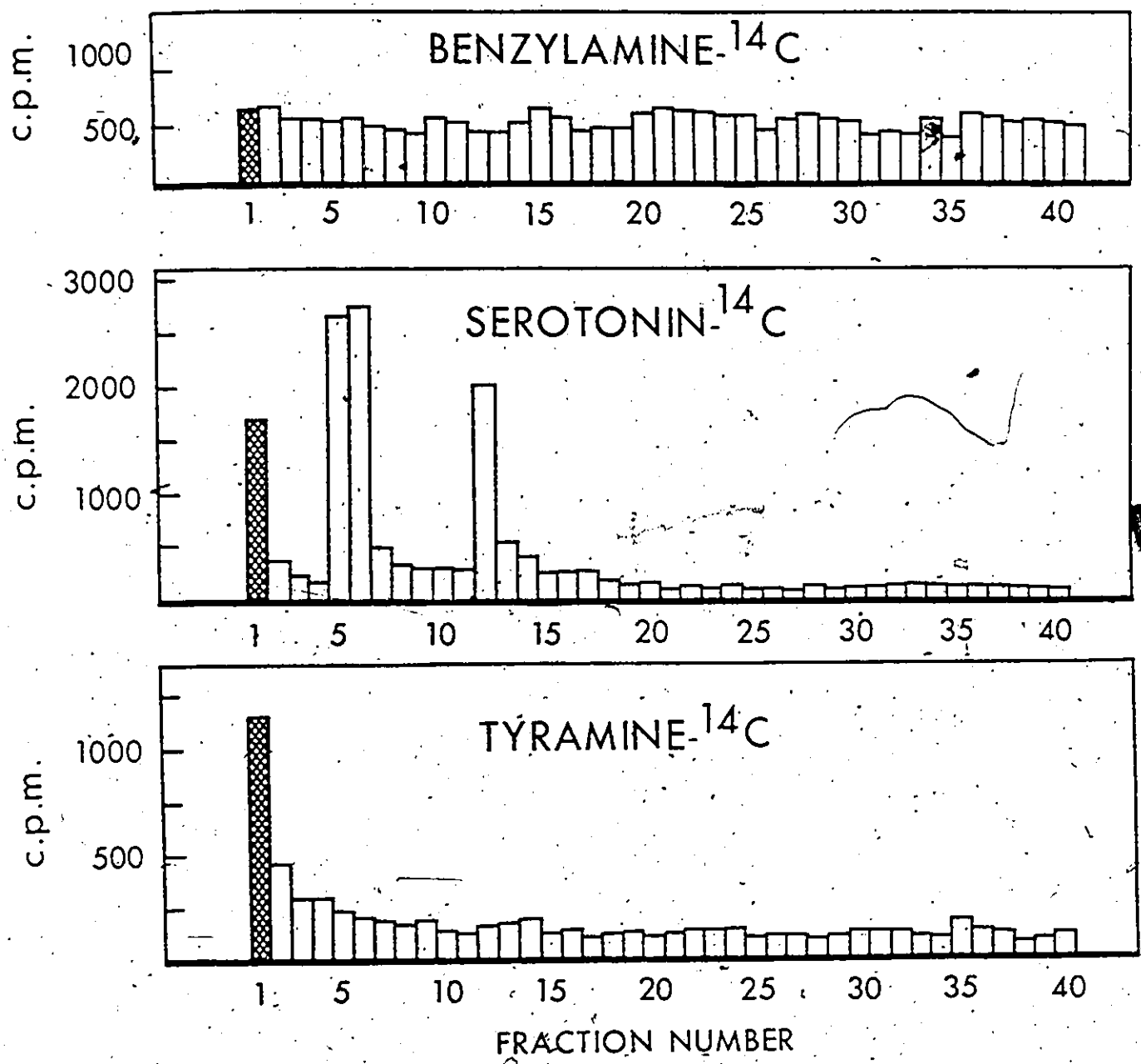

BENZYLAMINE-14C
c.p.m.
1000
500
1 5 10 15 20 25 30 35 40
SEROTONIN-14C
c.p.m.
3000
2000
1000
1 5 10 15 20 25 30 35 40
TYRAMINE-14C
c.p.m.
1000
500
1 5 10 15 20 25 30 35 40
FRACTION NUMBER

Figure 16.

Polyacrylamide gel electrophoresis of rat liver mitochondria. The electrophoresis was carried out for 120 minutes from anode to cathode with a current of 3 mA per gel. MAO activity was assayed directly on the gel with benzylamine (top graph), serotonin (middle graph) and tyramine (bottom graph) as substrates. The activity was expressed as c.p.m. in aliquot of 100 µl organic solvent/fraction/hour without blank subtraction, after extraction of the products of the MAO reaction with 200 µl of organic solvent. For more details see text.

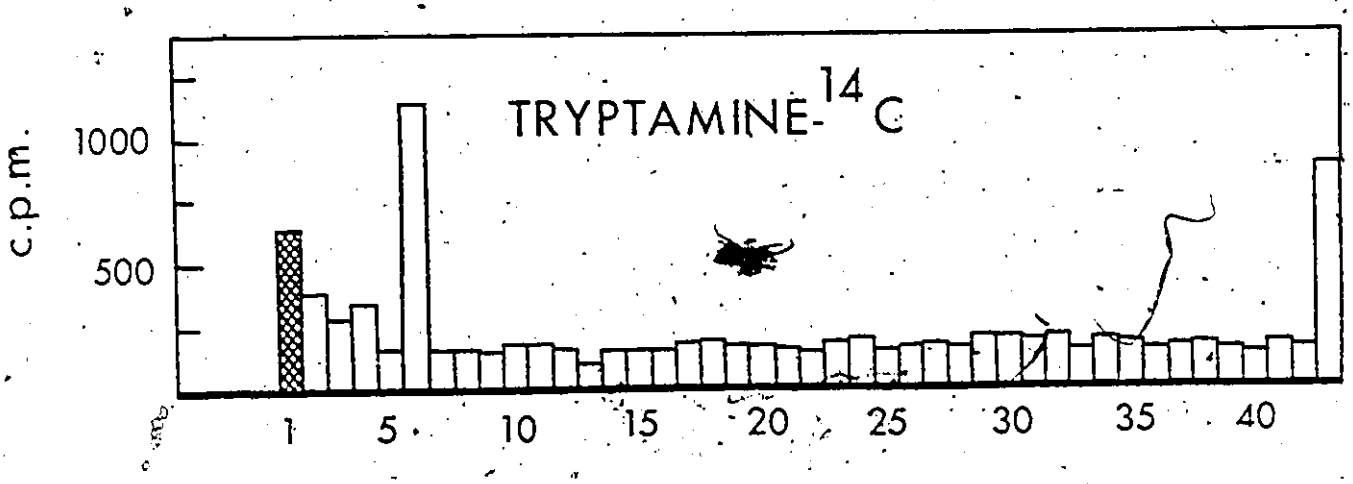

TRYPTAMINE-14C
c.p.m.
1000
500
1
5
10
15
20
25
30
35
40

Figure 17.

Polyacrylamide gel electrophoresis of rat liver mitochondria. The electrophoresis was carried out for 75 minutes from cathode to anode with a current of 2 mA per gel. MAO activity was assayed directly on the gel with tryptamine as substrate. The activity was expressed as c.p.m. in an aliquot of 100 µl of organic solvent/fraction/hour, without blank subtraction, after extraction of the products of the MAO reaction with 200 µl of toluene. For more details see text.

TABLE 4

Comparison of different solvents for the extraction of the products of the MAO reaction when tyramine is the substrate.

	Toluene		Ethyl acetate		Anisole		Ethyl ether	
	Blank	Sample	Blank	Sample	Blank	Sample	Blank	Sample
First extraction	244 (59)	9325 (54)	3105 (24)	27929 (72)	214 (44)	1359 (64)	441 (66)	20343 (90)
Four extractions	424 (100)	17287 (100)	12950 (100)	39009 (100)	486 (100)	21179 (100)	654 (100)	22677 (100)
Four extractions with blank subtracted		16862		26058		20692		22024

MAO activity is expressed as d.p.m./30 min. Each value represents the average of three assays. The samples were assayed as described in the text. A crude aldehyde dehydrogenase preparation was included in the assay medium. The values in brackets represent the percentage of radioactivity as compared with the radioactivity after four extractions of the blank and sample, respectively. The variation between triplicate samples was not greater than 7%.

but it is less efficient in the first extraction. Though ethyl acetate (169) seems to be efficient the blanks are higher and thus diminishes the accuracy of the method. Toluene (176) is the least efficient of the three solvents. On the other hand with diethyl ether the majority of the products are extracted in the first extraction.

The effects of both solubilized mitochondria and crude aldehyde dehydrogenase preparation on the assay of MAO with benzylamine, serotonin, tyramine and tryptamine as substrate are illustrated in tables 5 and 6. The presence of either solubilized mitochondrial protein up to a concentration of 40 µg or aldehyde dehydrogenase does not seem to affect benzylamine activity (table 5). However, this is not the case when 40 µg of solubilized protein and aldehyde dehydrogenase are added together. On the other hand there seems to be a reduction in serotonin activity when the amount of solubilized mitochondria is increased in the assay medium, but this effect can be overcome by adding aldehyde dehydrogenase. Furthermore, in the range of zero to 20 µg of solubilized mitochondrial protein, aldehyde dehydrogenase not only abolishes the effects of the solubilized protein but also increases the extraction of products compared to the control mitochondria without these additions. Like for serotonin MAO activity, the

TABLE 5

Influence of aldehyde dehydrogenase and solubilized mitochondrial preparation, in which MAO has been inactivated by heat, on the assay of MAO with benzylamine and serotonin as substrates.

Substrate	Control	Solubilized mitochondria (µg protein)			Solubilized mitochondria (µg protein) + crude aldehyde dehydrogenase			
		10 µg	20 µg	40 µg	0 µg	10 µg	20 µg	40 µg
Benzylamine	7429 (100)	7425 (100)	7214 (97)	7236 (97)	7788 (105)	7340 (99)	7115 (96)	5497 (74)
Serotonin	17521 (100)	15964 (91)	12329 (70)	4626 (26)	20992 (120)	20775 (119)	21668 (124)	7884 (45)

MAO activity is expressed as d.p.m./30 min. Each value represents the average of four assays. The MAO active preparation used was rat liver mitochondria suspended in distilled water. The figures in brackets indicate percentages in relation to the MAO activity of the control which was the rat liver mitochondrial suspension without addition of solubilized mitochondria and/or crude aldehyde dehydrogenase. The same amount of active MAO preparation was used for all the samples assayed with the same substrate. For more details see the text. The variation between quadruplicate samples was not greater than 7%.

