SOME STUDIES ON THE PURIFICATION AND THE PROPERTIES OF MONOAMINE OXIDASE

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ABSTRACT

Attempts were made to purify rat liver mitochondrial MAO in a single step by affinity chromatography. Except for tryptamine and serotonin-Sepharose, which irreversibly bound the enzyme, most adsorbents bound little if any of the enzyme. This was true for both detergent (triton X-100 and digitonin) and non-detergent (sonication and perchlorate) soluble enzyme preparations. However, the non-bound enzyme that eluted from either organomecurial or DTNB-Sepharose was purified 4 fold. Treatment of the triton X-100 or sonicated soluble MAO preparation with phospholipase A resulted in the binding of the enzyme to the DTNB-Sepharose but no increase in specific activity was observed on elution. Binding also occurred with the AB-15 absorbent but the enzyme could not be eluted in an active form. Using the triton X-100 solubilized enzyme, ammonium sulfate fractionation, DTNB-Sepharose and DEAE-Sephadex, a 15 fold purification was obtained with a 30% recovery of enzyme activity in under 3 hours.

The rat liver mitochondrial MAO could be differentiated into an A and B form with the irreversible substrate selective inhibitors Clorgyline, Deprenil and pargyline. The Clorgyline derivative Lilly 51618 was found to be reversible and to have a greatly reduced selectivity. In addition propargylamine, an acetylenic irreversible MAO inhibitor that lacks an aromatic moiety also had a greatly reduced substrate selectivity. Clorgyline was also shown to perturb the mitochondrial membrane structure through hydrophobic interactions unrelated to the inhibition of MAO.

Ferrous ion induced lipid peroxidation in mitochondrial suspensions inactivates MAO possibly by the interaction of lipid peroxides with protein SH groups. The altered membrane environment from lipid peroxidation however, does not affect the heat stability or the Deprenil inhibition pattern of MAO A or MAO B.

Treatment of the membrane bound or soluble MAO with phospholipase C had no effect on the enzyme activity or on the heat stability, tryptic digestibility, Arrhenius plots or the Deprenil inhibition pattern of MAO A and MAO B. Treatment of the membrane bound enzyme with phospholipase A decreased both the MAO activity by 23% and the overall heat stability of the enzyme; however the tryptic digestibility, Arrhenius plots and Deprenil inhibition patterns were unchanged. After lipid depletion of the soluble enzyme with phospholipase A, a similar loss of activity was observed as with the membrane bound enzyme but this preparation was more sensitive to inactivation by heat and trypsin. However, the different rates of inactivation of MAO A and MAO B were still observed and the Arrhenius plots and Deprenil inhibition patterns were similar to those of the membrane bound enzyme.

By labelling MAO with [^{14}C]pargyline the inactivated enzyme could be assayed, 30% of the enzyme was found in the mitochondrial structural protein fraction. In addition, when mild sonication was used to resolve the mitochondrial membranes, the MAO protein was found still associated with the membranes. The use of proteolytic digestion and DTNB-Sepharose showed a similar accessability to the enzyme from either side of the membrane.

The results are discussed in terms of the role of the enzyme environment and the relationship of the enzyme protein to the outer mitochondrial membrane.

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CONTENTS

1. INTRODUCTION

1.1 Classification and Substrate Specificity of Monoamine Oxidase (MAO)

The enzyme monoamine oxidase [Monoamine:O_2oxidoreductase (deaminating), E.C.1.4.3.4: MAO] oxidatively deaminates a large number of amines according to the equation:-

$$RCH_2NH_2 + O_2 + H_2O = RCHO + NH_3 + H_2O_2$$

The enzyme was first reported by Hare (1928) and within a year Best (1929) described an enzyme which oxidatively deaminated histamine according to the same equation. The first attempt to classify these enzymes was by Zeller, Stern and Wenk (1940), who distinguished two groups, the monoamine oxidases and the diamine oxidases (histaminase) by their substrate specificities. However, common substrates for both types of enzymes made further classification necessary and this was realized by the use of inhibitors. MAO is resistant to cyanide, semicarbazide and other carbonyl reagents whereas the diamine oxidases are sensitive to them (Blaschko, Friedman, Hawes and Nilsson, 1959; Zeller et al., 1959). More recently, distinction between the two groups of enzymes has proved possible by means of prosthetic groups, MAO is a flavoprotein (see Section 1.6) and the diamine oxidases contain copper and pyridoxal phosphate (see Zeller, 1972).

The oxidation of 2-phenylethylamine was found to be insensitive to cyanide and was followed by the production of ammonia and hydrogen peroxide (Hare, 1928). Since similar observations were made for the oxidation of tyramine, Hare (1928) suggested only one enzyme was involved. Blaschko, Richter and Schlossman (1937a) found that an enzyme in guinea-pig liver oxidized adrenaline and Pugh and Quastel, (1937a) found an enzyme that oxidized aliphatic amines in the rat brain and liver.

Using the method of mixed substrates, Kohn (1937) suggested that one enzyme oxidizes tyramine, adrenaline, hordenine and probably isopropylamine in the pig liver. Blaschko, Richter and Schlossman (1937b) reached the same conclusion for tyramine, adrenaline and aliphatic oxidases from inhibition, distribution and mixed substrate experiments. Using the rat liver as enzyme source, Pugh and Quastel (1937b) also suggested that one enzyme oxidizes tyramine, β-phenylethylamine and indolethylamine. The mixed or substrate competition experiments were extended by Kobayashi and Schayer (1955) using rat liver mitochondria as the enzyme source and using tyramine, isoamylamine, 2-phenylethylamine and tryptamine as substrates. These workers concluded that oxidation occurred at the same catalytic centre.

There are a large number of compounds that MAO acts upon and this has been extensively reviewed (Blaschko, 1952; Kapeller-Adler, 1970). The enzyme will oxidize in order of decreasing rate primary, secondary and tertiary amines of the general formula.

$$R^1 - CH_2 - NR^2 - R^3$$

where R^2 and R^3 must be either hydrogen atoms or methyl groups. Some of the series of straight chain aliphatic amines represented by

$$(CH_2)_n - NH_2$$

are substrates for MAO (Kohn, 1937; Pugh and Quastel, 1937a; Alles and Heegard, 1943). With the exception of methylamine which is not oxidized, oxidation increases with increasing chain length to N = 5 or 6 methyl groups after which oxidation decreases with increasing chain length. Guinea-pig liver MAO has been shown to oxidize isoamylamine, n-butylamine and isobutylamine but not sec-butylamine (Bhagvat, Blaschko and Richter, 1939). Short chain diamines are not substrates for MAO,

however, the enzyme from rabbit liver will oxidize the diamines with 4 to 13 methyl groups after which the rate decreases (Blaschko and Duthie, 1945). MAO will not oxidize histamine but some species N-methylate histamine producing N-methylhistamine which is a substrate (see Tipton, 1973).

Naturally occurring substrates identified to date are derivatives of phenylethylamine and indolethylamines. The diphenolic derivatives of phenylethylamine (catecholamines) are good substrates (see Blaschko, 1952). These include dopamine, adrenaline and noradrenaline. The work of Axelrod (see Axelrod, 1959) on catechol-O-methyl transferase led to the recognition that MAO oxidized m-O-methyladrenaline and m-O-methylnoradrenaline. Octopamine and tyramine both occur naturally and are substrates for MAO (Hare, 1928; Blaschko, 1974). Tryptamine (indolethylamine) is found in animals and is oxidized by MAO (Weissbach, King, Sjoerdsma and Udenfriend, 1959) and with the discovery of 5-hydroxyindolacetic acid in urine (see Udenfriend, 1958), MAO was found to be the enzyme deaminating 5-hydroxytryptamine (serotonin).

Randall (1946) studied the rate of oxidation of phenylethylamine derivatives in the guinea-pig and cat liver. Primary amines were oxidized more rapidly than secondary amines followed by tertiary amines. 2-Hydroxy phenylethylamine was oxidized less rapidly than the 3 and 4 position isomers. 2-Methoxyphenylethylamine was oxidized more rapidly than the 3 and 4 position isomers and 2,3 dimethoxyphenylamine has the most rapid oxidation rate of all the dimethoxy derivatives. Amines attached directly to a benzene ring are not substrates for MAO (Blaschko et al., 1937b). Benzylamine however, is a good substitute for MAO and can be used for the direct determination of enzyme activity (Tabor, Tabor and Rosenthal, 1954). Kynuramine a

non-physiological substrate for MAO, is also used to measure MAO activity (Weissbach, Smith , Daly, Witkop and Udenfriend, 1960).

Oxygen is a required second substrate for the oxidation of all amines. Tipton (1972) has shown that the K_m value for oxygen is about 0.2mM and that as the concentration of oxygen is decreased, the K_m value for the amine also decreases. This will render the enzyme insensitive to large fluctuations of oxygen levels in the cell.

1.2 Physiological Role of MAO

At present the functional significance of MAO has not been fully elucidated. One widely thought possible function of the enzyme may be the destruction of toxic amines which are ingested. This may be one reason for the high enzyme levels observed in the stomach, intestine and liver. Further support for this theory is indicated by the "cheese effect", a hypertensive reaction in patients treated with MAO inhibitors who then ingest food with a high tyramine content (see Knoll and Magyar, 1972). This reaction is believed to be due to tyramine releasing noradrenaline from nerve endings.

In nonneural tissue such as the liver or kidney, MAO inactivates catecholamines either before or after O-methylation. Tipton (1972) has shown that the K_m value of rat liver MAO deaminating metanephrine is tenfold lower than the value for adrenaline, suggesting the enzyme is more suitable for oxidation of methylated amines. In presynaptic neuronal tissue, MAO may oxidize released transmitter amines after reuptake. Trendelenburg, Draskoczy and Graefe (1972) have shown the process of reuptake, the main mechanism for inactivation of catecholamines, depends on the presence of MAO in the nerve endings.

They showed that when MAO is inhibited, the ability of the nerve ending to take up amines is impaired when the storage vesicles are saturated and as the level of the intraneuronal amine rises. Weiner and Bjur (1972) suggested that MAO may play a role in regulating noradrenaline synthesis by preventing a rise in the unbound intraneuronal noradrenaline, which may be responsible for the end-product feedback inhibition of tyrosine hydroxylase. MAO is present in the postsynaptic site, although its role is not known. Hendley, Taylor and Snyder (1970) have shown in the rat brain an extra neuronal uptake system and MAO may inactivate amines after they are taken up.

MAO is the main catalyst of serotonin oxidation (see Blaschko, 1974 for review). Similar to catecholamines, serotonin is synthesized, stored and released from neurous and MAO may have a similar role as described above for regulating amine levels in the neuron. Circulating serotonin in the blood is taken up by the platelets which contain MAO (Collins and Sandler, 1971) and the enzyme regulates the serotonin blood level.

Amine levels have been implicated to play a part in depression states (see Pare, 1972). Monoamine oxidase inhibitors have been used in therapy of mental depression, particularly the non-endogenous (reactive) depression with good results. However, some clinically similar patients fail to respond to MAO inhibitor treatment but the reasons for this are not known. It is also not certain that inhibition of MAO is the mode of action when these drugs affect mental states. Pare (1972) has shown however, that all MAO inhibitors used had a similar pharmacological effect on their endogenous depressed patients and that there was a correlation between the onset of clinical improvement and maximum monoamine levels in the brain. This suggested both

that amine levels are related to depression and that MAO inhibitors exert their action through the inhibition of MAO.

In addition to inactivating amines MAO may form new biologically active compounds. It has been suggested by Fischer, Schulz and Oliver (1966) that thyrodial MAO may function to provide hydrogen peroxide for iodothyronine synthesis. Gorkin and Orekhovitch (1967) suggested that the metabolites produced by MAO may regulate cellular oxidation reactions. This theory has received support from Tabakoff, Groskopf, Anderson and Alivisatoz (1974) who showed that biogenic amines added to brain homogenates, stimulated oxidation of glucose by the pentose phosphate shunt. In addition they showed that the aldehydes produced from the amines by MAO also stimulated glucose oxidation and that this was dependent on the oxidation of NADPH to NADP by aldehyde reductase. The oxidation products of the aldehydes (biogenic acids) were found to inhibit the aldehyde stimulation of glucose oxidation. The aldehydes produced from amine oxidation have also been suggested to be involved in sleep mechanisms (Jouvet, 1969). In the rat liver and heart muscle, α-amylase (acid α-glycosidase) has been shown to be stimulated by biogenic amines and inhibited by the oxidation products, suggesting a role for MAO in controlling α-amylase activity (Orlova, Sinyukhin, Popova, Gorkin and Rosenfeld, 1971).

Over the past few years, evidence has accumulated suggesting that MAO may be involved in migraine. Hanington (1967) found that in subjects affected by migraine induced by cheese, tyramine (which is present in most cheese) was able to initiate headache episodes. She suggested that some migraine sufferers may have a genetic MAO deficiency. Support for this theory comes from the work of Sandler, Youdim and Hanington (1974). They showed that phenylethylamine could induce

headaches in migraine subjects who are sensitive to chocolate (which contains phenylethylamine but no tyramine). Estimation of platelet MAO activity showed a significant decrease in the ability of the enzyme to oxidize phenylethylamine and tyramine as compared to control platelet activity. In addition there was a smaller but significant decrease in the enzymes ability to oxidize dopamine and serotonin.

1.3 Localization of MAO

The enzyme has a wide distribution in the body with relatively high levels in the liver, kidney, stomach, intestine, heart and brain (see Blaschko, 1952; Tipton, 1973). The blood plasma has little or no MAO activity but the enzyme does occur in the platelets (Collins and Sandler, 1971). Early studies with liver MAO indicated that the enzyme was associated predominantly with the mitochondria and a small fraction was also associated with the microsomes, but disrupted mitochondrial fragments could not be ruled out as the source of this fraction (Cotzias and Dole, 1951; Hawkins, 1952).

The controversy over the microsomal fraction is still not resolved. Roth and Stjärne (1966) found that a considerable fraction of MAO was present in the microsomes in close association with the amine storage particles. However, in a later re-examination of these results Stjärne, Roth and Giarman (1968) stated that they could separate the MAO activity in the microsomal fraction from the amine storage particles and suggested that this enzyme fraction may be an artifact due to the homogenization procedure. The same conclusion was suggested earlier by Jarrott and Iversen (1968) who found that reduced nicotinamide adenine dinucleotide ($NADH_2$)-cytochome C reductase, a marker enzyme for the outer mitochondrial membrane, had the same subcellular

distribution as MAO. However, De Champlain, Mueller and Axelrod, (1969) criticized the Jarrott and Iversen (1968) conclusions based on the findings of Green, Allmann, Harris and Tan (1968) that $NADH_2$-cytochrome C reductase is a microsomal enzyme that could contaminate the mitochondria. Also, they found MAO activity in the microsomes of the rat liver, heart, salivary gland and the vas deferens. On continuous sucrose gradients, the MAO microsomal particles were closely associated with the amine storage vesicles. In the rat brain, Rodriguez de Lores Arnaiz and De Robertis (1962) found about 12.5% of the MAO activity in the microsomal fraction and the rest sedimenting with the mitochondria. In subfractionation of the mitochondrial fraction, they found 37% in the synaptosomes and 60% associated with the free mitochondria.

In a number of innervated tissues, the enzyme may be present both intraneuronally and extraneuronally. Evidence for intraneuronal MAO includes the use of MAO inhibitors and measuring amine levels (Pletscher, 1958; Pletscher, Gey and Burkard , 1965); movement of MAO down the sciatic nerve (Dahlstrom,Jonason and Norberg 1969) and in experiments where enzyme activity falls after denervation (Jarrott and Iversen, 1971). The enzyme is largely extraneuronal in paraenchymal tissue such as salivary gland and liver (Jonason, 1969) and in sympathectomized adrenergic innervated organs, little or no decrease in enzyme activity compared to controls indicates extraneuronal MAO is present (Klingman, 1966; Horita and Lowe, 1972). Also, histochemical studies have indicated extaneuronal MAO in the rabbit ear artery (De la Lande, Hill, Jellet and McNeil, 1970)

In 1967, Schnaitman, Erwin and Greenawalt (1967) showed that graded treatment of rat liver mitochondria with digitonin can separate the outer mitochondrial membrane from the inner membrane plus matrix material. They found that the MAO activity was tightly bound to the outer mitochondrial membrane. Other workers using techniques besides digitonin treatment have confirmed these results (Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967; Tipton, 1967; Horita and Lowe, 1972).

Using a histochemical localization technique, Bloom, Sims, Weitsen, Davis and Hanker (1972) suggested that MAO of guinea-pig kidney was on the inner surface of the outer mitochondrial membrane. Racker and Proctor (1970) found that sonications of outer mitochondrial membranes at pH 9.6, four to eight times for 30 seconds followed by pH adjustment to 6.0 and centrifugation at 100,000 x g, yielded a resolved membrane preparation that contained no MAO activity. Addition of a purified MAO preparation to the resolved membranes at pH 7.5 resulted in reconstitution. Blaschko (1974) stated that from these results, MAO must be a minor constituent of the membrane protein and that MAO removal had not diminished the cohesion of the membrane. Partial support for this conclusion comes from the interesting work of Oreland and Olivecrona(1971) who found that extraction of pig liver mitochondria with methyl ethyl ketone yielded large amounts of phosphatidylcholine and phosphatidylethanolamine without liberating any MAO. A second extraction step in the presence of ammonium sulfate (0.05M) yielded anionic phospholipids (mainly cardiolipin) and the release of MAO soluble in buffer. It was suggested that the binding of the enzyme to mitochondria depended on the presence of anionic phospholipids. In a further report Olivecrona and Oreland (1971) found that soluble

MAO would bind to lipid depleted mitochondria in the presence of anionic phospholipids. The effect of cardiolipin on the binding of MAO to the mitochondria was studied, but no simple stoichiometry was found. Binding occurred also with lipid-depleted red cell and fat globule membranes.

It is now generally accepted that the outer mitochondrial membrane is not synthesized by the mitochondria and the membrane proteins are under nuclear deoxyribonucleic acid control (Baxter, 1971). However, the origin of MAO is still under consideration. By irreversibly inhibiting all of the MAO activity in the rat liver and then following the rate of enzyme regeneration, Erwin and Simon (1969) found that the rate of return was more rapid in the microsomal fraction than in the mitochondria indicating the enzyme originates in the microsomes and is transferred to the mitochondria. Furthermore, Erwin and Deitrich (1971) found the half-life of the microsomal enzyme to be about 1 day whereas the mitochondrial enzyme had a half-life of about 3.5 days. Also, cycloheximide prevented the regeneration of the microsomal enzyme but a significant amount was recovered in the mitochondria indicating that cycloheximide prevents the enzyme from binding to the microsomes and newly synthesized enzyme binds directly to the mitochondria. However, these results could also indicate that the microsomal and mitochondrial enzymes are two different proteins.

1.4 Solubilization and Purification

In order to perform detailed chemical and physical studies on MAO, highly purified preparations are needed. However, because of its tight binding to the outer mitochondrial membrane (see

Section 1.3), MAO has until recently resisted solubilization and purification.

Only in the past 10 to 15 years have methods been developed which solubilize* membrane bound proteins, to give consistent results (see Penefsky and Tzagoloff, 1971, Coleman, 1973, for reviews). These methods include low osmotic strength, high ionic strength, proteolytic digestion, phospholipases, solvent extraction, chaotropic agents, sonication and detergents. With few exceptions, the use of detergents and or sonication are needed to solubilize MAO.

Alterations in substrate and inhibitor specificity or other properties may change when a membrane bound enzyme is solubilized due to conformational changes. Ideally a solubilizing detergent should not change protein conformation and it has been shown that the anionic detergents sodium dodecyl sulfate and sodium deoxycholate will cause conformational changes correlated with solibilization using the erythrocyte membrane but nonionic detergents do not (Kirkpatrick and Sandberg, 1973). Triton X-100, a nonionic detergent has shown to cause little or no conformational changes with mitochondrial membranes (Kirkpatrick and Jacobs, 1970). At present little is known about how detergents solubilize proteins, but they have been shown to bind proteins (Helenius and Simons, 1972; Makino, Reynolds and Tanford, 1973) and triton X-100 can deplete lipoproteins of lipid as a function of detergent concentration and ionic strength in a displacement type reaction (Sun, Prezbindowski, Crane and Jacobs, 1968).

The majority of solubilization procedures shown in Table 1 are with detergent although some have successfully solubilized the enzyme with a combination of detergent plus sonication. The enzyme

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* Solubilization refers to proteins that do not sediment while centrifuging at 105,000 x g for one or more hours.

TABLE 1

Purified Preparations of Monoamine Oxidase

Source	Solubilization	Purification factor from:	Recovery %	Reference
Rat Liver	sonication & detergent	Homogenate 208	26	Youdim and Sourkes (1966).
Rat Liver	detergent	Homogenate 136	2	Akopyan, Veryorkina, Levyant, Moskvitina, Gorkin & Orekovitch (1971).
Beef Liver	detergent	mitochondria 61	5.1	Yasunobu, Igaue & Gomes (1968).
Beef Liver	detergent	mitochondria 51	0.9	Akopyan et al (1971).
Rabbit Liver	sonication & detergent	mitochondria 238	5	Gabay and Valcourt (1968).
Pig Liver	solvent extraction	Homogenate 530	5	Oreland (1971).
Pig Liver	detergent	Homogenate 287	-	Carper, Stoddard & Martin (1974).
Human Liver	detergent	Homogenate 17	23	Norstrand and Glantz (1973).
Bovine Kidney	digitonin	mitochondria 34.2	10	Erwin and Hellerman (1967).
Pig Brain	sonication & freeze-thaw	mitochondria 1000	22	Tipton (1967).
Human Brain	detergent	Homogenate 17	23	Nagatsu, Yamamoto and Harada (1969).
Beef Brain	detergent	mitochondria 36	0.04	Harada, Mizutani & Nagatsu (1971).
Beef Brain	detergent	mitochondria 15	3.7	Akopyan et al (1971).
Human Platelet	sonication & detergent	Platelets 12	10	Collins and Sandler (1971).

retained activity upon solubilization which is generally accepted to indicate that the enzyme has the same conformation as the membrane bound one. However, recent evidence indicates that sodium deoxycholate and triton X-100 may alter the conformation of MAO on solubilization (Diaz-Borges and D'Iorio, 1972; Severina and Zhivotova, 1973; Shih and Eiduson, 1973).

Weissbach, Redfield and Udenfriend (1957) reported a soluble form of guinea-pig liver MAO could be obtained by homogenizing the liver in water. The enzyme would not sediment at 100,000 x g and probably represents the enzyme on small membrane fragments due to the lytic action of water. No soluble form of the enzyme has been reported from other tissues by this procedure. In 1970 Hollinger and Oreland reported on a novel method for obtaining MAO from pig liver in 'true' solution. In the first step mitochondria were extracted with methyl ethyl ketone and washed with buffer. The membranes were extracted a second time with methyl ethyl ketone in the presence of ammonium sulfate, and up to 25% of the enzyme could then be released into buffer. However, organic solvent extraction was not successful on other tissue preparations (Cotzias, Serlin and Greenough, 1954; Barbato and Abood, 1963). With some preparations sonication alone has given good results, Guha and Krishna Murti (1965) reported on a solubilized preparation of MAO from rat liver by suspending mitochondria in buffer at pH 7.6 and exposing it to ultrasonic waves. Tipton (1968a) solubilized pig brain by a combination of freeze-thawing and sonication. Recently, other methods have been employed in an attempt to solubilize the enzyme from rat brain with little success, these included demyelinization, butanone extraction, lipase and tryptic digestion, osmotic shock, french press and nitrogen bomb (Shih and Eiduson, 1973). With the exception of the few procedures mentioned above,

MAO has been rendered suitable for purification by detergent and sonication or a combination of both (see Table 1).

The purification of membrane bound proteins presents added difficulties in obtaining highly purified preparations. This is usually due to the presence of bound lipid and membrane material. However, homogeneity is usually obtained with lower purification folds than with water soluble proteins. In some cases isolation of the cell membrane that contains the protein of interest will give a substantial increase in purity. Isolation of mitochondria increases the specific activity of MAO 2 or 3 times (Oreland, 1971) and the isolation of the outer mitochondrial membrane purifies the enzyme up to 123 fold (Schnaitman et al., 1967).

A list of some MAO purification factors is given in Table 1. With the exception of the human liver enzyme (Norstrand and Glantz, 1973), isolation of the mitochondria was the first step usually followed by solubilization. Purification was obtained by a combination of salt fractionation, gel-chromatography, DEAE ion exchange chromatography, adsorbtion chromatography, acid precipitation, sucrose gradients, electrophoresis and alcohol fractionation. As Table 1 shows there is a large variation in purification of the enzyme not only from tissues from different species and different tissues from the same species but also from the same tissue by different workers. The recovery of purified enzyme from the starting homogenates in most cases are low. The variations in purification and recovery reflect not only the different combination of purification procedures used but also the method employed to solubilize the enzyme. Although, some high degrees of purity are obtained with detergent solubilization methods, the highest degrees of purity are obtained with those preparations that do not use detergents for solubilization.

1.5 Affinity Chromatography

Methods for the conventional purification of enzymes depend on differences in size, charge and solubility of the protein. These differences are usually small so that purification is usually laborious and incomplete. Over the past few years a method has been refined that exploits the specificity of biological interactions for the isolation of specific proteins. This method is now referred to as affinity chromatography (Cuatrecasas, Wilchek and Anfinsen, 1968; Cuatrecasas, 1970). The technique is not new as Campbell, Leuscher and Lerman (1951) used affinity chromatography for anti-hapten antibodies and Lerman (1953) purified tyrosinase based on this method. Only recently has widespread use been realised with the development of suitable matrices and ligand coupling reactions.

The principle of affinity chromatography can be represented by the equation:-

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\,S \xrightarrow{k_2} E + P$$

The enzyme (E) interacts with the ligand (S) to form the enzyme-ligand complex E S . A necessary requirement is that the K_{diss} $\left(\frac{K_{-1}}{K_1}\right)$ should be small. In normal enzyme reactions the E S complex breaks down into the enzyme plus product, however, in purifying enzymes by affinity chromatography, it is best to use ligands that ensure the reversibility of the E S complex although in some cases it may be desirable to allow the breakdown to proceed to the E + P form.

In practice, the ligand is attached covalently to a suitable solid support system. The protein mixture is applied to the support and eluted with the ligand interacting with the specific protein retaining it

to the support system. The bound protein can then be eluted by a change that disrupts the ligand protein complex. Successful application of this technique depends on a close similarity of the ligand - protein interaction and that observed when the components are in free solution.