TABLE 6

Influence of aldehyde dehydrogenase and solubilized mitochondria in which MAO has been inactivated by heat, on the assay of MAO with tyramine and tryptamine as substrates.

Substrate	Control	Solubilized mitochondria (µg protein)			Solubilized mitochondria (µg protein) + crude aldehyde dehydrogenase			
		10 µg	20 µg	40 µg	0 µg	10 µg	20 µg	40 µg
Tryptamine	9530 (100)	8711 (91)	6483 (68)	4031 (42)	9648 (96)	9164 (101)	8966 (94)	8386 (88)
Tyramine	12450 (100)	10546 (85)	8750 (70)	5553 (45)	12640 (98)	12199 (99)	12368 (102)	8632 (69)

MAO activity is expressed as d.p.m./30 min. The figures in brackets indicate percentages in relation to the MAO activity of the control. All the conditions of the experiments are similar to those described in table 5.

activities for tryptamine and tyramine (table 6) appear to be diminish with different amounts of solubilized mitochondria and aldehyde dehydrogenase restores the activity to the original values. With tryptamine, even in the presence of 40 µg of solubilized mitochondrial protein the recovery of the enzymic products is comparable to the control. The same is not the case with tyramine as the substrate. Solubilized mitochondria did not interfere with MAO assay the separation of the products from the substrate is obtained on an Amberlite CG-50 column (table 7). A comparison between the separation of radioactive products by the column procedure and by extraction with organic solvents is illustrated in table 8. It may be observed that more radioactivity is recovered with the use of Amberlite CG-50 column when tryptamine is the substrate but with benzylamine and tyramine the opposite is true. When serotonin is the substrate both methods appear to have similar efficiency. The blanks for all the four substrate are similar for the two methods.

Figures 18-23 show the effects of substrate concentration, enzyme concentration and different times of incubation on the activity of MAO for benzylamine (figs.18, 19), serotonin (fgs.20 and 21), tyramine (figs.22 and 23) and tryptamine (figs.24 and 25). It is possible to observe that the reaction is linear for all the substrates over a period of 30 minutes. In the case of tyramine and tryptamine

TABLE 7

Influence of solubilized mitochondrial preparation in which MAO has been inactivated by heat on MAO assay with Amberlite CG-50.

Substrate	Control	Solubilized mitochondria (40 µg of protein)
Tyramine	20812	19364
Tryptamine	13758	14367
Serotonin	27497	28986

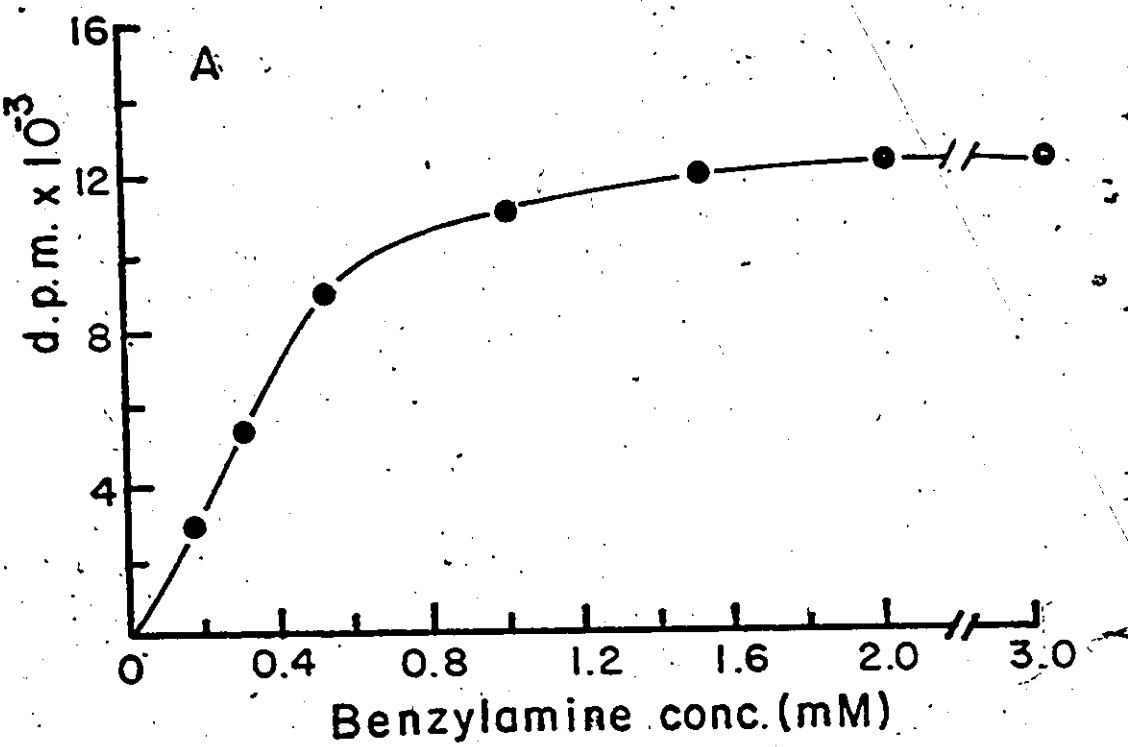
MAO activity is expressed as d.p.m./30 min. Each value represents the average of three samples. The MAO active preparation was rat liver mitochondria suspended in distilled water. For more details see text. The variation between triplicate samples was not greater than 7%.

TABLE 8

Comparison between the separation of radioactive products of MAO reaction by Amberlite CG-50 column and by organic solvents.

Substrate	Amberlite CG-50 column	Organic solvent
Benzylamine	17101 (100)	30565 (179)
Serotonin	111413 (100)	99287 (89)
Tyramine	66479 (100)	82554 (124)
Tryptamine	42187 (100)	30565 (72)

MAO activity is expressed as d.p.m./30 min. Each figure represents the average of four assays. The figures in brackets indicate percentages in relation to the activity of samples assayed with the same substrate by means of Amberlite CG-50 columns. When organic solvents were used for the extraction of the products four extractions were made and a crude aldehyde dehydrogenase preparation was included in the assay medium. For more details about the assays see text. The variation between quadruplicate samples was not greater than 7%.


A
d.p.m. x 10^{-3}
16
12
8
4
0
0.4
0.8
1.2
1.6
2.0
3.0
Benzylamine conc.(mM)

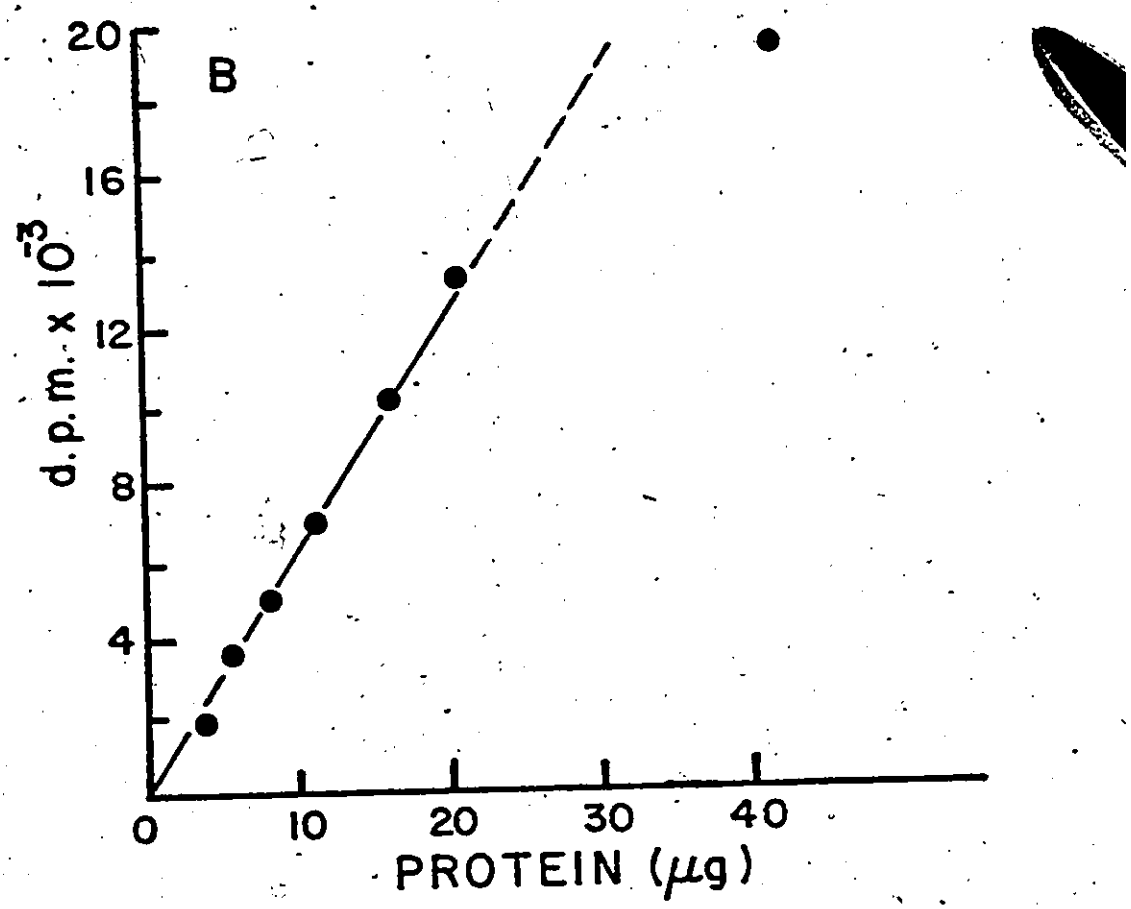

B
d.p.m. x 10^{-3}
20
16
12
8
4
0
10
20
30
40
PROTEIN (μg)

Figure 18.

A. Effect of benzylamine concentration on MAO activity. B. Effect of different concentrations of enzyme (µg of mitochondrial protein) on benzylamine MAO activity. The incubation was carried out at 37° for 30 minutes. The activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of toluene. The final concentration of substrate in the incubation medium in B was 1 mM. Each experimental point represents the average of three samples.

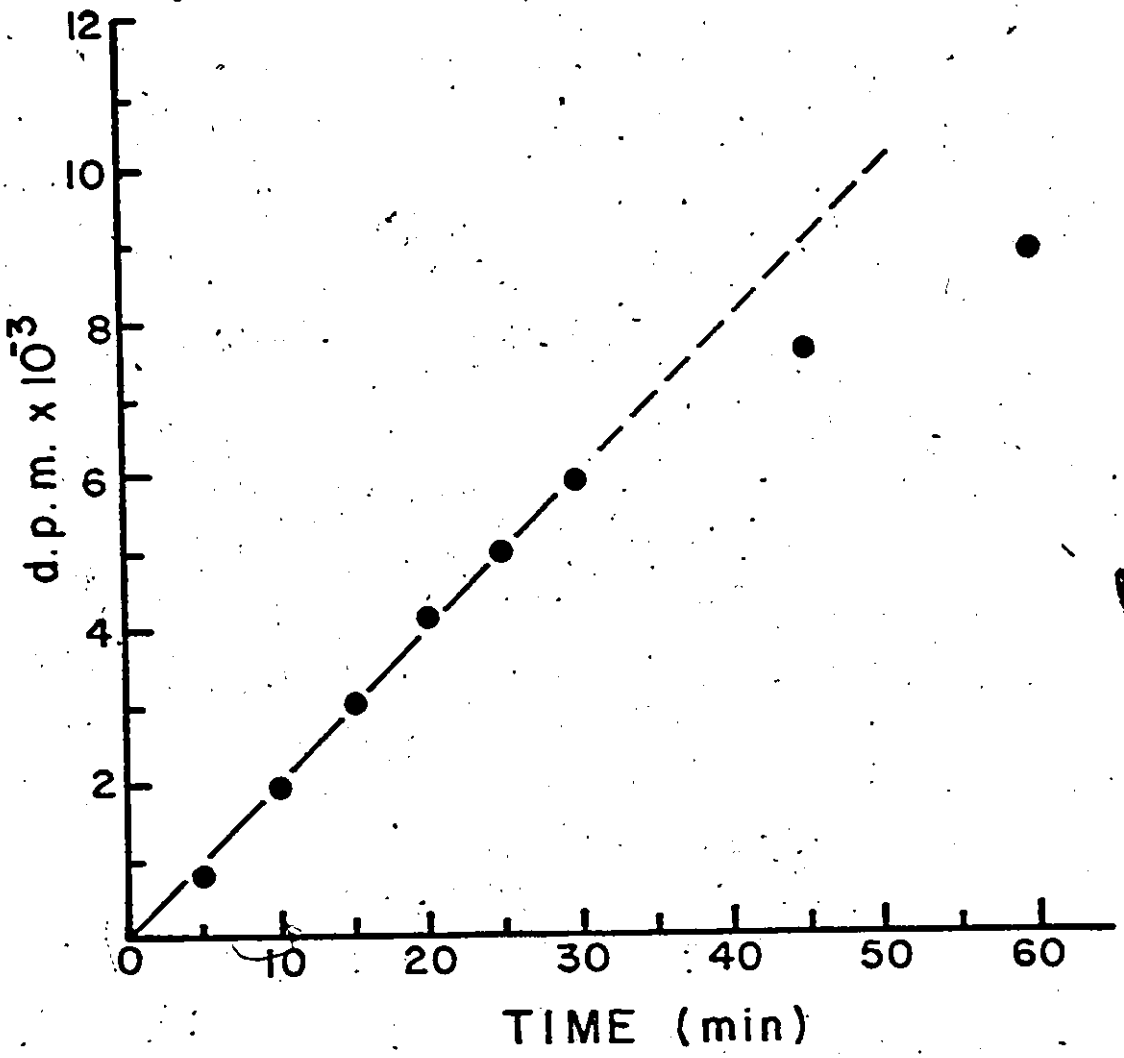
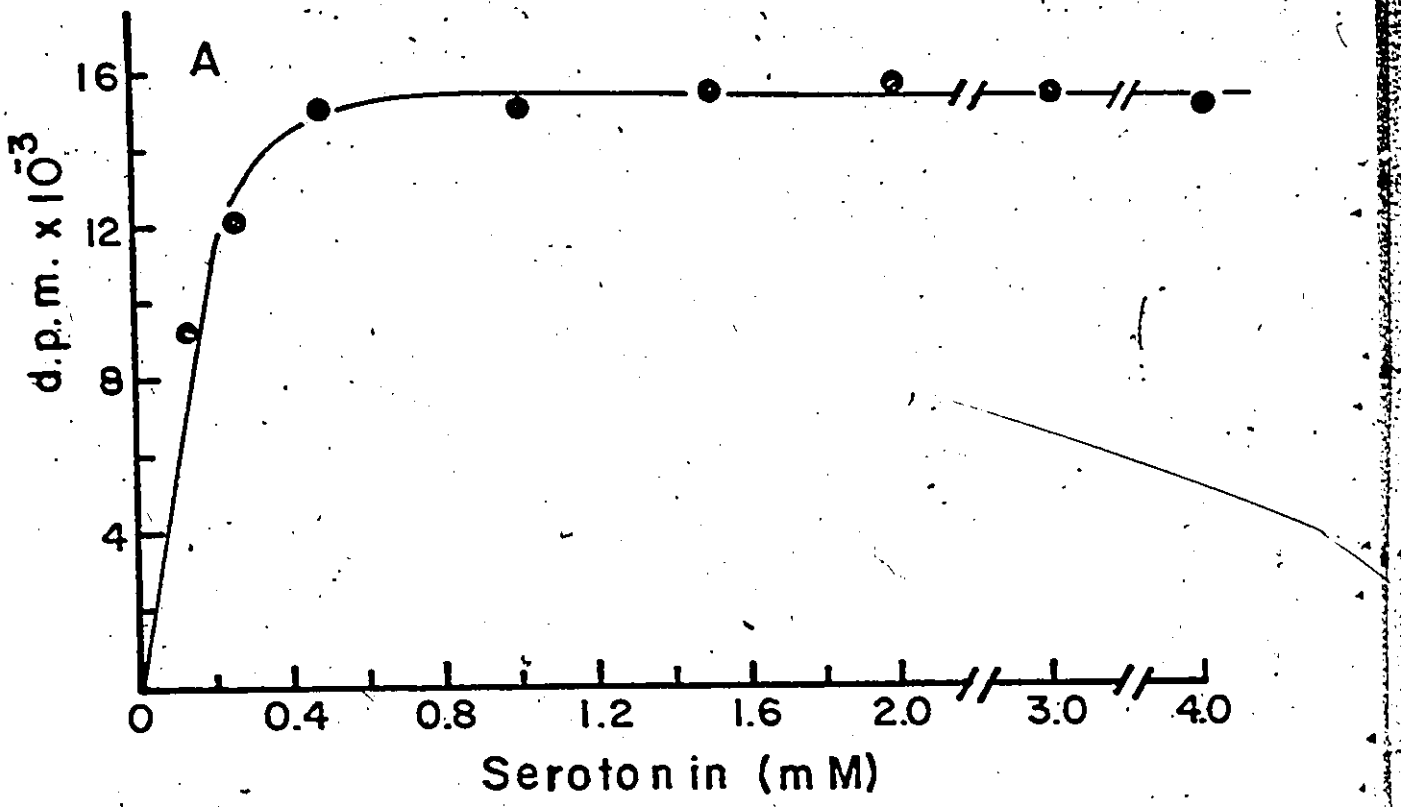

12
10
8
6
4
2
d.p.m. x 10^{-3}
0
10
20
30
40
50
60
TIME (min)

Figure 19.

Effect of different time of incubation on benzylamine MAO activity. The incubation was carried out at 37° with a final concentration of substrate of 1 mM. The activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of toluene. Each experimental point represents the average of three samples.


A
16
12
8
4
d.p.m. x 10⁻³
0
0.4
0.8
1.2
1.6
2.0
3.0
4.0
Serotonin (mM)

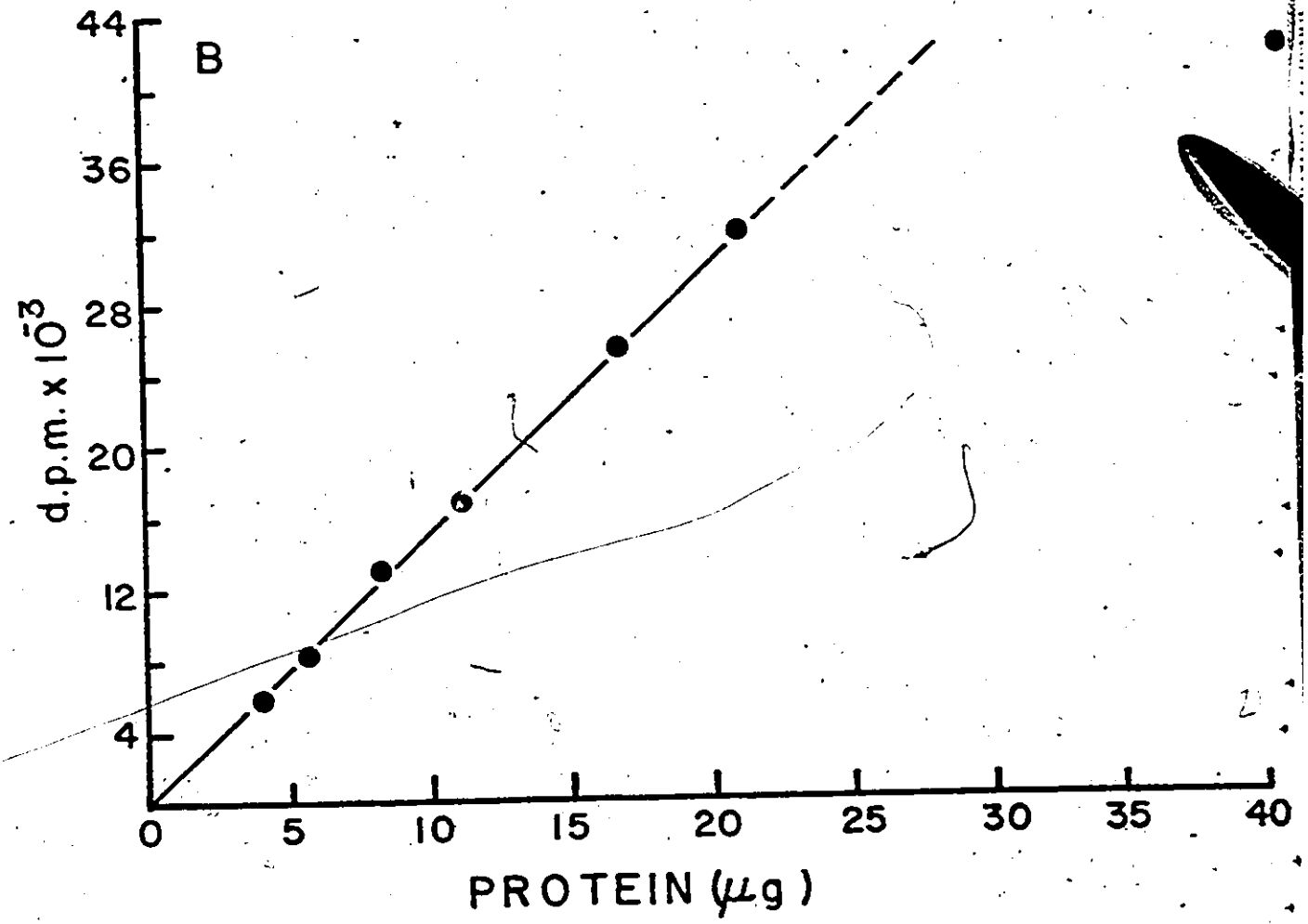

B
44
36
28
20
12
4
d.p.m. x 10⁻³
0
5
10
15
20
25
30
35
40
PROTEIN (μg)

Figure 20.