Close attention must therefore be made to the solid support system, the structure and means of attachment and the conditions of adsorbtion and elution.

The soluble support matrix should have the following properties: it must interact weakly with proteins to minimise non-specific adsorption it must have a loose porous network which permits the entry and exit of large macromolecules, there must be chemical groups suitable for reaction in attachment of ligands and be mechanically and chemically stable to the coupling, adsorption and elution conditions and it must retain good flow characteristics (Cuatrecases and Anfinsen, 1971).

Several materials have been used for solid supports including hydrophilic cellulose derivatives (Lerman, 1953), polyacrylamide beads (Inman and Dintzis, 1969) and glass beads (Weetall and Hersch, 1969). However, the most widely used support systems are the beaded derivatives of agarose (Hjerten, 1962) which has most of the properties for an ideal adsorbent.

The molecule to be covalently attached to the insoluble matrix must have affinity for the protein to be purified. The ligand - protein complex must be reversible and are therefore usually competitive inhibitors, cofactors and in some cases substrates. The molecule must contain a reactive group for attachment to the matrix without abolishing its affinity for the protein. In some cases it may therefore be necessary to synthesize special molecules for attachment (see e.g. Dudai , Silman, Kalderon and Blumberg, 1972; Edmondson, Massey, Palmer, Beacham and Elion, 1972).

When a macromolecule is penetrating a solid support matrix for interaction with a directly attached ligand, the matrix itself may block the interaction. It appears that this steric interference occurs mostly with proteins of high molecular weight and with low affinity systems (Lowe, Harvey, Craven and Dean, 1973). This interference can be minimised by inserting chemically a long hydrocarbon chain between the matrix and ligand (Cuatrecasas, 1970). Insolubized D-tryptophan methyl ester is ineffective compared to ε-aminohexanoyl-D-tryptophan methyl ester in purifying α-chymotrypsin (Cuatrecasas, Wilchek and Anfinsen, 1968). Direct attachment of p-aminophenyl-β-D-thiogalacto-pyranoside results in no affinity for E.coli β-galactosidase whereas the ligand about 21 Å from the matrix results in the enzyme being strongly adsorbed (Steers, Cuatrecasas and Pollard, 1971). A thorough study on the binding effects of extension arms has been made by Lowe, Harvey, Craven and Dean (1973), using interactions of kinases and pyridine nucleotide-dependent dehydrogenases with derivatives of ATP and NAD^+. They showed that affinity increases with increasing arm length to a maximum and then decreases with further arm lengthening. This decrease in affinity could be explained by an increase in flexibility and folding of the extension arm at distance (Lowe and Dean, 1974).

Sepharose 4B (Pharmacia) is the most widely used agarose derivative for affinity chromatography. Compounds that contain primary aliphatic or aromatic amines can be coupled direct by activation of the agarose with cynanogen bromide at alkaline pH (Cuatrecasas, 1970). Spacer arms such as diaminoethane or diaminohexane can be attached by this procedure. This amino-agarose preparation can then be used in a variety of ligand attachment procedures. Ligands that contain carboxylic groups can be coupled to amino-agarose with water soluble carbodiimides or the amino derivative can be converted to the carboxylic derivative with

succinic anhydride which will attach ligands containing aliphatic or aromatic amine groups again with carbodiimides. Bromoacetyl-agarose can be prepared from amino-agarose with O-bromoacetyl-N-hydroxy-succinimide. This derivative can then be used to couple ligands containing sulfhydryl, amino, phenolic or imidazole groups. Diazonium derivatives can be prepared by treating the amino-agarose with p-nitrobenzoyl azide. This derivative will couple ligands containing phenolic or imidazole groups and can be cleaved from the matrix by treatment with dithionite. Amino-agarose treated with N-acetyl-homocysteine can react with ligands via thiol ether or ester linkage and can be readily cleaved under basic conditions. In some cases ligands may not be soluble in water, therefore use can be made of up to 50% ($^{v}/_{v}$) of dimethylformamide, ethylene glycol or ethanol in the coupling procedure without altering the properties of the agarose. This briefly describes some of the most widely used coupling procedures and several reviews describe them in more detail (Cuatrecasas, 1970; Cuatrecasas and Anfinsen, 1971; Lowe and Dean, 1974; Parikh, March and Cuatrecasas, 1974).

Affinity Chromatography can be performed using batch techniques if the ligand-protein interaction has a high affinity or if the solution contains particulate protein which would impede column flow rates. However, this is usually rare and the majority of applications is performed with column chromatography procedures.

Adsorption to the matrix will be dictated by the optimum conditions for the protein-ligand interaction. The column equilibrating buffer must be at the optimum pH, ionic strength and temperature for the interaction. The protein solution should be prepared under the same conditions or dialyzed against the column equilibrating buffer before application to the column.

Elution of specifically bound protein requires a change such that the affinity of the ligand-protein complex decreases enough to allow elution. This can usually be achieved by a change in pH, ionic strength or temperature of the eluting buffer (Cuatrecasas and Anfinsen, 1971). If the protein is very tightly bound to the matrix use of denaturants such as urea or guanidine hydrochloride may be needed. The eluted protein should be dialysed immediately after elution to restore the original conformation. However, in some cases the change will be irreversible. Elution can also be achieved using solutions of inhibitors, either a higher concentration of the one bound or one with a higher affinity. Another method is to use a solution of substrate to release the bound enzyme. In some cases it may be desirable to remove the matrix from the column and dilute it in a particular buffer, reducing the effectiveness of the complex (Cuatrecasas, 1972a). In cases where the ligand has been coupled by azo linkage, thiol or alcohol ester bonds, elution can be achieved by cleavage of the ligand-protein complex from the matrix under mild conditions (Cuatrecasas and Anfinsen, 1971).

Although the main use of affinity chromatography is in enzyme purification, these procedures can in principle, be applied to antibodies, antigens, vitamins, drug-binding proteins, and biological receptors. The techniques can be used for concentrating a protein solution and separating biologically active from denatured protein (see Cuatrecasas, 1972a).

Aldehyde oxidase was purified 100 fold by a single passage of protein through an agarose column coupled with N-benzyl-6 methyl nicotinamide (Chu and Chaykin, 1972). The bound enzyme was eluted at high pH and salt concentration. The ligand coupled close to the matrix reduced the purification 10-15 fold. Breslow and Sloan (1972) described

a method for purifying arylsulfatase A from human urine on a column of psychosine sulfate-agarose. The enzyme was strongly bound to the matrix and could only be eluted in the presence of triton X-100. Lipoprotein lipase was purified 2000 fold by binding heparin to agarose (Olivecrona, Egelrud, Iverius and Lindahl, 1971). The bound protein could be eluted by low ionic strength. This simple purification procedure gave an enzyme with a higher specific activity than any previously reported using conventional techniques. Cuatrecasas and Illiano (1971) described a procedure for purifying neuraminidase from Vibro cholerae by affinity chromatography. Purification of up to 450 fold could be achieved on a agarose column containing covalently bound N-(4-aminophenyl) oxamic acid. The enzyme bound to the matrix at pH 5.5 and could be eluted at pH 9.

Lefkowitz, Haber and O'Hara (1972) covalently attached noradrenaline 30 Å from the agarose matrix and purified the cardiac beta-adrenergic receptor protein. Other receptors purified by these methods with success are the insulin receptor (Cuatrecasas, 1972b) and the 5-hydroxytryptamine binding protein (Shih, Eiduson, Geller and Costa, 1974).

In general affinity chromatography is a specific technique. Each protein to be purified must have a specific adsorbent made for its binding. However, there are some ligands that can be covalently coupled that will bind specific sites on many proteins. Cuatrecasas (1970), described a method for attaching p-chloromercuribenzoateto agarose and this derivative can be used to separate thiol containing proteins. Sluyterman and Wijdenes (1970) used bound p-amino-phenylmercuric acetate bound to agarose to separate mercaptopapin from non-mercaptopapin. Another system useful for binding peptides containing free sulphydryl groups is 5-5' dithiobis-(2-nitrobenzoate) bound to agarose (Lin and Foster, 1975). Recently, proteins have been purified by ligands attached to agarose and interacting

hydrophobically. Er-el, Zaidenzaig and Shaltiel (1972) purified glycogen phosphorylase using hydrocarbon coated agaroses. The enzyme did not bind to the single carbon (C_1) chain length, was slightly retarded on the C_2 adsorbent and tightly bound on the C_4-C_6 columns. A deforming buffer was used to elute the enzyme and in some cases denaturation was necessary. Lipids can be separated from blood plasma on a column of dodecylamine-agarose (Deutsch, Fogleman and von Kaulla, 1973). A disadvantage of hydrophobic ligands is their possible detergent like action causing some proteins to denature (Hofstee, 1973). However, Yon (1972) has reduced the hydrophobicity of long chain hydrocarbons by introducing ionic groups at the end of the chain. Concanavalin A bound to agarose can be used to adsorb polysaccharides and glycoproteins containing α-D-glucosyl, α-D-mannosyl or sterically similar residues. Aspberg and Porath (1970) studied the adsorption of serum glycoproteins to agarose bound concanavalin A and Rush, Thomas, Kindler and Udenfriend (1974) purified dopamine-β-hydroxylase up to 67 fold on this adsorbent.

Although there have been no reported attempts to purify MAO by affinity chromatography, this approach may be suitable in purifying the enzyme. Solubilized preparations for use in affinity column procedures can be obtained by detergent and or sonication (see Section 1.4). The enzyme has sulfhydryl and hydrophobic groups (see Section 1.6) which may interact with immobilized reagents specific for these groups and thus may help in purification. The competitive inhibitors, proflavine (Gorkin, Komisarova, Lerman and Veryovkina, 1964) and 1-m-amino-phenyl-2-cyclopropylaminoethanol, AB-15 (Huszti, 1972) have groups for covalent attachment to agarose and may be quite specific for retaining the enzyme on the column. Some of the substrates for MAO may also be covalently immobilized for use in this technique.

1.6 Properties of MAO.

With the advent of purified preparations of MAO, the physical properties of the enzyme could be investigated. The molecular weight of MAO has been determined for a number of enzyme preparations and these have varied widely (see Table 2). It is now recognized that soluble MAO has a marked tendency to aggregate which may explain the variations seen in Table 2 when molecular weight values are obtained by gel filtration. In an attempt to overcome this problem, filtration has been performed in the presence of large amounts of detergent. Oreland (1971) found that purified pig liver MAO, when applied to a column of Sephadex G-200, eluted as a single excluded peak. If the gel filtration was performed in the presence of 2.5% sodium cholate, only a small fraction of the enzyme was excluded and most of the activity eluted in a new retarded peak with a small fraction retarded even more. Increasing the concentration of cholate to 5% and the most retarded peak increased at the expense of the excluded peak and at a concentration of 10% cholate, the excluded peak disappeared with a further increase in the most retarded peak. The molecular weights of the two included peaks were calculated to be 275,000 and 115,000 daltons. This deaggregation of MAO in the presence of detergent proved to be reversible as re-filtration of the most retarded peak without detergent resulted in an excluded peak and only very small amounts of the included peaks. Using a different solubilization procedure with pig liver MAO, Carper, Stoddard and Martin (1973), obtained two enzyme peaks of 146,000 and 1,200,000 daltons. Houslay and Tipton (1973) showed that a partially purified preparation of rat liver MAO treated with the chaotropic agent, sodium perchlorate, eluted as 50% in one excluded and 50% in one retarded peak on a column of Sepharose 4B. In the presence of 0.01% sodium deoxycholate, more than 90% of the enzyme eluted in the retarded peak with a molecular weight of 390,000 daltons.

Table 2

Some Properties of Purified Preparations of Monoamine Oxidase (from Tipton, K. F. (1972) Brit. Med. Bull. 29, 116-119).

Source	Molecular Weight (daltons)	Flavin Fluorescence	FAD Content (mol/10^5g)	Metal Content	References
Beef liver	450,000; 1,250,000	No	1.0	No Cu,Fe, Mn,Co or Mo	Yasunobu et al 1968; Gomes et al, 1969.
Pig liver	100,000	Yes	1.0	No Cu; some Fe	Oreland, 1971
Rat liver	150,000; 300,000	Some preparations	0.7	No Cu; Fe present	Sourkes, 1968; Youdim and Sourkes, 1972
Beef kidney	290,000	Yes	1.0	No Cu	Erwin and Hellerman, 1967
Pig brain	100,000	Yes	1.0	No Cu or Fe	Tipton, (1968, a,b)
Human brain	400,000	Yes	0.7	-	Nagatsu et al, 1972

Although this molecular weight value was in close agreement with the enzyme from beef liver (Gomes, Igaue, Kloepfer and Yasunobu, 1969) and human brain (Nagatsu, Nakano, Mizutani and Harada, 1972) it is not in agreement with the results of Youdim and Sourkes (1966) who obtained values of 260,000 daltons by gel filtration and 150,000 daltons by ultracentrifugation also for the rat liver enzyme. This tendency to aggregate may also have affected the molecular weight values obtained for the rabbit liver enzyme, 200-300,000 daltons (Gabay and Valcourt, 1968), the beef brain enzyme, 400,000 daltons (Harada, Mizutani and Nagatsu, 1971, Nagatsu et al., 1972) and human platelet MAO, 235,000 daltons (Collins and Sandler, 1971). Although the molecular weight of the enzyme bound to the mitochondria is still speculative, the minimum molecular weight of the enzyme based on its flavin content, has been found similar in all cases (see Table 2) at about 100,000 daltons. Also, the molecular weight determined by active site directed inhibitors for the beef-kidney (Hellerman and Erwin, 1968) and pig brain (Tipton, 1968) enzymes, have been shown to be close to 100,000 daltons.