A. Effect of serotonin concentration on MAO activity. B. Effect of different concentrations of enzyme (µg of mitochondrial protein) on serotonin MAO activity. The incubation was carried out in the presence of aldehyde dehydrogenase at 37° for 30 minutes. MAO activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of diethyl ether. The final concentration of substrate in the incubation medium in B was 1 mM. Each experimental point represents the average of three samples.

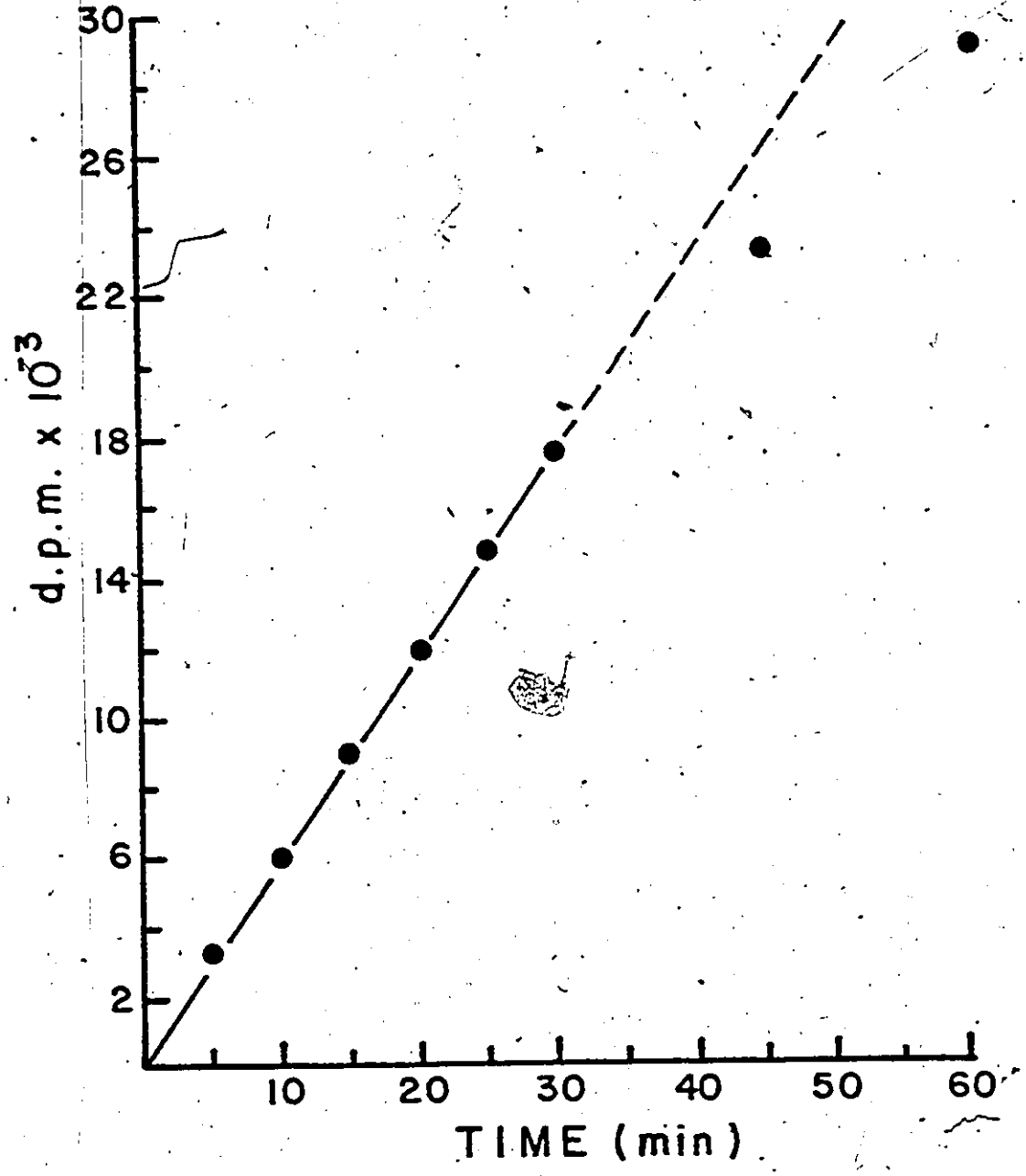

30
26
22
18
14
10
6
2
d.p.m. x 10^{-3}
10
20
30
40
50
60
TIME (min)

Figure 21.

Effect of different time of incubation on serotonin MAO activity. The incubation was carried out in the presence of aldehyde dehydrogenase at 37° with a final concentration of substrate of 1mM. The activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of diethyl ether. Each experimental point represents the average of three samples.

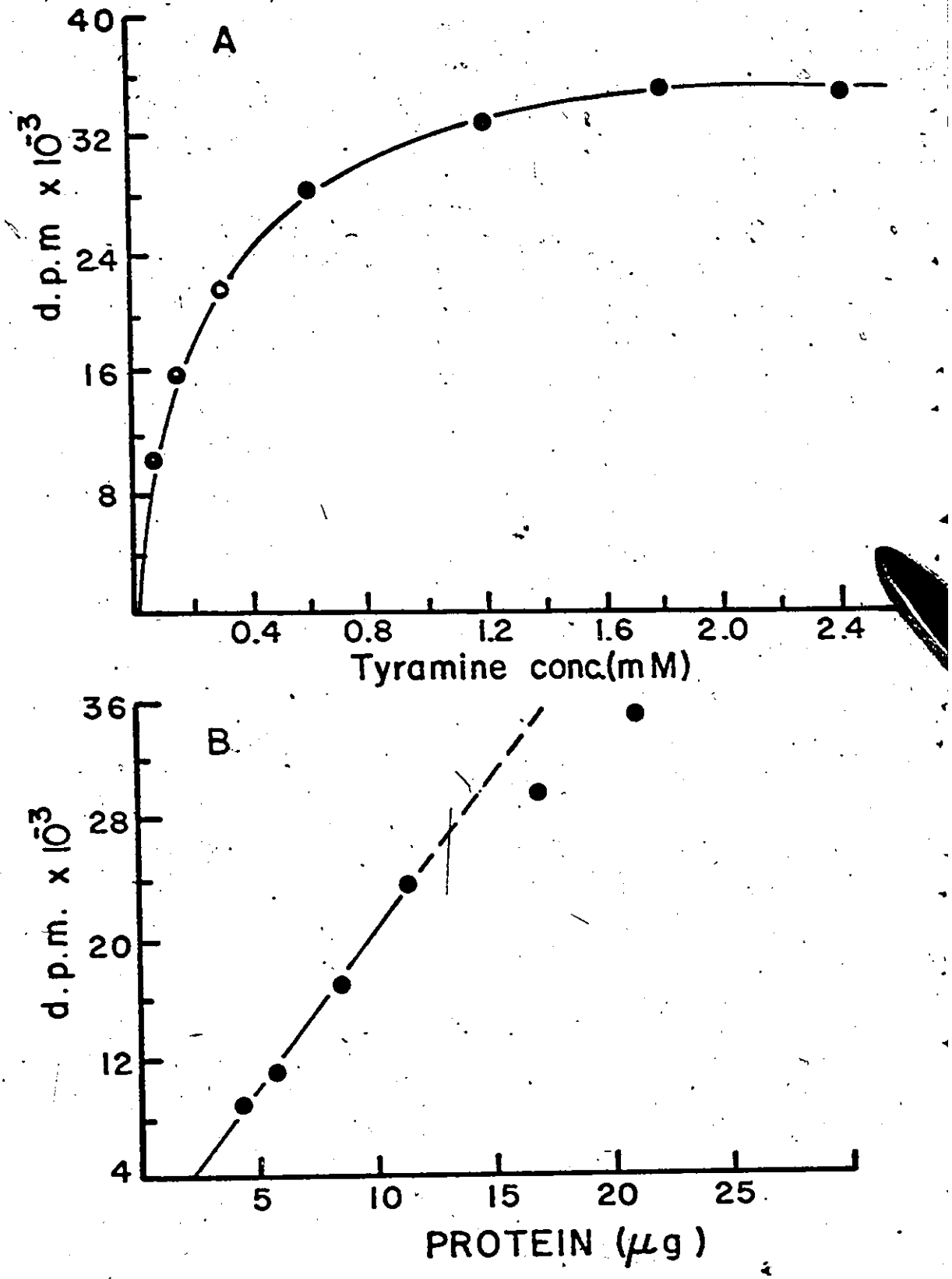

A
d.p.m x 10^{-3}
40
32
24
16
8
0.4
0.8
1.2
1.6
2.0
2.4
Tyramine conc.(mM)
B
d.p.m. x 10^{-3}
36
28
20
12
4
5
10
15
20
25
PROTEIN (μg)

Figure 22.

Effect of tyramine concentration on MAO activity. B. Effect of different concentrations of enzyme (µg of mitochondrial protein) on tyramine MAO activity. The incubation was carried out in the presence of aldehyde dehydrogenase at 37° for 30 minutes. The activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of diethyl ether. The final concentration of substrate in the incubation medium in B was 1.8 mM. Each experimental point represents the average of three samples.

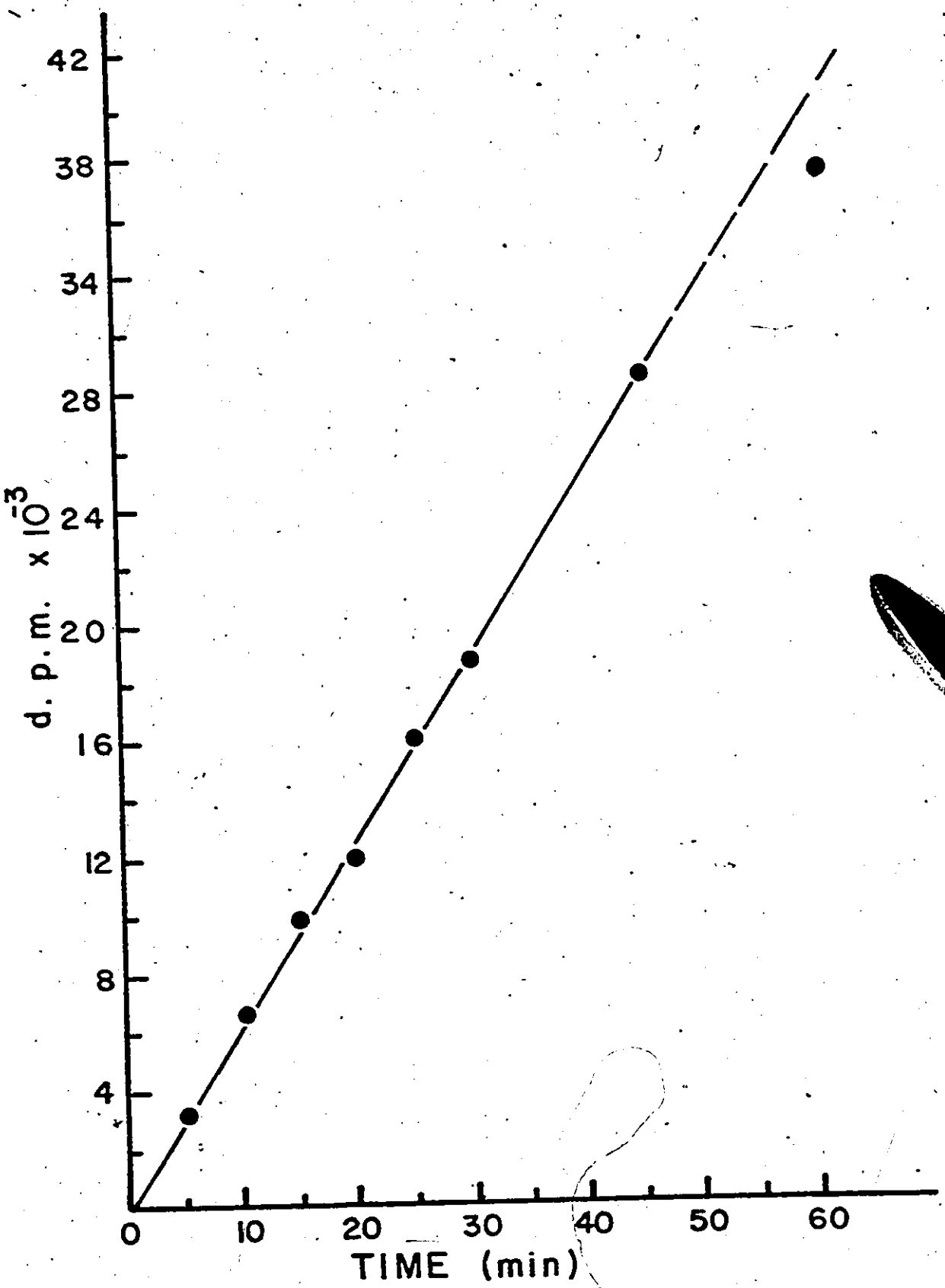
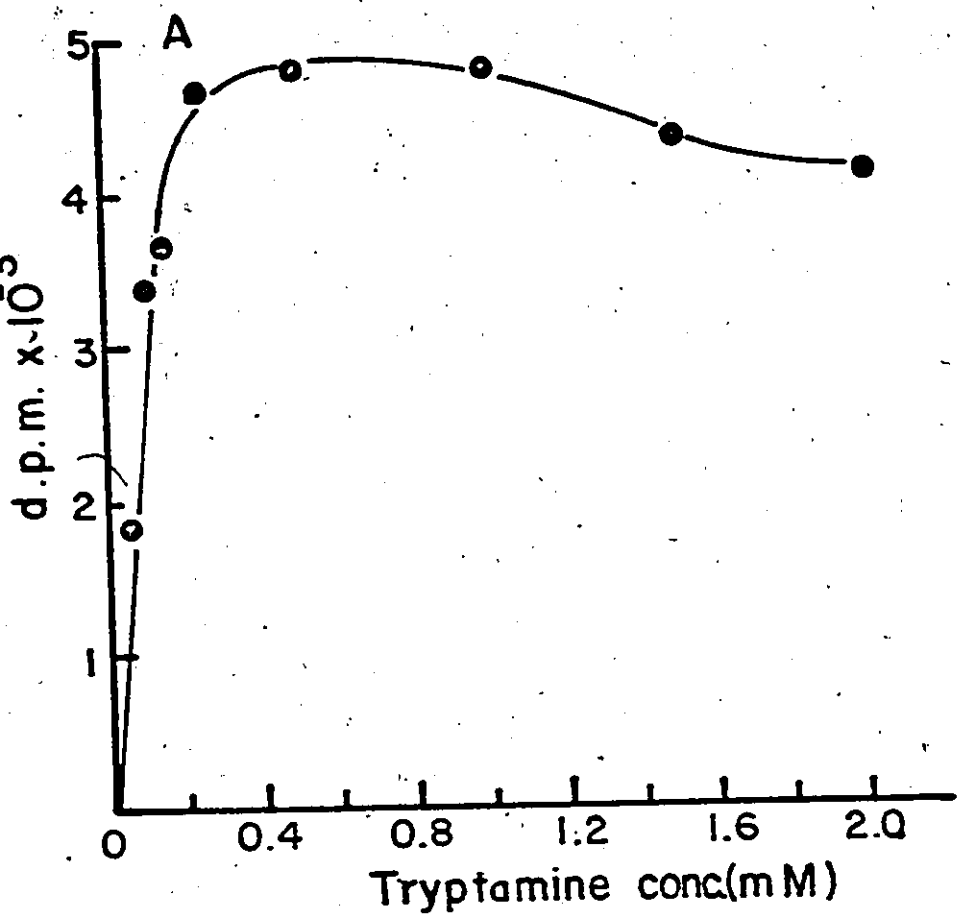

42
38
34
28
24
20
16
12
8
4
d. p. m. x 10^{-3}
0
10
20
30
40
50
60
TIME (min)

Figure 23.

Effect of different time of incubation on tyramine MAO activity. The incubation was carried out in the presence of aldehyde dehydrogenase at 37° with a final concentration of substrate of 1.8 mM. The activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of diethyl ether. Each experimental point represents the average of three samples.


A
d.p.m. x 10⁻³
5
4
3
2
1
0
0.4
0.8
1.2
1.6
2.0
Tryptamine conc.(m M)

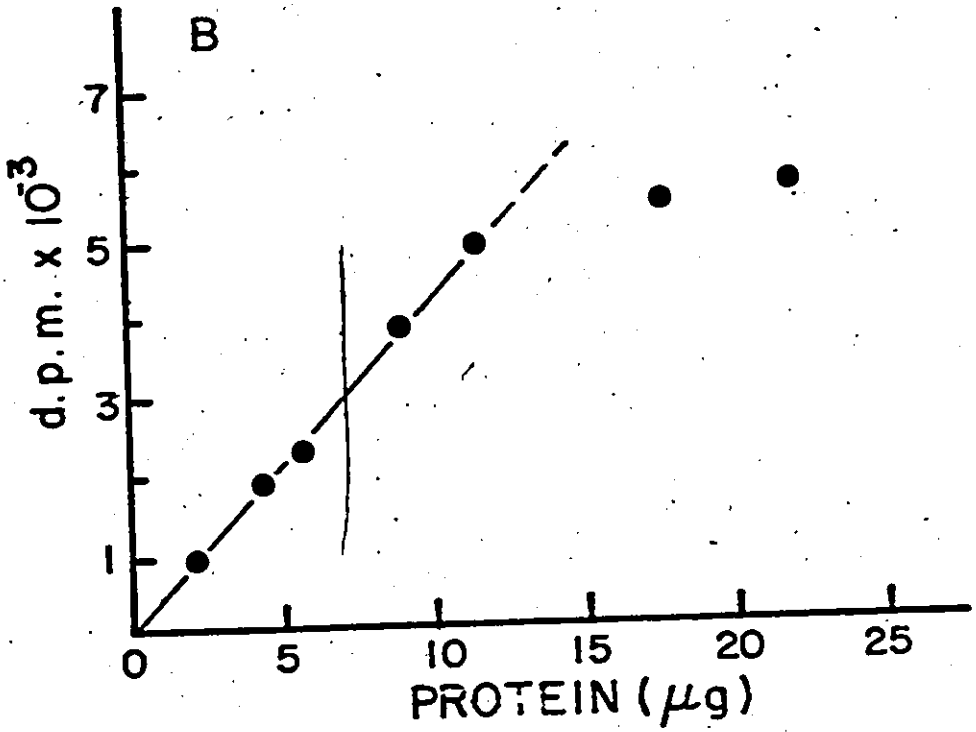

B
d.p.m. x 10⁻³
7
5
3
1
0
5
10
15
20
25
PROTEIN (μg)

Figure 24.

A. Effect of tryptamine concentration on MAO activity. B. Effect of different concentrations of enzyme (µg of mitochondrial protein) on tryptamine MAO activity. The incubation was carried out in the presence of aldehyde dehydrogenase at 37° for 30 minutes. The activity is expressed as d.p.m. after three extractions of the radioactive products with 200 µl of toluene. The final concentration of substrate in the incubation medium in B was 0.5 mM. Each experimental point represents the average of three samples.