Experiments have also been performed to determine the subunit molecular weight of MAO. Youdim and Collins (1971) found that partially purified preparations of rat liver MAO had a molecular weight of about 306,000 daltons when measured by gel filtration. Incubation of the enzyme in the presence of 1% sodium dodecyl sulphate and mercaptoethanol yielded a molecular weight of 76,740 daltons by gel filtration in the presence of 1% SDS. Similarly incubation of the enzyme with 8M urea in the presence of mercaptoethanol and the molecular weight determined by polyacrylamide gel electrophoresis, gave a value of 78,000. As the 78,000 dalton subunits prepared by the two methods were enzymatically inactive, these may represent subunit dimers or tetramers. The molecular

weight of pig liver MAO has been shown to be about 115,000 daltons (Oreland, 1971). Gel filtration of the purified enzyme on Sepharose 4B in the presence of 6M guanidine and 0.1M mercaptoethanol and analytical ultracentrifugation of the enzyme with 1% SDS and 0.1% mercaptoethanol gave molecular weights of 55,000 and 63,000 daltons respectively. SDS polyacrylamide-gel electrophoresis in the presence of mercaptoethanol yielded one band of about 60,000 daltons. In the absence of mercaptoethanol, electrophoresis yielded two bands of 60,000 daltons both of which contained an active site when labelled with [^{14}C] pargyline, a MAO inhibitor blocking the active site of the enzyme in a 1:1 molar ratio (see Hellerman and Erwin, 1968). The results indicated that the enzyme contained two subunits of identical size (60,000 daltons) of which only one contains the active site (Oreland, Kinemuchi and Stigbrand, 1973). These experiments were repeated for the rat liver enzyme by Collins and Youdim (1975) and similar results and conclusions were reported.

In an early study of MAO, Richter (1937) suggested that the enzyme may act in a similar manner as D-amino acid oxidase which was later shown to be a flavoprotein. Hawkins (1952) compared the MAO and D-amino acid oxidase activity in livers of riboflavin deficient rats. After 2 weeks on the deficient diet, the D-amino acid oxidase activity had fallen to one fifth the control value, whereas the MAO activity had fallen to one half. Feeding riboflavin to the deficient rats resulted in a rapid rise of the D-amino acid oxidase levels but the rise in MAO activity was very small. These results indicated that if MAO was a flavin containing protein, it was less ready to give the flavin up than D-amino acid oxidase or that the flavin was required for the synthesis of the enzyme. A fall in MAO activity by riboflavin deficiency has been

confirmed by a number of reports (Wiseman and Sourkes, 1961; Wiseman-Distler and Sourkes, 1963, Youdim and Sourkes, 1965; Symes, Missala and Sourkes, 1971). In a recent study Youdim and Sourkes (1972) gave rats in their daily diet galactoflavin, an antimetabolite of riboflavin. Although there was a marked decrease of MAO activity over the 42 day experiment, there was no significant difference with these animals as to animals compared with simple riboflavin deficiency. There was a much greater lowering of the liver activity than the brain activity indicating either two different enzymes or galactoflavin had a limited access to the brain. Other indirect evidence that MAO may contain a flavin was reported by Lagnado and Sourkes (1956) who studied the enzymatic reduction of tetrazolium salts by amines and suggested the electron transfer process involves a flavoprotein.

With the advent of purified preparations of MAO, the enzyme was tested directly for flavin content (see Table 2). The fluorescence properties of the enzyme indicated that a flavin was present although some preparations did not fluoresce (Table 2). This may be due in part to quenching impurities in the enzyme preparations (Youdim and Sourkes, 1972). In some preparations the flavin has been found to be bound in a covalent nature to the apoenzyme (Erwin and Hellerman, 1967; Oreland, 1971). Only treatment of MAO with proteolytic enzymes could release the flavin which was found to be in the form of flavin-adenine dinucleotide (FAD) (Igaue, Gomes and Yasunobu, 1967; Oreland, 1971). However, Sourkes (1968) found that about one half of his purified rat liver preparation released a fluorescing material with trichloroacetic acid extraction indicating two forms of binding of the flavin component. A similar finding was reported for the human and beef brain enzyme in which part of the flavin material could be released by boiling the enzyme in a

solution of zinc sulfate at pH 7.0 (Nagatsu et al. 1972). Tipton (1968b) reported that the flavin component of purified pig brain MAO could be released by either heating an enzyme solution at 100°C for 12 minutes or by extraction with trichloroacetic acid. The flavin component was identified as FAD by ascending chromatograpy with pure FAD and by titration with FAD-specific D-amino acid apooxidase. Also, after extraction of the FAD with acid, the apoenzyme could be reactivated by the addition of FAD but not flavin mononucleotide (FMN) indicating that FAD was the flavin component of MAO. Other preparations of MAO have been shown to contain flavin in the form of FAD and there is good agreement between workers of one mole FAD per 10^5g of enzyme (see Table 2).

Binding of the flavin component to the apoenzyme has recently been studied by Kearney, Salach, Walker, Seng,Kenney, Zeszotek and Singer (1971). Using purified preparations of beef liver mitochondrial MAO, digested with trypsin and α-chymotrypsin yielded riboflavin covalently linked to a peptide chain. It was further found that the flavin was attached via the 8α-carbon of the riboflavin. Unlike succinate dehydrogenase, another flavoprotein which is linked via the 8 α-carbon through an imidazole nitrogen (Walker and Singer, 1970), MAO was found to be a cysteinyl-8 α-riboflavin (Walker, Kearney, Seng and Singer, 1971). Furthermore, a synthetic cysteinyl-8 α-riboflavin has been prepared (Ghisla and Hemmerich, 1971) and is identical with the component isolated from MAO (Walker et al. 1971).

The possible involvement of metal ions as a cofactor for MAO has been investigated both directly and indirectly with varying results. Lagnado and Sourkes (1956) found that some metal ions activated rat liver MAO in moderate concentrations (10^{-4}M) suggesting a metal ion may be a cofactor. Rat liver and brain MAO were found to be reversibly inhibited

by the chelating agents 8-hydroxyquinone, plumbone, diethyldithiocarbonate and cyclohexanediamine tetraacetate (Gorkin, 1959). It was postulated that zinc or copper may be a cofactor for the enzyme. Green (1964) made a comparative study on the inhibition of MAO by hydrazine derivatives and their decomposition catalyzed by cupric ions. Also, 8-hydroxyquinone was shown to be a competitive inhibitor of the enzyme and protected it from iproniazid inhibition. These results suggested that cupric ions are in or near the active site of MAO and activate hydrazine derivatives before they can inhibit the enzyme. Support for the involvement of copper as a cofactor came from the findings of Cog and Baron (1967) who inactivated the rat liver enzyme by extensive dialysis and complete reactivation was achieved by addition of cupric ions. However, Youdim and Sourkes (1965) found that D-penicillamine and diethyldithiocarbamate, relatively specific chelating agents for cupric ions, inhibited MAO only at high concentrations. Furthermore, they found that the enzyme activity was not changed in livers of copper deficient rats suggesting that if a metal ion is a cofactor of the enzyme, it probably is not copper. The metal deficiency study has been repeated by Symes, Sourkes, Youdim, Gregriadis , and Birnbaum (1969) and Symes et al. (1971), who found that the enzyme activity of copper deficient rats was unchanged from controls whereas the livers from iron deficient rats had lowered MAO levels. This indicated a possible role for iron in the enzyme.

With the advent of purified preparations and the direct determination of metal content, clarification of the issue was still not possible. Some early studies with purified preparations of beef liver MAO suggested it was a copper containing protein (Nara, Gomes and Yasunobu, 1966), in an even purer preparation Yasunobu, Igaue and Gomes (1968) could only detect small amounts of the metal and little copper if any could be

detected on other preparations of the enzyme (see Table 2). Some purified preparations contained iron; Youdim and Sourkes (1966) reported 0.12% iron in the rat liver enzyme and a similar amount was found for the pig liver enzyme (Oreland, 1971). In a recent report, Carper et al. (1974) found 0.0434% copper in the pig liver enzyme, however, this may be an impurity as this preparation was not as pure as the one reported by Oreland (1971).

Even with purified preparations, some authors use the inhibitory action of metal chelators to suggest that the enzyme contains a metal cofactor (Barbato and Abood, 1962; Nara et al. 1966; Gabay and Valcourt, 1968; Kinemuchi, 1971). However, in studies with purified preparations of beef kidney MAO, Erwin and Hellerman (1967) showed that of all the chelating agents used only 8-hydroxyquinone produced marked inhibition and that similar types of inhibition (competitive with substrate) were produced with compounds similar to the hydroxyquinones suggesting the inhibition was due to the phenolic nature of the compound and not their chelating ability. A similar finding was reported by McEwen, Sasaki and Jones (1969a) who pointed out that most copper chelating agents may be considered substrate analogs and inhibition may therefore not be related to their chelating ability.

It has been shown that MAO is inhibited by sulfhydryl reagents suggesting the enzyme contains thiol groups (Singer and Baron, 1945; Lagnado and Sourkes, 1956). With purified preparations of beef liver MAO, Gomes, Naguwa, Kloepfer and Yasunobu (1969) found seven cysteine residues per 100,000g of MAO. Similar values were reported for purified preparations of rat liver MAO (Klyashtorin and Gridneva, 1966) and beef kidney enzyme (Erwin and Hellerman, 1967). In a study on the sulfhydryl groups of beef liver MAO, Gomes, et al. (1969) found that the enzyme

retained 70-80% of its activity after step wise addition of p-chloromercuribenzoatein amounts sufficient enough to react with all sulfhydryl groups. Further, they found that the inhibition produced by sulfhydryl reagents was non-competitive. These results indicated that the SH groups of MAO were likely for conformational stability rather than being involved in catalysis. However, McEwen, Sasaki and Jones (1969b) found that p-chloromecuribenzoate was a competitive inhibitor of human liver MAO. In a further study on the SH groups of beef liver MAO, Yasunobu and Oi (1972) showed using methylmecuric chloride to titrate the SH groups, that in the presence of substrates only five SH groups reacted per 100,000 g of protein whereas seven reacted in the absence of substrate, indicating that the active site of the enzyme contains two cysteine residues. Furthermore, they determined possible amino acid residues involved in catalysis by determining the effect of temperature on pK values and from these calculated the heats of ionization. These results suggested that a cysteine residue may be involved in amine binding and that a histidine residue may be involved in catalysis.

Besides thiol groups the active site of MAO has been reported to contain both a hydrophobic and polar regions (Severina and Sheremet'evskaya, 1967). It has been suggested that since MAO oxidizes amines without aromatic rings, the hydrophobic region that interacts with the rings is non-specific and outside the catalytic site. However, from kinetic data on human liver MAO, McEwen, Sasaki and Lenz (1968) and McEwen et al. (1969a) suggested that the hydrophobic region was located in the active centre in close proximity to an electrophilic binding site for substrates and inhibitors. A similar conclusion was also reported for the catalytic site of the pig liver enzyme (Severina, 1973).

1.7 Substrate Selective Inhibitors and the Binary Enzyme Hypothesis.

It is now generally accepted that in certain tissues MAO exists in more than one form. Although little is known about the nature of these forms, our present understanding of them comes mostly from studies with substrate selective inhibitors. Evidence that the enzyme was present in more than one form, was at first indirect. Studies on the substrate specificity (Blaschko et al.,1937b; Alles and Heegard, 1943; Barlow and Khan, 1959; Weiner, 1960) pH maxima (Alles and Heegard, 1943; Barbato and Abood, 1962; Youdim and Sourkes, 1965) anion inhibition (Van Woert and Cotzias, 1966) and heat stability (Gorkin, 1963; Oswald and Strittmatter, 1963; Youdim and Sourkes, 1965; Squires, 1968) all suggested more than one form of the enzyme.

Using the irreversible inhibitor iproniazid, Hardegg and Heilbronn (1961) determined the second order rate constant for MAO inactivation with tyramine and serotonin. Since the two constants were different (870 $\underline{M}^{-1}$ min^{-1} for tyramine and 1380 $\underline{M}^{-1}$ min^{-1} for serotonin), they suggested that there were at least two sites for amine oxidation as only one site would give the same constant for both substrates. They further suggested that serotonin was oxidized by one site and tyramine was oxidized at two sites. Determination of rate constants for irreversible MAO inhibitors to distinguish between different oxidation sites has also been reported by Tipton (1969) and Fuller, (1972). The compound N-methyl-N-2 propynyl-1 indanamine was found to inhibit tyramine oxidation at lower concentrations than serotonin oxidation in the rat tissues both in vivo and in vitro (Maitre, 1967). This indicated that there may be at least two sites for amine oxidation, although it should be pointed out that the differences in inhibition between the two substrates were small.

The first irreversible substrate selective inhibitor Clorgyline [N-methyl-N-propargyl-3-(2,4 dichlorophenoxy) propylamine]; was reported by Johnston (1968). Working with rat brain mitochondrial MAO, he observed that a plot of percent inhibition against Clorgyline concentration showed a single sigmoid curve for serotonin as substrate and a double sigmoid curve with tyramine as substrate with a plateau of invarient inhibition joining the two sigmoid curves (see Fig. 1). Johnston (1968) proposed that the enzyme contained two components, a relatively sensitive A enzyme and a more resistant B enzyme. Serotonin is a substrate for the A enzyme and tyramine a substrate for both enzymes. This hypothesis has been expanded by Hall, Logan and Parsons, (1969) who found also with the rat brain enzyme, that dopamine and tryptamine gave a plateau shaped curve similar to tyramine and are substrates for both enzymes and that benzylamine has a single sigmoid curve, specific for enzyme B (see Fig. 1). Using the inhibitor Clorgyline to show more than one site of oxidation Hall, Logan and Parsons (1969) found only a single site in cat, dog, pig, ox and rabbit liver and the pig brain whereas two oxidation sites were detected in the cat, dog, ox and rabbit brain and in both rat and human brain and liver tissue. This binary system of MAO based on the inhibitor Clorgyline, is now used by several workers as a criterion for multiplicity (Goridis and Neff, 1971; Jarrott, 1971; Squires, 1972; Houslay and Tipton, 1974). The list of preferred substrates has been extended by Goridis and Neff (1971) and Yang and Neff (1973) for the rat brain enzyme and by Houslay and Tipton (1974) for the rat liver enzyme. Some preferred and common substrates for the A and B forms of MAO are shown in Table 3. Houslay and Tipton (1974) classified a large number of preferred and common substrates for MAO and found that with the exception of serotonin, all compounds tested were substrates for species B unless they possessed a β-hydroxyl group.

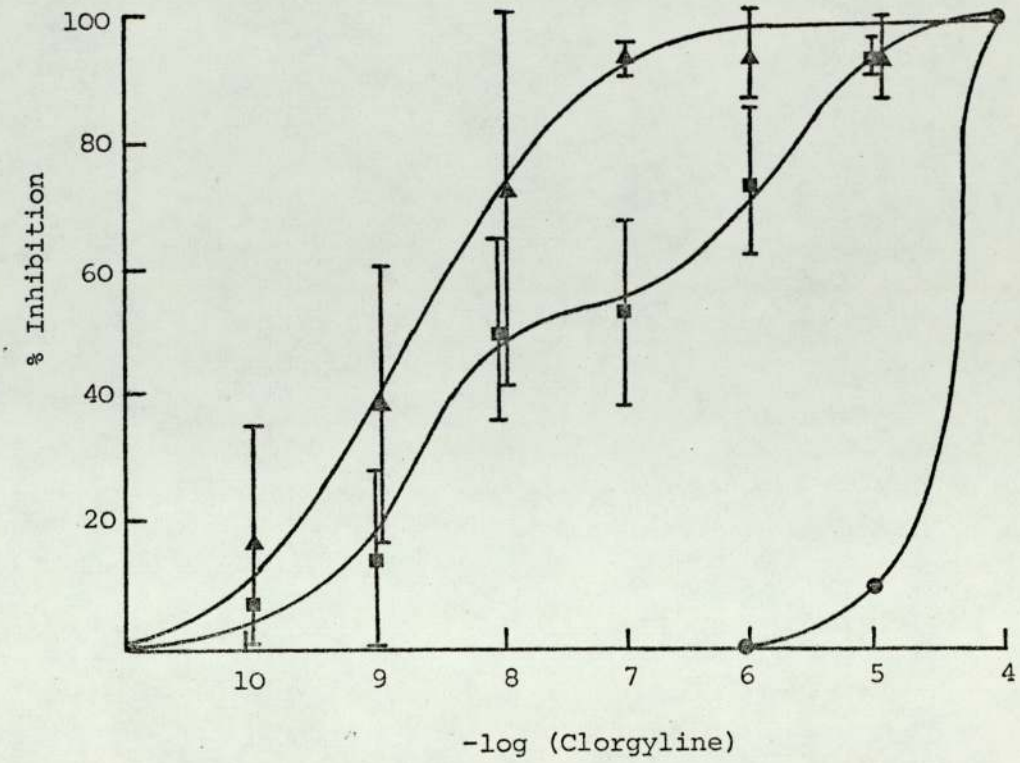


Fig. 1. Inhibition of rat liver MAO with Clorgyline. The substrates used were tyramine (■), serotonin (▲) and benzylamine (●). Redrawn from Hall, Logan and Parsons (1969).

TABLE 3.

Substrate specificity of rat liver mitochondrial MAO (from Houslay and Tipton, 1974).

Substrates for 'Species A' activity only	Substrates for 'Species B' activity only	Common substrates
Serotonin Adrenaline 3-O-Methyladrenaline	Benzylamine 4-Hydroxybenzylamine 4-Hydroxy-3-methoxybenzylamine (Vanillylamine)	Tyramine Dopamine
Noradrenaline 3-O-Methylnoradrenaline Octopamine	2-Phenylethylanine 3,4-Dimethoxy-2-phenylethylamine 4-Methoxy-2-phenylethylamine Tryptamine 5-Methoxytryptamine	4-Hydroxy-3-methoxy-2-Phenylethylamine

They found that tyramine, dopamine and 3-methoxy-tyramine were common substrates and that tryptamine was a substrate for the species B only. Tryptamine was shown to be a common substrate for the rat brain enzyme (Hall, Logan and Parsons, 1969) indicating that the substrate specificity of the two forms may differ depending on the enzyme source. Further support comes from Lyles and Callingham (1974) who found that in the rat heart tyramine is oxidized by the A enzyme only, while benzylamine is oxidized by both the A and B forms. Recent evidence also suggests the possibility of a C form of the enzyme in the rat brain (Gascoigne, Williams and Williams, 1975). Using a histochemical technique to determine the enzymes distribution and Clorgyline to distinguish the different forms, they found the A and B forms broadly distributed and a third type of MAO (C) which like the A, used serotonin as substrate, but was relatively insensitive to Clorgyline inhibition and was predominantly circumventricular in distribution.

Other irreversible MAO inhibitors have been developed which are substrate selective and support the binary enzyme hypothesis. Taylor, Wykes, Gladish and Martin (1960) found that pargyline (N-methyl-N-benzyl-propynylamine) is a potent irreversible inhibitor of MAO and Squires (1968) showed that it may preferentially inhibit the type B enzyme. Fuller (1968; 1972) showed that the compound N-[2-(O-chloro-phenoxy)-ethyl]-cyclopropylamine; Lilly 51641 and certain of its derivatives act in a way similar to Clorgyline, i.e., selectively and irreversibly inhibiting serotonin oxidation. Another compound Deprenil (phenylisopropylmethylpropinylamine) has been shown to inhibit in a reverse manner to Clorgyline (Knoll and Magyar, 1972). The substrate tyramine shows the double sigmoid dose response curve for both rat liver and brain MAO, however, it selectively inhibited benzylamine oxidation and was very much less efficient against serotonin oxidation. Thus

Deprenil is a selective inhibitor for the B enzyme. One other possible irreversible substrate selective inhibitor is 2-phenylethyl-hydrazine shown by Tipton, Youdim and Spires (1972) in the beef adrenal medulla, to have a similar time course of inhibition for kynuramine, tyramine, serotonin and benzylamine as substrates whereas dopamine oxidation is quite resistant over the same time period. However, Christmas, Coulson, Maxwell and Riddell (1972) in their work on substrate selective MAO inhibitors classified 2-phenylethyl-hydrazine as a nonselective inhibitor.

In addition to the substrate selective irreversible inhibitors, there are several reversible inhibitors that show substrate selectivity. The alkaloid harmine and α-methyl-tryptamine have been shown to selectively inhibit serotonin oxidation before tyramine oxidation (Gorkin and Tatyanenko, 1967; Gorkin, Tatyanenko, Suvorov and Neklyudor, 1967). It has been suggested by Belleau and Morgan (1963) that harmine may inhibit the enzyme by forming charge-transfer complexes with the flavin group in the enzyme. A similar mode of action has been suggested for proflavine (3,6-diamino-acridine) which also inhibits serotonin oxidation before other substrates (Gorkin, Komisarova, Lerman and Keryovkiva, 1964). A more recently introduced compound, AB-15 (1-m-amino-phenyl-2-cyclopropylaminoethanol) has been shown to also be a selective inhibitor of serotonin oxidation both in vitro and in vivo (Huszte, 1972).

The method of mixed substrates can be used to distinguish between one enzyme acting on two substrates and two enzymes with different specificities (Dixon and Webb, 1958). A number of workers have used this method with MAO, indicating that only one enzyme oxidized the variety of substrate pairs tested (Blaschko et al, 1937b; Kohn,

1937; Pugh and Quastel, 1937a; Kobayashi and Schayer, 1955; Oswald and Strittmatter, 1963; Houslay and Tipton, 1973). Recently, Houslay, Garrett and Tipton (1974) have extended the theory of mixed substrates to include systems in which two enzymes are each active towards two different substrates. They showed that regardless of the difference in the K_m values of each enzyme for the two substrates, if the K_m values of the two enzymes are similar for each substrate, the experimental results will indicate only one enzyme is present. This may explain why only a single enzyme was detected for MAO by this method.

Substrate competition can also be used to study molecular forms of an enzyme (Dixon and Webb, 1958). Houslay and Tipton (1975a) using the two site model they developed for rat liver MAO (Houslay and Tipton, 1974), presented some steady state rate equations for the oxidation of substrate mixtures and from these they could predict the type of inhibition one MAO substrate had on the oxidation of another. They concluded that the observations from substrate competition experiments can be accounted for in the Johnston (1968) binary enzyme theory. This approach has also been used by White and Wu (1975) to detect different sites in the human brain enzyme and by Edwards and Chang (1975) to show at least two sites for amine oxidation in the human platelets. It is also clear from substrate competition experiments that the presence of one amine may affect the oxidation of other amines and that this could have a central importance in the control of amine levels in vivo.

A large amount of work has been reported on the mechanism of action of the irreversible MAO inhibitor pargyline which is important not only in revealing the chemical properties of the enzyme, from which new and better inhibitors can be developed, but also may help elucidate the nature of the multiple forms. It was first suggested by Belleau and Morgan (1963) that the acetylenic group of pargyline may form a

covalent bond possibly with thiol groups in the enzyme. However, Vina, Gorkin, Gridneva and Klyashtorin, (1966) and Hellerman and Erwin (1968) found that after the enzyme was inhibited, there was no decrease in the number of protein thiol groups. Youdim and Sourkes (1965) found the inhibition with pargyline decreases with increasing pH, similar to some chelating agents, suggesting that it may react with a metal cofactor in the enzyme. In a detailed study using highly purified beef kidney MAO, Hellerman and Erwin (1968) found that pargyline can act as an electron donor for the enzyme and reduce the flavin moiety. They also showed that pargyline formed a very tight, probably covalent bond with the flavoenzyme and is specific for the catalytic site of MAO in a ratio of 1 mole of inhibitor per 10^5 grams of protein, the same ratio as the flavin content. The authors suggested that pargyline may be acted upon like a substrate with the removal of two electrons from the α carbon atom transferred to the flavin group. This would activate the pargyline molecule and it would react either directly with or near the flavin moiety. Support for this comes from Oreland, Kinemuchi and Yoo (1973) who digested the $[^{14}C]$-pargyline labelled enzyme from pig liver mitochondria with pronase. It was found that pargyline was in close association if not covalently bound to the flavin group. Similar results were obtained for the beef kidney enzyme by Chuang, Patek and Hellerman (1974) who also showed that the purified pargyline-enzyme complex had a highly characteristic spectrum which coincided with the spectrum of a product

resulting from photoreduction of flavin with pargyline (Zeller, Gartner and Hemmerich, 1972). The structure of this product has been elucidated by Gartner and Hemmerich, (1975) and is shown in Fig. 2.

1.8 Separation of the Multiple MAO forms.

Although early indirect evidence suggested the possibility of MAO existing in more than one form, physical separation could not be attempted because the enzyme was tightly bound to the mitochondrial membrane (see Section 1.3). With the advent of suitable solubilization procedures, many workers have reported on the separation of more than one MAO form (for review see Sandler and Youdim 1972). Some cases of reported separated forms may be due to polymerization of the enzyme. Gomes, Igaue, Kloepfer and Yasunobu (1969) obtained two beef liver MAO fractions from a column of hydroxylapatite, however from studies on their substrate specificity, FAD content and molecular weight it was suggested that they were aggregates of the same enzyme. Aggregation may also explain the two forms of beef liver separated by ammonium sulfate precipitation by Kinemuchi (1971), as the substrate and inhibitor specificity were similar.

By far the most frequently used technique to separate the different forms of MAO has been electrophoresis and a summary of reported separations is shown in Table 4. In some cases, most pronounced the rat liver, there is a wide variation on the number of reported forms of the enzyme. However, detailed studies have been performed on some separated fractions in attempts to establish their validity.