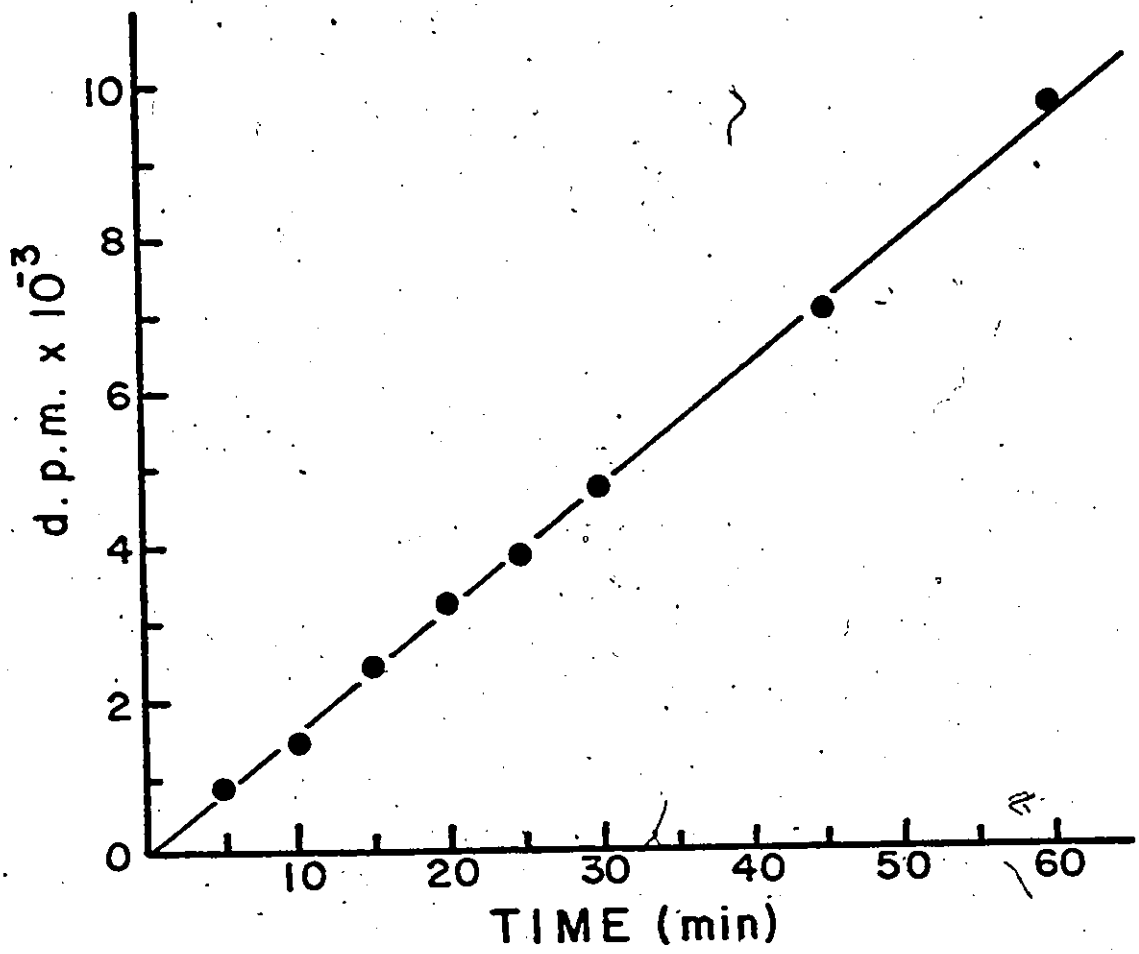

d.p.m. x 10^{-3}
0
2
4
6
8
10
10
20
30
40
50
60
TIME (min)

Figure 25.

Effect of different time of incubation on tryptamine MAO activity. The incubation was carried out in the presence of aldehyde dehydrogenase at 37° with a final concentration of substrate of 0.5 mM. The activity is expressed as d.p.m. after three extractions with 200 µl of toluene. Each experimental point represents the average of three samples.


ty
t
s
b

Figure 26.

Detection of MAO activity after polyacrylamide gel electrophoresis by the method of Glenner et al.(290) as described by Youdim et al.(388). Rat liver solubilized mitochondria were subjected to polyacrylamide gel electrophoresis under conditions identical to those described in Figure 12. Benzylamine (b), serotonin (s), tryptamine (t) and tyramine (ty) were used as substrates.

the reaction was linear up to 45 and 60 minuts, respectively. Care was taken to see that the period of incubation and the amount of enzyme did not exceed the requirements for eliciting a linear response (figs. 18B, 19, 20B, 21, 22B, 23, 24B, 25). The exceptions to this were the experiments reported in table 8 where the concentration of enzyme was high and the others where the incubation of 1 hour was used in order to ascertain the detection of MAO activity in the polyacrylamide gels. In the first case the same conditions were adequate in order to get the same amount of products for comparing the efficiency of the methods for separating the products from the substrates. In the second case the method of assay was used only to localize the enzyme activities on the gels.

The recovery of products in the first extraction in relation to that obtained with three extractions was the following: benzylamine 75%, serotonin 76%, tyramine 89% and tryptamine 71%. The variation between duplicate samples was not greater than 7%.

Figure 26 shows gels stained with tetrazolium salt for the localization of MAO activity after 75 minutes of electrophoresis run from cathode to anode with a current of 2 mA per gel. A broad band was observed when serotonin, tyramine and tryptamine were used as substrates. This band was localized at the end of the

acrylamide gel and at the beginning of the acrylamide-agarose gel. It was not present when benzylamine was the substrate or when the preparation was incubated for 15 minutes with 10^{-4}M isocarboxazid (a gift of Hoffman-La Roche Limited, Montreal) prior to the electrophoresis. A positive reaction with similar properties was observed in the top of the acrylamide gel. A narrow purple band between the acrylamide gel and acrylamide-agarose gel and a weak positive reaction in the top of the acrylamide gel were noticed when the current was increased to 3 mA and the electrophoresis conducted for 2 hours. If the current was reversed and the electrophoresis lasted for 75 minutes (2 mA per gel) or 2 hours (3 mA per gel), it was not possible to detect any band with similar properties to those described above. The staining of the gels using serotonin as substrate showed a dark purple background whereas the other three substrates gave yellowish backgrounds with a light purple colour. This staining of the gels appeared darker or lighter in some zones than the surrounding areas giving the appearance of other bands. These bands were visible even if the sample was pre-incubated with isocarboxazid.

The effects of solubilization with Lubrol and sonication, as described in methods of this section, on MAO activity for tyramine and tryptamine are illustrated in table 9. The recoveries of both activities are similar though these

TABLE 9

Effect of Lubrol W and sonication on tyramine and tryptamine MAO activities in rat liver mitochondria.

	Tyramine	Tryptamine
Lubrol W (12.5%)	92.4 (76.4 102.7)	89.5 (68.3 104.7)
Lubrol W (12.5%) and sonication	72.8 (41.1 104.6)	73.8 (52.9 95.9)

The values are expressed as the percentage of MAO activity of the original mitochondria suspended in distilled water. MAO activity was measured by oxygen consumption. Benzylamine and serotonin to a final concentration of 3 mM were added to the samples with Lubrol. The samples were dialyzed before the MAO assay for 36 hours. All procedures before the assay were carried out at pH 8.8 and 4°. The sample was treated as indicated for the solubilization of the samples in methods of this section. Each value is the mean of 5 experiments for tyramine and tryptamine, respectively. Values in brackets represent the range.

are lower than the recoveries obtained with benzylamine, serotonin or kynuramine (table 3). Preliminary experiments showed that the change in the strength of the Tris-HCl buffer from 0.01 M to 0.1 M is without effect on the recovery after solubilization of benzylamine and serotonin MAO activities. The values of the activity for catalase were 891.2 ± 124.8 (S.E.) Sigma units/mg protein and 540.5 ± 69.7 (S.E) Sigma units/mg protein for mitochondrial and solubilized samples, respectively.

DISCUSSION

The preelectrophoresis of the gels, mentioned elsewhere, produced a system of two gels which at the beginning of the electrophoresis had conditions different to those described by Ornstein (424) and Davis (425). The buffer in both the acrylamide (spacer) and the acrylamide-agarose (separation) gel would be Tris-glycine instead of the original Tris-HCl buffer (424). The pH in the acrylamide gel would be about 8.3 while in the acrylamide-agarose gel it would be 9.5. The buffer in the acrylamide gel would have a lower ionic strength than that of the acrylamide-agarose gel. Since there is not a leading ion, chloride, and a trailing ion, glycine, the system would tend to lose some of the stacking effect described by Ornstein. At the same time other factors could contribute to sharper starting zones. Among these factors could be mentioned the difference in the concentration of acrylamide in both gels (424, 437), the presence of agarose in the acrylamide-agarose gel (426) and differences in ionic strength and pH (424). On the other hand the use of the acrylamide gel would prevent clogging of the surface of the acrylamide-agarose gel (438). Since a complex preparation was used there might be complexing and precipitation as the protein concentrates on the surface of the gel. In fact it is possible to observe some clogging (acrylamide gel) in the gels shown in figure 26. Some

experiments were carried out where the same sample was subjected to electrophoresis on gels prepared as described by Sierens and D'Iorio (272, 281) and on gels prepared by the procedure described above. At the end of the electrophoresis the gels were stained with Coomassie brilliant blue R250 (439) following the procedure described in ORTEC application note AN32 (452) for the localization of proteins. In the gels subjected to preelectrophoresis the bands were somewhat less sharp and wider. But this broadening of the band might not have any effect on the MAO localization due to the size of the fractions used for the MAO assay. Perhaps, in view of the tendency of the enzyme to aggregate (219, 233, 243, 245, 262) and the crudity of the preparation, the high concentration of the sample obtained in the procedure of Ornstein (424) and Davis (425) would not be desirable. Enzymes containing sulphydryl groups and subjected to polyacrylamide gel electrophoresis have been found to be affected by ammonium persulfate (428-430) while nonsulphydryl proteases are not affected (430). MAO is considered a sulphydryl enzyme in which the sulphydryl groups are essential for activity (chapter VI, this thesis). It is possible that in our experiments ammonium persulfate, when used as a catalyst, could have contributed to the inactivation of the enzyme activity.

The assay of MAO with radioactive substrates is a

specific method with a relatively high sensitivity that permits the localization of traces of enzyme activity. But the detection of the enzyme activity on the gels was only qualitative. Therefore, the improvement in the recovery of the products with the use of aldehyde dehydrogenase could not be critical but the presence of this enzyme in the assay medium contributed to the uniformity of the assay in the different gel fractions.