The molecular weight of purified rat liver MAO was determined to be 155,000 daltons by ultracentrifugation and 300,000 daltons by gel

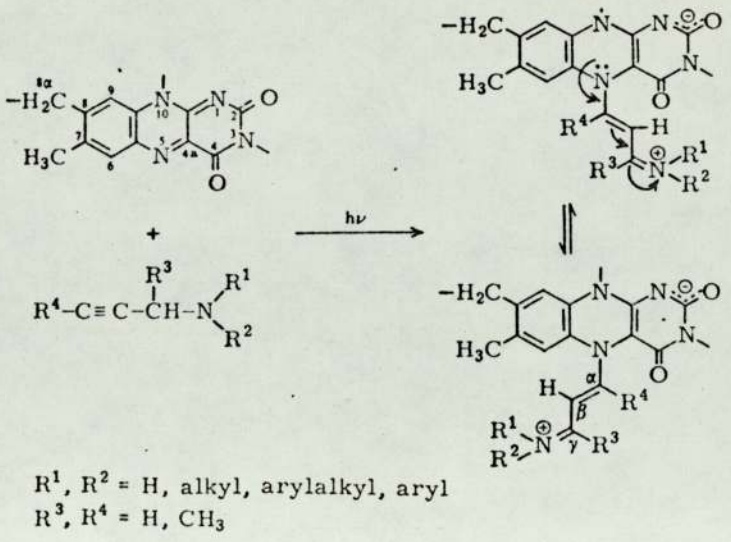


Fig. 2. The photoaddition of acetylene compounds to the flavin nucleus. Reproduced from Gartner and Hemmerich (1975).

TABLE 4 .

The Electrophoretic Pattern of Multiple Forms of Solubilised Mitochondrial MAO from a Variety of Different Species and Tissues (from Sandler and Youdim, 1972).

Tissue	"Anodic"	"Cathodic"	References
Rat Liver	2,3,4,7.	1	Akopyan et al, 1971; Collins et al, 1968; Kim and D'Iorio, 1968; Youdim et al, 1970; Shih and Eiduson, 1971.
brain	4,3	1	Shih and Eiduson, 1971; Youdim et al, 1969.
uterus	1	2	Southgate, 1972.
heart	4	0	Shih and Eiduson, 1971.
Human brain	3,2	1	Collins et al, 1970; Nagatsu et al. 1972.
liver	4	1	Collins et al, 1968.
endometrium	2	1	Southgate, 1972.
placenta	2	1	Youdim and Sandler, 1967.
platelets	1	0	Collins and Sandler, 1971.
Pig brain	1	0	Tipton and Spires, 1968.
liver	3		Oreland, 1971.
Beef brain	2		Harada et al, 1971.
liver	1	2	Gomes et al, 1969.
adrenal medulla	4	1	Tipton et al, 1972
Monkey intestine	1		Murali and Radhakrishnan, 1970.
Chick brain	5,6	1	Shih and Eiduson, 1969; Shih and Eiduson, 1971.
Xenopus larvae	3.2		Baker, 1971.

The figures indicate the number of bands of activity claimed by various authors to migrate towards the anode or the cathode.

filtration (Youdim and Collins, 1971). Electrophoresis of the purified preparation separated five MAO forms four of which had molecular weights of 300,000 daltons by gel filtration. When dissociated by 8M urea in the presence of mecaptoethanol followed by dialysis, each fraction reassociated to form the original five forms suggesting that the different forms are conformational enzymes. This conflicts in part with the findings of Ragland (1968) who partially separated enzyme fractions from rat, beef and rabbit liver that possessed varying activity towards different substrates on Sephadex G-200 columns. These results suggest that the different forms vary in their molecular weights. The results reported on the substrate specificity of the different forms of rat and human liver (Collins, Youdim and Sandler, 1968; Youdim, Collins and Sandler, 1970), rat brain (Youdim, Collins and Sandler, 1969) and human brain MAO (Collins, Sandler, Williams and Youdim, 1970) are inconclusive. The bands of activity that migrate towards the anode showed activity with all substrates tested andthe ratio of tyramine: tryptamine: kynuramine: benzylamine: dopamine was fairly constant with only minor exceptions. The heat stability of the separated forms from human liver show no differences whereas, the rat liver enzyme has a wide variation for the different components (Collins, Youdim and Sandler, 1968). Wide variation in pH optimums and inhibition patterns have also been reported for the separated components (Youdim, Collins and Sandler, 1969; Youdim, Collins and Sandler, 1970). However, there was little correlation between the differential effect of inhibitors on the separated forms in vitro and the pattern observed in vivo (Collins, Youdim and Sandler, 1972).

Particular interest has been made of the MAO component that migrates toward the cathode. This component is seen with human brain (Collins et al, 1970), rat brain (Youdim, 1972), beef adrenal medulla (Tipton, Youdim and Spires, 1972) rat liver (Tipton, 1972; Youdim, 1972) and rat uterus (Collins and Southgate, 1970). The enzyme is resistent to hydrazine type inhibitors, is more heat stable, has a different pH optima and because it has a very much lower K_m value for dopamine than the other components, it has been referred to as a specific dopamine MAO (see Youdim; 1972, 1973a). However, Houslay and Tipton (1974) in kinetic studies of membrane bound rat liver MAO, could find no evidence for the presence of an enzyme with a low K_m value for dopamine before electrophoresis. It was suggested that this cathodic component may be produced as an artifact of the electrophoretic system, since it is unusual for a protein that has been purified by adsorption to DEAE cellulose at pH 7.2, to show a positively charged component on electrophoresis at pH 9.2.

The possibility that the separated forms of MAO could be due to the binding of varying amounts of membrane material was first suggested by Verevkina, Gorkin, Mityushin and El'piner (1964). However, Collins, Youdim and Sandler (1968) pointed out that if fragments of membranes were being observed, they were remarkably consistent. This is supported by Kim and D'Iorio (1968) who found similar results whether sonication or detergent is used to break up the mitochondria. The method used by Youdim and co-workers to solubilize MAO, sonication plus detergent, may on electrophoresis show different enzyme species due to varying phospholipid contents. This contention was examined by Tipton, Youdim and Spires (1972) for beef adrenal medulla and Tipton (1972) for

the rat liver enzyme. They found varying phospholipid contents between the separated components but could find no correlation between the electrophoretic mobility and phospholipid content. This does not, however, rule out the possibility that lipids are responsible for the observed forms. Tipton (1972) showed that electrophoresis in the presence of 1.25% triton X-100 resulted in only one band migrating toward the anode but treatment of the enzyme with organic solvents and phospholipase A to remove lipids, resulted in enzyme inactivation. In the elgant study on the separation of the rat liver components, Houslay and Tipton (1973) showed that treatment of the partially purified enzyme with the chaotropic agent, sodium perchlorate, resulted in only a single band migrating towards the anode. They concluded that since the chaotropic agent reduced the amount of bound lipid, the separated components are artifacts of the solubilization and purification procedure. This observation is supported by the finding that electrophoresis of the outer mitochondrial membrane from rat liver revealed only a single band of MAO activity (Houslay and Tipton, 1973) and it seems unlikely that numerous MAO's are represented in the dozen or so major proteins of the outer mitochondrial membrane (Schnaitman, 1969).

Some workers have reported the separation of some MAO fractions that have greatly reduced or even have no enzyme activity for certain substrates which, if the oxidation sites are on different proteins, would be in accordance with Johnstons (1968) binary enzyme theory. Sierens and D'Iorio (1969) reported that they could separate two bands of rat liver MAO, one which was active towards benzylamine alone and the other active towards both benzylamine and serotonin. The enzyme was solubilized with the ionic detergent deoxycholate, which rendered the

enzyme unstable, imparing the serotonin activity more than the benzylamine activity. Further experiments with [^{14}C] deoxycholate excluded the possibility of detergent bound to one fraction and not the other but inhibition of serotonin oxidation indicated that the detergent caused irreversible damage to one fraction and not the other (Diaz Borges and D'Iorio, 1972). Fractions of rat brain MAO, solubilized by triton X-100 could be separated by both electrophoresis and gel filtration (Shih and Eiduson , 1973). One fraction oxidized both benzylamine and serotonin while the other had a greatly reduced serotonin oxidizing ability. In a further experiment the enzyme was labelled with [^{14}C] pargyline and the label was followed during solubilization. At the same time an unlabelled fraction was assayed for enzyme activity. About 86% of the enzyme was solubilized when assayed for pargyline and about 77% when assayed with benzylamine, however, only 56% was solubilized when assayed for serotonin activity indicating appreciable MAO activity was lost on solubilization possibly due to a detergent induced conformational change.

Immunochemical methods have been found to be useful for comparing related proteins. Antigenic differences or similarities are known to correlate well with protein structure. Hidaka , Hartman and Udenfriend (1971) found that mitochondrial MAO from beef brain cross-reacted with antibody to liver MAO. It was possible to precipitate immunologically 80% of the brain enzyme. The authors suggested that the 20% that didn't precipitate was a detergent solubilized antigen-antibody complex. However in a further study Hartman and Udenfriend (1972) found that the 20% that didn't crossreact represents a different enzyme with regard to several enzymatic parameters. This finding has

been confirmed by McCauley and Racker (1973) who found that 90% of the benzylamine activity from beef brain could be precipitated by antibodies to the liver enzyme, whereas 70% of the tyramine activity remained in the supernatant. The two forms differed in their heat stability and inhibitor specificity. The authors suggested that these two forms are similar to the binary system of Johnston (1968), however more detailed evidence is needed before this is confirmed as detergents were used to solubilize the enzyme and may have impaired the oxidizing capacity of one fraction as in the cases mentioned above.

The possibility that the different forms of MAO may be present in different mitochondria has been suggested by Youdim (1972a). Using sucrose density gradients to separate rat brain mitochondria, Kroon and Veldstra (1972) suggested that dopamine and serotonin are oxidized by a type of MAO located mainly in large synaptosomes containing mitochondria. Kynuramine was mainly degraded by an MAO in mitochondria free from neuronal and glial cells. Youdim (1973b, 1974) obtained results from ficoll-sucrose gradients also suggesting that the different forms of MAO from rat liver and brain may be located on different mitochondria.

Although, at present the precise nature of the amine oxidation sites is not known, results from some experiments has led to some interesting speculations. Houslay and Tipton (1973) showed that treatment of partially purified rat liver MAO with the chaotropic agent sodium perchlorate, abolished the heat stability and inhibitor specificity with various substrates. They found a reduction in enzyme bound phospholipid material after perchlorate treatment suggesting that the different enzyme forms may result from similar enzyme proteins in different membrane environments. Similar results were shown for human

brain MAO with the same conclusion (Tipton, Houslay and Garrett, 1973).

In the rat brain MAO B is more sensitive to heat and resistant to trypsin digestion than MAO A (Yang, Goridis and Neff, 1972; Yang and Neff, 1973). These differences may be due to phospholipids bound to the enzyme as Oreland and Estedt (1972) showed that phospholipid affected the heat stability and tryptic digestion of MAO from pig liver. The aromatic moiety of MAO substrates and inhibitors were suggested to interact with non-specific sites on the enzyme surface and not with the catalytic site (Belleau and Morgan 1963). From these observations Neff and Yang (1974) suggested that phospholipid may influence the non-specific sites forming the different MAO forms from enzyme protein with the same catalytic site. They also suggested that MAO B contains more phospholipid than MAO A and is thus more hydrophobic. Support for this theory also comes from the chemical nature of the substrates and inhibitors as suggested by Neff and Yang (1974). Serotonin and Clorgyline, which interact with MAO A, have more polar aromatic rings than benzylamine and Deprenil, substances that interact with MAO B. This theory can explain most experimental observations at present but it also suggests that both the membrane and the environment in relation to the enzyme must be examined in more detail.

1.9 Aims of Thesis.

While a number of highly purified preparations of MAO have been reported (see Table I), the purification procedures used were in most cases laborious, time consuming and usually resulted in a low enzyme recovery. It was proposed in the present study to attempt to purify MAO in a single step by the method of affinity chromatography.

An examination of the literature reveals that there are a large number of compounds that interact with MAO and several of these are suitable for covalent attachment to an insoluble matrix, which may make them useful in this purification method. In order to reduce non-specific interactions of the enzyme with the adsorbent columns possibly involving detergents, it was planned to use MAO preparations that were solubilized both with and without detergents.

It was proposed to use a number of acetylenic substrate selective MAO inhibitors to classify the different forms of rat liver mitochondrial MAO. In addition it was planned to use some derivatives of these compounds to study their mode of action and the nature of their selectivity.

Recent evidence indicates that the membrane environment of MAO may help to confer some of the properties to the enzyme and may be responsible for the formation of the multiple forms (for a review see Neff and Yang, 1974). It was proposed to study the effect of lipid peroxidation, phospholipases and chaotropic agents on the activity of MAO using a sensitive radio assay with several substrates. Furthermore, it was planned to use the above mentioned methods to alter the membrane environment and to reduce the amount of enzyme bound lipid in order to investigate the effect of the environment on the heat stability, tryptic digestibility, Arrhenius plots and Deprenil inhibition pattern of MAO A and MAO B.

Finally, it was proposed to study the relationship of the enzyme protein to the outer mitochondrial membrane by a variety of techniques including the isolation of mitochondrial structural protein, resolution of the membrane by mild sonication and by the effects of proteolytic digestion and DTNB-Sepharose on intact and lysed mitochondria.

2. MATERIALS AND METHODS.

2.1 Materials.

(Side chain-2-^{14}C) tyramine hydrochloride (50 µCi; 55 mCi/mmol) and 5-hydroxy (side chain-2-^{14}C) tryptamine creatinine sulfate (50 µCi; 55 mCi/mmol) were obtained from the Radiochemical Centre, Amersham U.K. (Side chain-2^{14}C) tryptamine bisuccinate (50 µCi; 47 mCi/mmol) and Protosol tissue solubilizer were purchased from New England Nuclear, Boston, Mass. U.S.A. (7-^{14}C) Benzylamine hydrochloride (0.1 mCi; 5.6 mCi/mmol) and the ion exchange resin, Amberlite CG-50; 100-200 mesh were obtained from Mallinckrodt Chemical Works, St. Louis Mo. U.S.A. To each radioactive substrate, 1 ml of deionized-distilled water was added and the stock solutions were stored at -20°C. The following chemicals were obtained from Sigma Chemical Co. Ltd., Surrey, U.K., non-labelled tryptamine, tyramine, serotonin and benzylamine, 2, 4, 6 trinitrobenzene sulfonic acid, bovine serum albumin, digitonin, proflavine hemisulfate, mercaptoethanol, nitro blue tetrazolium, DL-α-phosphatidylethanolamine dipalmitoyl, DL-α-phosphatidylcholine dipalmitoyl, phospholipase A (bee venom), phospholipase C (Cl.welchii), 2-thiobarbituric acid, Sephadex G-50 and G-200, DEAE Sephadex, Sepharose 4B, 5,5'-dithiobis-(2-nitrobenzoate), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and trypsin (bovine pancreas). CN-Br Sepharose and AH and CH Sepharose were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Aquacide I was obtained from Calbiochem AG, Lucerne, Switzerland. Mono-propargylamine hydrochloride was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wis. U.S.A. 1-Anilinonaphthalene-8-sulphonic acid magnesium salt and all other reagents of the

highest purity were obtained from BDH Chemicals Ltd., Poole, U.K. N-Methyl-N-propargyl-3-(2, 4 dichlorophenoxy) propylamine hydrochloride (Clorgyline) was obtained from Dr. D. R. Maxwell, May and Baker Ltd., Dagenham, U.K. AB-15, 1-m-aminophenyl-2-cyclopropylaminoethanol dihydrochloride was a gift from Dr. Z. Huszti, Research Institute for Pharmaceutical Chemistry, Budapest, Hungary. Deprenil (phenylisopropylmethylpropinylamine hydrochloride, E-250) was a gift from Dr. J. Knoll, Semmeliveis University of Medicine, Budapest, Hungary. N-(2-(2, 4-dichlorophenoxy)-ethyl)-3-methyl-1-butyn-3-amine hydrochloride (Lilly 51618) and N-methyl-N- β -(2, 4-dichlorophenoxy)ethyl)-propargylamine hydrochloride (Lilly 50122) were a gift from Dr. R. W. Fuller, The Lilly Research Laboratories, Indianapolis, Indiana, U.S.A. Pargyline (N-methyl-N-(2-propynyl) benzylamine) and (^{14}C) pargyline were a generous gift from Dr. Witherspoon, Abbott Laboratories, Surrey, U.K.

2.2 Determination of Enzyme Activity.

MAO activity towards tryptamine, tyramine, serotonin and benzylamine was assayed by a similar procedure described by Wurtman and Axelrod (1963) and Christmas et al. (1972). Assay test tubes in ice contained 50 µl enzyme (0.1-2.5 mg protein/ml), 100 µl of 0.1 M sodium phosphate buffer, pH 7.4 or pH 8.0 containing 2 mM-diaminoethane tetraacetic acid (EDTA) and 100 µl of substrate. Unless otherwise indicated, final substrate concentrations were 1 mM containing 50 nCi of either (^{14}C)benzylamine or (^{14}C) serotonin or 25 nCi of either (^{14}C) tyramine or (^{14}C) tryptamine. For inhibitor studies 100 µl of buffer was replaced by 50 µl of 0.2 M buffer containing 4 mM-EDTA and 50 µl of inhibitor in water. Incubations were performed

in air at 37°C in a shaking water bath for 20 min. At the end of the incubation 0.3 ml of 2 N HCl was added to each tube to stop the reaction. Five mls of toluene were added to each tube and shaken vigorously for 30 seconds followed by centrifugation at 500 x g for 2 min to separate the layers. Aliquots of the toluene layer were transferred to a vial or the tubes were kept at -20°C for three hours and the toluene poured into the vial followed by 5 ml of scintillation fluor [5 g 2, 5-diphenyloxazole and 0.3 g 1,4-di(2, 5-phenyloxazole) benzene, per 1 of toluene] and the vials counted for 5 to 20 min in a Beckman LS-230 liquid scintillation spectrometer. The counting efficiency was found to be 83% for ^{14}C by the internal standard method (see page 72). Blank values were obtained by using a boiled enzyme (100°C, 5 min) or distilled water in place of the active enzyme and were subtracted from the enzyme counts. Enzyme activity was linear for at least 20 min and linear through 1.5 mg of protein per ml final concentration with all substrates used.

When tris buffers were used and other experiments as noted in results, MAO activity towards tyramine, tryptamine, benzylamine and serotonin was determined by a modification of the method described by Robinson, Lovenberg, Keiser and Sjoerdsma (1968). The ion exchange resin Amberlite CG-50 was prepared for use as described by Pisano (1960). Assay mixtures were composed of the same components as described above except that the final substrate concentration of 1 mM contained 10 µCi of (^{14}C) tryptamine and (^{14}C) tyramine or 20 µCi of (^{14}C)benzylamine or (^{14}C) serotonin. At the end of the incubation 5 ml of a mixture of 20 g Amberlite per 100 ml of water was added to each tube and shaken for 10 s. After the resin had settled for 5 min

and the tubes centrifuged at 500 x g for 2 min , 2.5 ml of the aqueous supernatant was transferred to a vial and 10 ml of Bray's (1960) scintillation fluor was added. The vials were counted for 5 to 20 min and efficiency was calculated at 76% for ^{14}C by the internal standard method (see page 71). Blank values were obtained as described in the first method and subtracted from the enzyme counts. This assay system was linear through 2.5 mg of protein per ml final concentration and for at least 20 min with all substrates. Using ascending paper chromatography and solvent systems described by Wurtman and Axelrod (1963), the reaction products of tryptamine oxidation were identified as indoleacetic acid and indoleacetalydehyde.

2.3 Preparation of Mitochondria.

Mitochondria were prepared essentially by the method of Hunter, Scott, Hoffsten, Gebicki, Weinstein and Schneider (1964). Male Wistar rats were decapitated by guillotine and the livers were removed immediately, blotted,weighed and placed in ice cold 0.25 M sucrose. Portions of chopped liver were homogenized with about 9 volumes of cold 0.25 M sucrose in a glass homogenizer with a loose fitting teflon, motor driven pedestal. The homogenate was made 10% with sucrose and centrifuged at 600 x g for 10 min (MSE High Speed 18; 4°C), the supernatant was then centrifuged at 10,000 x g for 10 min. The crude mitochondrial pellet was washed twice with 0.25 M sucrose and then used as described in the following sections.

2.4 Protein Determination.

The protein concentrations were determined by the method of Lowry , Rosebrough, Farr and Randall (1951) with bovine serum albumin as standard.

2.5 Solubilization Procedure.

2.5.1. Sonication.

Mitochondria were suspended and washed twice in 0.01 M phosphate buffer, pH 7.5 and resuspended in 0.25 M sucrose which contained 0.05 M phosphate buffer, pH 7.5 to a protein concentration of 25 mg/ml. A suspension of 60 ml was then sonicated for 100 min with a Dawe Soniprobe (Dawe Instruments Ltd., London) fitted with a 13 mm (0.5 inch) diameter flat-profile probe at an amperage output of between 6 and 7. Sonication was performed in a cold room at $4^{\circ}C$ and the suspension was surrounded by an ice bath to dissipate heat. In some experiments as indicated in the results, sonication was performed in the presence of benzylamine and glass beads. At the end of the sonication period the suspension was left for 30 min at $4^{\circ}C$ and then centrifuged at 105,000 x g (MSE Superspeed 50; $4^{\circ}C$) for 1 hour. The supernatant, which contained the enzyme, was stored frozen and before use was thawed and dialyzed against the appropriate buffer.

2.5.2 Digitonin.

Digitonin was solubilized by heating a suspension of the compound in 0.25 M sucrose almost to a boil. When the suspension clears, it was cooled to $4^{\circ}C$ and used within 30 min. Mitochondria washed twice with 0.25 M sucrose were suspended in sucrose to give a protein concentration of 75 mg/ml. Enough 0.25 M sucrose containing

the digitonin was added to give a final protein concentration of 50 mg/ml. Digitonin was added in concentrations of 1.5 or 2.5 mg digitonin per 10 mg protein. After stirring on ice for 3 min, the suspension was centrifuged at 40,000 x g for 20 min and the supernatant contained the MAO for further use.

2.5.3 Chaotropic Extraction.

Sucrose washed mitochondria were suspended in distilled water at 50 mg/ml and dialyzed against 15 l of distilled water for at least 10 hours. The dialyzed mitochondria were then centrifuged at 30,000 x g for 30 min and the pellet resuspended in distilled water to 35-40 mg protein/ml. The suspension was made 1 mM in tyramine, to protect the enzyme, 10 mM in mercaptoethanol, to protect the SH groups, and 0.5 M in sodium perchlorate ($NaClO_4$). The suspension was incubated for 8 min at 25°C, cooled on ice to 0°C and centrifuged at 100,000 x g for 1 hour. The supernatant was discarded and the pellet was resuspended in the original volume of distilled water. Perchlorate treatment was performed as described above. The pellet after centrifugation was suspended to the original volume in 0.05 M tris buffer, pH 8.2 and made 5 mM in tyramine and 10 mM in mercaptoethanol. Enough 7 M sodium perchlorate was added to give a final concentration of 0.5 M and the suspension was incubated for 8 min at 25°C and then centrifuged at 105,000 x g for one hour. The supernatant was dialyzed against either 0.05 M tris buffer, pH 8.2 or 0.05 M phosphate buffer, pH 8.0 (3 changes of 12 l each over 15 hours). The perchlorate treatment in tris buffer at pH 8.2 was repeated four or five times and the supernatant contained the MAO activity. After dialysis the three highest specific activity fractions were pooled in

dialysis bags and packed in aquacide I to concentrate the protein solution to about one quarter of the original volume. In some cases, a precipitate formed after concentration, which was removed by centrifugation at 20,000 x g for 20 min. The enzyme preparation was stored at 4°C and used further within three days.

2.5.4 Triton X-100 Solubilization.

Mitochondria were washed with 0.05 M phosphate buffer, pH 7.2 and then lysed by suspension in distilled water. After centrifugation at 20,000 x g for 20 min the pellet was suspended in the same buffer and solid ammonium sulfate was added to give a 10% (w/v) solution. The suspension was stirred on ice for 20 min and centrifuged at 25,000 x g for 20 min and the supernatant was discarded. The pellet was suspended in 0.1 M phosphate buffer, pH 8.0 to a protein concentration of 10 mg/ml. A solution of 10% (v/v) triton X-100 was added dropwise to give a final concentration of 0.18% and the suspension was slowly stirred on ice for a further 30 min followed by centrifugation at 40,000 x g for 30 min. The supernatant was made 15% (w/v) in ammonium sulfate, stirred on ice for 20 min and centrifuged at 20,000 x g for 20 min. The pellet resuspended in 0.05 M phosphate buffer, pH 8.0 was dialyzed against 15 l of the same buffer and was then ready for further use.

In some experiments, MAO was solubilized and treated with ammonium sulfate by the method of Youdim and Sandler (1968).

2.5.5 Other Solubilization Methods.

Attempts to solubilize MAO were also tried by a variety of other techniques. Distilled water washed mitochondria were treated with acetone, butanol, ethanol and methyl ethyl ketone, as described

by Hollunger and Oreland (1970) for releasing the enzyme from pig liver mitochondria. Water washed mitochondria were treated with phospholipases and trypsin as described in Sections 2.17 and 2.18 respectively and centrifuged at 40,000 x g for 30 min. The pellets were suspended in 0.05 M phosphate buffer, pH 8.0 and all fractions were assayed for MAO activity. Solubilization was also attempted by incubating water washed mitochondria (10 mg protein/ml) with 50% (v/v) solution of ethylene glycol and 0.1 M phosphate buffer, pH 8.0 for two hours at room temperature. After centrifugation at 40,000 x g for one hour, 2 ml samples of the supernatant were passed through a 2.2 cm x 15 cm column of Sephadex G-50 equilibrated with 0.05 M phosphate buffer, pH 8.0. The pellet was washed with the same buffer and all fractions assayed for MAO activity.

2.6 Preparation of Affinity Adsorbents.

Unless otherwise indicated, the coupling reactions described and the 2,4, 6-trinitrobenzene sulfonic acid (TNBS) colour test are essentially those described by Cuatrecasas (1970).

2.6.1 Coupling of Amine.

CN-Br activated Sepharose was swollen and washed with 2 l of 1 mM HCl per six grams of dry Sepharose. The washed gel was added to an equal volume of cold distilled water containing 2 mmol of diaminohexane per ml which had previously been adjusted to pH 10.0 with 6N HCl. The slurry was allowed to react for 16 h at 4°C and then washed with large volumes of water. The TNBS colour test gave an orange colour.

2.6.2 Succinylation of Amine.

The aminated derivative was added to an equal volume of distilled water containing 2 mmol of succinic anhydride per ml. The

pH was raised to and maintained at 6.0 with 20% NaOH. When the pH no longer changed, the slurry was left for a further 5 h at 4°C. Complete reaction of the amino groups was indicated by change from orange to yellow with the TNBS colour test.