The preliminary studies carried out with the assay of MAO, using radioactive substrates, were made with the purpose of standardizing this method for further experiments where the different MAOs could be purified. From this data it is clear that the recovery of the products of the oxidative deamination of benzylamine by extraction into toluene was better than obtained with chromatography on Amberlite CG-50. The extraction with toluene is more effective and at the same time it is not affected by the presence of solubilized protein in the sample. With the other substrates the method of extraction is more difficult to select though a method can be more efficient than another in extracting the products of a particular substrate. The recovery of radioactive products with organic solvents is affected by solubilized mitochondria. In the case of serotonin, this recovery is also apparently affected by the presence of mitochondria suspended in distilled water since

aldehyde dehydrogenase can improve the recovery of products even in the absence of solubilized mitochondria. Though the presence of up to 40 µg of solubilized protein in the incubation medium had no effect on the assay of MAO activity with Amberlite CG-50, this procedure is less sensitive and more complicated as compared to the extraction by means of organic solvents. With the organic solvent all of the incubation medium is subjected to extraction and 50% of the total products extracted are measured in the liquid scintillation counter. On the other hand when the Amberlite CG-50 was used only 2/3 of the incubation medium was applied to the column and only 25% of the total elution volume was subjected to radioactive counting. Furthermore the efficiency of the radioactive counting with the Bray solution (about 67.5%) was lower than the counting efficiency found with the solution of Omnifluor in toluene (about 84%). Some of these factors can be modified in order to increase the sensitivity of the method. It is necessary to see that the changes do not complicate the procedure, and at the same time they must be evaluated since it seems that the amount of protein interferes with the method (177, 347).

The extraction of the products of tryptamine oxidation into toluene has been found to be adequate when the source of MAO is not a solubilized preparation (341, 345) while the solubilized preparation produces inconsistent

results (347). When assay mixtures which contained homogenized tissues were incubated in the presence of aldehyde dehydrogenase, the number of radioactive products extracted in toluene was unchanged, notwithstanding that the ratio between aldehyde and acid was changed. This is similar to the results reported in table 6 where aldehyde dehydrogenase was without effect on the MAO assay when tryptamine was the substrate and lysed mitochondria the source of the enzyme. On the other hand Southgate and Collins (347) obtained erratic results when they added aldehyde dehydrogenase to the incubation mixture containing solubilized enzyme. These authors did not mention the presence of appropriate cofactors in the incubation mixture. Jain et al.(346) recently reported a study on the assay of MAO with radioactive substrates using rabbit and liver mitochondria. For the assay with Amberlite CG-50 they terminated the reaction with HCl before applying the sample to the column. This has the advantage that the reaction can be stopped instantaneously but at the same time, as suggested by Southgate and Collins, the denatured protein could increase the non-specific binding of the radioactive metabolites.

Jain et al.(346) found toluene suitable for extracting tryptamine oxidation products and diethyl ether for the oxidation products of all the other substrates (tyramine, dopamine, serotonin and norepinephrine) which is

in accordancewith the experimental data shown in this section. They also suggested the use of aldehyde dehydrogenase although was pointed out that in some situations this may be undesirable.

Ungar et al. (434) found a substantial amount of the products of biogenic amines formed by the action of MAO to be bound to tissue. They suggested that the data derived by means of the radioisotopic assay might be used for comparative purposes, with similar enzyme preparations and for the same substrate only because the phenomenon of binding would lead to underestimation of enzyme activity and errors in determination of kinetic constants. Their data also indicated that the presence of a free (unsubstituted) hydroxyl as a constituent of the aromatic ring would be important in the orientation and firm attachment of the aldehydes to tissues. The absence of any influence of the solubilized mitochondria on the MAO assay with benzylamine could be explained on the basis of the above idea.

The results with polyacrylamide gel electrophoresis show that when the electrophoresis is run from cathode to anode benzylamine activity remains in the acrylamide gel and can be separated from serotonin activity which penetrates the acrylamide-agarose gel. Tyramine activity cannot be separated from benzylamine activity excepting one peak

illustrated in figure 12 which penetrates the acrylamide-agarose gel. This peak has not been found to coincide with the peaks of serotonin MAO activity. Tryptamine activity under the electrophoretic conditions described in figure 12 seems to migrate similar to serotonin activity. If the current is reversed benzylamine activity could not be detected while the other activities are found along the gels in several bands. Undoubtedly the migrations described are dependent upon the conditions of the electrophoresis and under different conditions results may be different . For example in some experiments in which the gels were prepared with ammonium persulfate and without preelectrophoresis, the solubilized mitochondrial sample was subjected to electrophoresis under the same conditions as described in figure 12. It was possible to find benzylamine activity in the acrylamide gel and at the end of the acrylamide-agarose gel while serotonin activity was localized in the middle of the acrylamide-agarose gel. When the electrophoresis was run under the same conditions but with the current reversed, benzylamine activity could be detected in the acrylamide-agarose gel. As mentioned elsewhere these results were not reproducible and the MAO activities were either low or were not observed.

The individual patterns of migration of the activity for each one of the substrates obtained in our experiments could be explained on the basis of either incomplete

solubilization of the corresponding enzyme (section I, this thesis) or polymerization of the same basic subunit or association with some kind of subparticles, rather than on the basis of the existence of isoenzymes (160, 289, 397, 420, 422). Furthermore, as mentioned above, the pattern of migration in these experiments depends on the electrophoretic conditions. Moreover the use of crude preparations could give rise to artifacts. More important than the separation of different bands of the activity for the same substrate is the complete independence of electrophoretic migration between the different enzymatic activities. The present results constitute strong evidence for the existence of completely different MAO enzymes and are consistent with those described in section II, using a different procedure. The obvious differences in the electrophoretic migration between the distinct enzyme activities might not be imputed to artifacts for several reasons. The solubilization treatment did not alter in a selective way the activities for the substrates studied. Serotonin activity is more labile than benzylamine activity and there are fractions where this activity can be isolated devoid of benzylamine activity. Furthermore the difference in migration of the different activities using different methods of separation is in accordance with the reports of other authors who have used different methodology (269, 383, 386, 390).

The results reported here also seem to indicate the existence of an overlapping substrate specificity for the deamination of tyramine between the enzyme that deaminates benzylamine and that which deaminates serotonin. The only exception apparently is the band with tyramine activity illustrated in figure 12 which is localized in the middle of the acrylamide-agarose gel where the other activities cannot be detected. This overlapping in the substrate specificity for tyramine is in agreement with the results obtained with the inhibitor clorgyline by several authors (115, 116, 375-378, 440). Tryptamine seems to be deaminated by the same enzyme that deaminates serotonin but this substrate was studied only under one specific electrophoretic condition (fig. 17) and further studies would have been necessary.

After the first electrophoretic separation of MAO by Kim and D'Iorio (271, 384) and Youdim and Sandler (287) the localization of MAO in polyacrylamide gels by staining with tetrazolium salts has been a standard technique (76, 160, 161, 282, 284, 289, 359, 385, 387-389, 392, 393, 395, 400) although in some reports MAO have been localized by other methods (272, 281, 391, 421-423). A series of factors seems to complicate the interpretation of the results when tetrazolium salts are used. Lagnado and Sourkes (228, 441) and Sourkes and Lagnado (442) have described the requirement for a heat-stable supernatant factor in the

reduction of tetrazolium salts by rat brain and liver suspensions. This factor can be replaced by purine, hypoxanthine and related compounds. Weissbach et al.(364) observed a lag phase when they used tetrazolium salts for the assay of MAO in vitro and this lag phase was not present when the enzyme was assayed with the manometric method. A lag phase was also observed by Lagnado and Sourkes who used other tetrazolium salts (228). Brooke and Engel (443) warned against the use of phenazine methosulfate with nitroblue tetrazolium in the same system because of the risk of non-enzymatic reduction of the tetrazolium salt. Falkenbergs et al.(444) suggested that a non-specific staining observed by different authors in gel electrophoresis (starch, polyacrylamide, cellulose acetate), after staining with tetrazolium salts, and called "nothing dehydrogenase", could be due to the presence of lactic dehydrogenase isoenzymes in the sample and groups similar to lactate in the supporting medium. It has been considered that the reduction of the tetrazolium salt may depend on the aldehyde of the MAO reaction (290, 445). Whether the aldehyde reduces the tetrazolium to formazan directly or through some other enzyme e.g. aldehyde dehydrogenase has been discussed (363, 364). Since indol-3-acetaldehyde reduces certain tetrazolium salts directly while aldehyde such as benzaldehyde and acetaldehyde do not (445), Glenner et al.(445) and Boadle and Bloom (363) agree that in all probability the indolacetaldehyde produced by the

oxidative deamination of tryptamine does indeed reduce the tetrazolium directly. According to Boadle and Bloom (363), for the localization of MAO with tetrazolium salts it is necessary to rest on the assumption that the aldehyde does not diffuse far from the reaction site before encountering a molecule of tetrazolium.

In view of the wide use of tetrazolium salts for the localization of MAO in studies on its multiplicity and the facts mentioned above, it was found desirable to compare the findings obtained with the use of a specific method for the localization of MAO to those obtained by the use of tetrazolium staining. For this staining four substrates, benzylamine, tyramine, serotonin and tryptamine were used. Benzylamine has been reported to be unsuitable as substrate (358, 363, 445) since benzylamine did not reduce tetrazolium. This is in accordance with the negative results obtained in our experiments with this substrate. On the other hand reports exist where MAO could be localized with this substrate (271, 359,384, 385, 393) but phenazine methosulfate and cyanide were present in the incubation medium. This, according to the report of Brooke and Engel (443), would invalidate these results. Tyramine has been reported as a poor substrate for the tetrazolium reduction while serotonin and tryptamine are good substrates (290, 364). In our results with the use of tetrazolium

staining, tyramine, serotonin, and tryptamine seem to migrate identically. The bands observed with this method did not correspond to those detected using radiometric techniques. Therefore the reaction with tetrazolium salts in our observed bands appears unspecific for MAO activity and extremely unreliable. While the tetrazolium staining is inhibited by isocarboxazid a wellknown MAO inhibitor, this does not necessarily mean that the staining is specific for the MAO reaction.

Youdim, Collins, Sandler, and collaborators (161, 287-289, 387-389, 392, 400, 160) have reported the separation of MAO activity in several tetrazolium staining bands after polyacrylamide gel electrophoresis of a partially purified MAO preparation. The enzyme was eluted and studied. These experiments, according to the authors, constituted a direct confirmation of the existence of multiple MAOs and designated them as isoenzymes.

In 1971, Youdim and Collins (387) reported the dissociation and reassociation of rat liver mitochondrial MAO after treatment of the purified preparation with urea and sodium dodecyl sulfate. The denaturing treatment produced a single protein band devoid of enzymatic activity after polyacrylamide gel electrophoresis. If the enzyme preparation was dialysed prior to the electrophoresis the

separation pattern was identical to the non-denatured protein. When the individual bands of activity were exposed to the same denaturing treatment and dialysis, the electrophoretic pattern of each band was similar to that of the purified preparation of mitochondrial MAO with one exception. The molecular weight of the purified MAO was 150,000 and 300,000, respectively if determined by ultracentrifugation or gel filtration. The molecular weight of the different electrophoretic bands of MAO activity fractions determined by gel filtration was 300,000. Treatment of either a partially purified MAO preparation or of the various electrophoretic bands with a denaturing agent (such as urea) produced a lowering of the measured molecular weight (approximately 78,000). The molecular weight was, however, determined only in four of the five bands of activity. The above observations could be an indication of the similarity of the different bands. The authors suggested that the multiple MAO forms are conformational enzymes. Later Lagnado et al.(446) and Youdim and Lagnado (447) reported that sites of MAO enzyme activities detected on polyacrylamide gels by the tetrazolium method do not necessarily coincide in all cases with bands of MAO activity measured by radioassay. They observed this phenomenon when staining the gels using tyramine and tryptamine as substrates and assaying the zones of enzyme activity with the same substrates by radiometric techniques.

They concluded that "it is evident that the use of tetrazolium salts for the detection of multiple forms of MAO in electrophoretic experiments or in histological studies can lead to an erroneous assessment of MAO activity depending on the nature of the substrates tested, and on other yet unidentified factors which appear to control reduction of these salts". They also observed an inhibition of MAO activity by 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl-2H-tetrazolium chloride. On this basis Sandler and Youdim (160) criticized the results of other authors (385, 393, 395) who used tetrazolium salts to localize MAO activity.

Tipton et al.(284) reported the similarity between 24-fold purified MAO from beef adrenal medulla and a purified MAO from rat liver (278). When the beef adrenal medulla preparation was submitted to electrophoresis the bands of activity were similar to those described for rat liver mitochondria (388). They found different amounts of phospholipids in each band, but the gradation of phospholipid content does not parallel the electrophoretic mobility. A single band which moved towards the cathode on polyacrylamide gel electrophoresis was found to have a relatively high specific activity with dopamine as the substrate and to differ from the remainder of the enzyme in being relatively insensitive to inhibition by 2-phenylethyl-hydrazine, iproniazid and isocarboxazid. No separable bands

of enzyme activity could be obtained when the electrophoresis of rat liver MAO was carried out in the presence of Triton X-100 (76). This according to Tipton would support the contention that the multiple forms may represent a single species to which different amounts of membrane material are attached as proposed by Veryovkina et al.(275). Recently, Houslay and Tipton (420) using rat liver mitochondria found an indication that one of the bands reported by Youdim et al.(388, 392) and Youdim (288) which remained in the origin was an artifact due to the method used to apply the sample. When a crude preparation of rat liver mitochondrial outer membranes or a partly purified rat liver MAO after treatment with sodium perchlorate were subjected to polyacrylamide gel electrophoresis only one band was observed. This would suggest that the presence of electrophoretically separable bands of MAO activity in the partly purified preparation of MAO could be an artifact of the purification procedure.

Guha et al.(448, 449) and Sen et al.(450) have suggested the presence of a monoamine tetrazolium reductase system distinct from MAO on the basis of different properties such as sensitivity to inhibitors, substrate specificity, influence of temperature, influence of anaerobiosis and pH activity curves. The presence of this system would give rise to more questions about the specificity of tetrazolium salts for the localization of MAO. Therefore in summary

the reports on the electrophoretic separation of several MAO with the use of crude preparations and tetrazolium salts (271, 359, 385, 393, 395) are unreliable. In view of the findings of Houslay and Tipton electrophoretic separation using purified preparation (76, 160, 161, 282, 284, 387-389, 392, 400) would still give rise to dubious results even if tetrazolium staining was specific.

Recently, D'Iorio et al.(423) and Kandaswami et al.(451) have obtained preliminary results which confirm the results shown in section I and II. D'Iorio et al.(423) subjected rat liver mitochondrial preparation to several chromatographic steps and obtained three fractions with different substrate specificities. The procedure was as follows: the high speed supernatant of a solubilized preparation was treated with DEAE-cellulose (diethylaminoethyl-cellulose). This solubilized preparation was prepared as described in section II in presence of dithiothreitol and ethylenediamine-tetracetate. These two substances, which were also present in all the solutions used, increased the recovery of the activities for the substrates studied and in particular the serotonin activity. After centrifugation the DEAE-cellulose was discarded and the supernatant was passed through a column of Sephadex G-200. The MAO activity was eluted immediately after the void volume (M.W. = 450,000). No significant separation of the MAO

activities for the different substrates was obtained in these steps. The fractions containing MAO activity were pooled, concentrated and dialyzed against 0.01 M Tris-HCl buffer, pH 7.4, treated with DEAE-cellulose and centrifuged. The supernatant was filtered (fraction I). The resin was washed with distilled water and the eluate was separated (fraction II). Further elution of the resin with 0.1 M NaCl in 0.01 M Tris-HCl buffer, pH 7.4, yielded another fraction (fraction III). These fractions were found to have differences in their substrate specificity as illustrated in table 10. Serotonin activity is predominantly in fraction II and III while benzylamine is predominantly in fraction I. Serotonin activity is scanty in fraction I. These results indicate the occurrence of at least two enzymes with varying substrate specificity. Unfortunately the recovery of the enzyme activities in the different fractions was low and inconsistent, notwithstanding that the substrate specificity of the fraction was always similar. By adopting another method a fraction which exhibits a high activity towards serotonin has been isolated by Kandaswami et al.(451). The procedure for the isolation of this enzymic fraction consisted of extraction of rat liver mitochondria with Triton X-100, followed by ammonium sulphate fractionation, treatment with alumina Cγ gel and molecular sieving on Sepharose 6B. In the presence of this fraction serotonin was preferentially oxidized whereas little activity towards benzylamine was

TABLE 10

MAO activity in different fractions of DEAE-cellulose*

	Benzylamine	Serotonin	Tyramine
Fraction I	100	7	52
Fraction II	204	394	148
Fraction III	87	251	105

*The values are expressed as the percentage of benzylamine MAO activity in fraction I. MAO was assayed by oxygen consumption (291) in presence of catalase (299). One-hundred was equivalent to 0.775 µatoms of oxygen per hour per mg protein. For more detail see text (p. 225).

detected. Other substrates like tyramine and tryptamine were also oxidized by the enzyme. This fraction appeared to be stable.

CONCLUSION

Studies on substrate specificity, kinetics, selective inhibition by MAO inhibitors or physical agents and distribution of the activity of MAO with various substrates under different experimental conditions suggested the presence of either more than one enzyme or multiple forms of one enzyme. On the other hand many of these data can be diversely interpreted. Many attempts have been made in order to separate these MAOs but many questions concerning methodology still have to be resolved before a precise identification of the enzymic fractions is obtained.

Although an evaluation of the degree of liberation of MAO, obtained with the technique described in this thesis, is made difficult by the presence of detergent, this technique of solubilization when applied to rat liver mitochondria compares favourably with the results of other authors. At the same time the substrate specificity of the original mitochondria was maintained, at least for the five substrates studied. Several factors such as the presence of detergent and substrates, alkaline pH and sonication after equilibration of the sample with H_2 was simultaneously used. Some of them have been found to facilitate the detachment of MAO while others prevent its inactivation.

When a solubilized rat liver mitochondrial preparation was subjected to sucrose gradient electrophoresis the pattern of distribution of the activity for serotonin was different from those of benzylamine and kynuramine. Serotonin activity was predominant in some fractions even though it was more labile than benzylamine and kynuramine activities. These results together with other reports found in literature strongly militate in favor of at least two different activities, one for serotonin and the other for benzylamine and kynuramine.

The results obtained with sucrose gradient electrophoresis were further confirmed by the use of polyacrylamide electrophoresis. The enzyme activity was detected on the gels by means of radiometric methods instead of tetrazolium staining, which has been shown to be unreliable and unspecific. For the assay of MAO with radioactive substrates the use of aldehyde dehydrogenase and appropriate cofactors in the assay medium is recommended when tyramine, serotonin or tryptamine are used as substrates. In the presence of aldehyde dehydrogenase there was an increase in the products, extracted but this was not found when benzylamine was the substrate. By means of polyacrylamide gel electrophoresis, the activities for serotonin, tryptamine and tyramine can be separated in several bands which migrate to either cathode or anode, while benzylamine activity was

detected as only one band which could be isolated devoid of the other enzyme activities. As with sucrose gradient electrophoresis the separation in multiple bands of the activity for the same substrate could be due to artifacts rather than to MAO multiplicity. However, the separation of the activity for one substrate from the other could not be ascribed to such factors. Though benzylamine and serotonin appear to be deaminated by different enzymes, the possibility of an overlapping substrate specificity for tyramine and benzylamine activity and also for tyramine and serotonin activity is not discarded.

SUMMARY

A crude mitochondrial preparation was treated with a nonionic detergent and subjected to sonication in order to liberate MAO from the mitochondrial membranes. With this treatment the substrate specificity of the original mitochondria was maintained unchanged for five substrates (benzylamine, serotonin, kynuramine, tyramine and tryptamine). After this treatment the mitochondrial preparation was submitted to electrophoresis on sucrose gradient and on polyacrylamide gel. With sucrose gradient electrophoresis it was possible to show that the pattern of migration of serotonin activity was different from those of benzylamine and kynuramine activity. Polyacrylamide gel electrophoresis confirmed these results. The MAO activity was localized directly on the gel with radioactive substrates (serotonin, benzylamine, tyramine and tryptamine). Serotonin and tyramine MAO activities separated in several bands which migrated either to the cathode or to the anode whereas benzylamine activity was localized in one band. The latter could be separated devoid of the activity for serotonin though not from the tyramine activity. Tryptamine activity was studied only under one particular electrophoretic condition in which it migrated in a similar way to that of serotonin activity. All these results indicate the occurrence of different enzymes for the oxidative deamination of benzylamine and serotonin. The possibility of

overlapping substrate specificity for tyramine activity with those of serotonin and benzylamine activities is not discarded. When tetrazolium salts were used to detect MAO in the gels the localization observed was different from that obtained with radioactive substrates. The use of tetrazolium staining for the detection of MAO was found to be unreliable and unspecific. When MAO is assayed by radioactive techniques with serotonin, tyramine and tryptamine as substrates, the use of aldehyde dehydrogenase and an appropriate cofactor is advisable because it gives rise to an increase in the products extracted, particularly when a solubilized MAO preparation is used.

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