2.6.3 Coupling of Amine to Carboxyl Group.

The succinylated agarose was washed with 5 l of water per 25 ml of Sepharose and suspended in an equal volume of distilled water containing 2 mmol per ml of diaminohexane and the pH was adjusted to 4.8 with 6 N HCl. The slurry was then made 0.05 M with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and the pH was maintained at 4.8 per 1 h with 0.1 N NaOH. The gel was then stirred gently for another 20 h at room temperature and then washed with large volumes of water. Attachment was shown by a change from yellow to orange with the TNBS colour test. This derivative was then used to attach some carboxylic containing ligand or succinylated as described in Section 2.6.2 and then reacted with amine containing ligands.

2.6.4 Organomercurial-Sepharose.

The aminated Sepharose was suspended in 2 volumes of 40% dimethylformamide and 0.1 mmoles of p-chloromercuribenzoate was added per ml of packed Sepharose. The pH is adjusted to 4.8 and the slurry was made 0.05 M in EDC. The pH was maintained at 4.8 for one hour and gently stirred for a further 20 h at room temperature. The gel was then washed with large volumes of 0.1 M $NaHCO_3$, pH 8.8 for 8 hours. Complete reaction of the agarose amino groups was indicated by loss of orange colour with TNBS.

2.6.5 DTNB-Sepharose.

This adsorbent was prepared by a similar procedure described by Lin and Foster (1975). Thirty ml of amino-agarose was added to a 50% (v/v) solution of dioxane containing 1.5 mmol of 5, 5'-dithiobis-(2-nitrobenzoic acid). The pH was adjusted to 5.5 and the slurry was made 0.05 M in EDC and maintained at pH 5.5 for three hours. The slurry was gently stirred for a further 20 hr at room temperature and then washed with 3 l of 0.05 M phosphate buffer, pH 8.0 containing 0.5 M NaCl and then with 3 l of distilled water. The amount of DTNB bound was determined by adding 100 mg of the DTNB-Sepharose to 4 ml of 0.1 M phosphate buffer, pH 8.0 containing 0.01 M mercaptoethanol. The slurry was incubated for 5 min. at 37°C, the Sepharose was allowed to settle for 5 min and the 5-thio-2-nitrobenzoate (TNB) anion in the supernatant was read at 412 nm in a Pye Unicam SP500 spectrophotometer.

2.6.6 Dodecylamine-Sepharose.

This adsorbent was prepared by a similar procedure described by Deutsch, Fogleman and von Kaulla (1973). Dodecylamine (1 g) dissolved in 60 ml of ethanol, was added to 30 ml of CN-Br activated Sepharose in 30 ml of 0.3 M Na_2CO_3-HCl buffer, pH 10.2 and the slurry was slowly stirred for 20 h at 5°C. The suspension was warmed to room temperature to dissolve any precipitated dodecylamine and then washed with 3 l of ethanol and 5 l of water. Attachment of dodecylamine was shown by the TNBS colour test, which gave an orange colour.

2.6.7 Tyramine and Benzylamine-Sepharose.

Aminohexane-Sepharose (30 ml) which had been succinylated (see section 2.6) was suspended in an equal volume of distilled water and 1 mmol of tyramine or benzylamine containing 1 μCi of (^{14}C) tyramine

or (^{14}C) benzylamine were added to the suspension. The pH was adjusted to 4.8 with 1 N HCl and 10 mmoles of EDC was added. The slurry was maintained at pH 4.8 for at least 1 h and slowly stirred for a further 20 h at room temperature. The gel was washed with 5 l of 0.5 M NaCl and 5 l of water. The amount of tyramine or benzylamine bound was determined by placing a preweighed amount of gel in a vial, followed by 10 ml of NE-260 scintillation fluor and the vial counted. Results were expressed as μmol bound per ml of Sepharose.

2.6.8 Proflavine and AB-15-Sepharose.

CH Sepharose, an adsorbent containing 6 carbon spacer groups and a free carboxylic group for binding was swollen and washed with 3 l of 0.5 M NaCl per 6 grams of dry Sepharose, followed by 2 l of distilled water. The Sepharose was then suspended in an equal volume of distilled water containing 1 mmol of proflavine hemisulfate or 1-m-amino-phenyl-2-cyclopropylaminoethanol dihydrochloride (AB-15) per 20 ml of settled Sepharose. The pH was adjusted to 4.8 and the suspension was made 0.05 M in EDC. The pH was maintained at 4.8 with 1 N HCl for 1 h and the suspension was slowly stirred for a further 20 h at room temperature followed by washing the gel with 8 l of 0.5 M NaCl and 4 l of water. The amount of ligand bound, expressed as μmol/ml of Sepharose was determined by the method described by Lowe and Dean (1974). The derivativized gel was suspended by ethylene glycol in 4 mm cells and read against underivatized gel in a double beam spectrophotometer (Pye Unicam Sp-8000). The proflavine-Sepharose was read at 442 nm and the AB-15-Sepharose at 285 nm. They have molar extinction coefficients of 26,300 and 1700 M^{-1} cm^{-1} respectively.

2.6.9 Tryptamine and Serotonin-Sepharose.

These two MAO substrates were covalently bound to Sepharose 4B by the Mannich reaction, similar to that described by Ranadive and Sehon (1967). The reaction slurry contained 20 ml of aminohexane-Sepharose in an equal volume of distilled water, 7.0 ml of 3 M sodium acetate, 10 ml of 7.5% formaldehyde and 2 mmol of tryptamine or serotonin which contained 1 μCi of the (^{14}C) ligand. The slurry was stirred gently for 24 h at room temperature and then washed with 3 l of 0.5 M NaCl and 3 l of distilled water. The amount of ligand bound was estimated by counting a preweighed sample suspended in NE-260 scintillation fluor and expressed as μmol per ml of Sepharose.

2.7 Affinity Chromatography Procedure.

A 5 cm x 2.2 cm bed of affinity adsorbent was equilibrated at room temperature with 0.005 M phosphate buffer, pH 6.8 to 8.0. The soluble enzyme preparation dialyzed against the column equilibrating buffer, was applied to the column and eluted with the starting buffer at 0.5 ml per min. When all unbound protein was eluted from the column, as detected with a Uvicord II (LKB Instruments Ltd., Surrey, U.K.) at 280 nm, the buffer was changed to elute any bound protein.

2.8 Treatment of Soluble MAO Preparations with Perchlorate.

This procedure was similar to the one described by Houslay and Tipton (1973). Solubilized preparations of MAO (0.8 ml, 20-30 mg protein/ml) in 0.05 M tris buffer, pH 8.1 were incubated at 25°C for 8 min with 0.1 ml of 10 mM tyramine, 5 μl of mercaptoethanol and 0.1 ml of 7 M sodium perchlorate. At the end of the incubation, the tubes were cooled to 4°C in an ice bath. The mixture was then applied to a 12 cm x 2 cm column of Sephadex G-50 that had been equilibrated with

0.05 M phosphate buffer, pH 8.0 containing either 0.05% sodium deoxycholate or 0.05% triton X-100 and was eluted at room temperature.

2.9 Gel Filtration Experiments.

Gel filtration was carried out on 40 x 2.5 cm columns of Sepharose 4B and Biogel A-150 m with flow rates of about 20 and 25 ml per h respectively. Columns were equilibrated with 0.05 M phosphate buffer, pH 8.0 which contained in some experiments, 0.05% sodium deoxycholate or triton X-100. All gel filtration experiments were performed in a cold room at 4°C and a fraction collector was used to collect about 3.2 ml per tube.

2.10 Ion Exchange Chromatography.

A 12 x 2 cm column of DEAE A-50-120 Sephadex was equilibrated with 0.01 M phosphate buffer, pH 8.0 at room temperature. The protein solution, in the same buffer, was applied to the column and eluted at about 0.5 ml per min. Bound protein and other material was eluted by passing a solution of 0.1 M phosphate buffer, pH 7.2 containing 0.8 M KCl through the column.

2.11 Electrophoresis.

Polyacrylamide gels were made 7.5% (w/v) by the method of Davis (1964) and used in the Shandon disc electrophoresis apparatus (Shandon Scientific Co., Ltd., Surrey, U.K.) with a continuous buffer system consisting of 0.05 M tris-HCl buffer, pH 8.5. Since polymerization was accomplished in the presence of ammonium persulfate, pre-electrophoresis was performed at 5 mamps/tube for one hour. The sample (100 to 200 µg protein in a volume of not more than 30 µl) was made 1% (w/v) with sucrose and applied directly to the top of the gel. No

sample or spacer gel was used. Electrophoresis was carried out at room temperature for 40-60 min at 5 mamps per tube (5.5 x 74 mm). After the gels were removed from their glass running tubes, they were stained for MAO activity by the method of Glenner, Burtner and Brown (1957) as modified by Youdim, Collins and Sandler (1970). Gels were incubated with a freshly prepared solution containing tryptamine (15 mg), sodium sulfate (9 mg) and nitro blue tetrazolium (3 mg) in 10 ml of 0.05 M phosphate buffer, pH 7.4. Incubations were from 30-60 min at 37°C and the MAO activity appeared as blue-mauve bands. When tryptamine was substituted by other substrates in the staining mixture, the same molar concentrations were used. Gels were stained for protein by the method of Hartman and Udenfriend (1969) using 8-anilinonaphthalene-1-sulphonate (ANS) or by staining the gels with a 1% (w/v) solution of Amido Black in 7% acetic acid. Excess stain was removed by soaking the gels in 7% acetic acid.

In some experiments, electrophoresis was carried out in gels where the bottom half contained a Sepharose derivative. These gels were prepared by adding a half volume of settled sepharose to the acrylamide solution then pouring it into the gel tubes. Polymerization took between 20 and 30 min by which time the Sepharose had settled to about one half the gel length. Control gels were prepared by adding underivatized Sepharose to the acrylamide solution. Electrophoresis was carried out as described above.

2.12 Estimation of Phospholipid Content.

Phospholipids were determined by a modification of the method developed by Raheja, Kaur, C., Singh, A., and Bhatia, I.S. (1973). The chromogenic solution was prepared as described by Vaskovsky and Kostetsky (1968) and the

phospholipids were extracted from the protein samples by the method of Folch, Ascoli, Lees, Meath and LeBaron (1951). The chloroform-methanol extract was evaporated to dryness and 0.4 ml of chloroform and 0.1 ml of chromogenic reagent was added to the tube. A blank was prepared with chloroform and chromogenic solution only. The tubes were placed in a boiling water bath for 1 to 1.5 min and cooled to room temperature. After standing for 5 min , 1 ml of chloroform and 2 ml of 1, 2 dichloroethane were added and the tubes shaken for 1 min followed by centrifugation at 500 x g for 3 min. The organic layer was then read at 710 nm against the blank. A calibration curve was prepared (see Fig. 3) using phosphatidylcholine and phosphatidylethanolamine as standards and was linear through 300 µg of phospholipid.

2.13 Determination of SH Groups.

SH groups were measured by a modification of Ellman (1959) method. Assay mixtures contained 3.8 ml of 0.05 M phosphate buffer, pH 8.0 containing 1% (w/v) sodium dodecyl sulfate (SDS) and 0.5 mg/ml of EDTA, 0.1 ml of protein (5 to 10 mg/ml) and 0.1 ml of DTNB (40 mg per 10 ml of 0.1 M phosphate buffer, pH 8.0). Protein and DTNB blanks were prepared at the same time. The mixtures were incubated at 37°C for 15 min and read at 412 nm in a spectrophotometer. A standard curve was prepared using cysteine as standard and results are expressed as µg cysteine per mg protein.

2.14 Lipid Peroxidation.

Peroxidation experiments were performed by a modification of the McKnight, Hunter and Oehlert (1965) method. Isolated mitochondria were washed twice with 0.025 M tris buffer, pH 7.4 containing 0.175 M KCl. The mitochondria were stored at 0°C and used within 3 hours. Stock

solutions of mitochondria were added to 600 ml of 0.025 M tris buffer, pH 7.4 containing 0.175 M to a final concentration of 200 µg protein/ml. Peroxidation was initiated by addition of 1 ml of freshly prepared ferrous ammonium sulfate in 0.175 M KCl to give a final concentration of 20 µM. The suspension was incubated at 4°C or room temperature for one hour at which time enough EDTA was added to give a concentration of 50 µM. In some experiments the incubation mixture contained 0.01 M mercaptoethanol or 0.2 mg of bovine serum albumin (BSA) per ml. The suspension was centrifuged at 10,000 x g for 10 min and the pellet was washed twice in 0.05 M phosphate buffer, pH 7.4 and resuspended in the same buffer.

At certain time intervals, aliquots of the peroxidation incubation mixture were assayed for lipid peroxide by a modification of the Hunter, Gebicki, Hoffsten, Weinstein and Scott (1963) method. One millilitre of the reaction mixture was mixed with 1 ml of 20% (w/v) trichloroacetic acid in 0.5 M HCl and 1 ml of 2% (w/v) 2-thiobarbituric acid (TBA) and placed in a boiling water bath for 10 min. After cooling to room temperature and centrifuging at 500 x g for 5 min, the TBA colour formed was read at 532 nm against a blank reaction without protein. Values were plotted directly as absorbance without conversion to malonaldehyde equivalents.

Turbidity changes of mitochondrial suspensions were followed at 520 nm. The SH content during peroxidation was followed essentially by the procedure described in Section 2.12 except 1 ml of reaction mixture was added to 2.8 ml of 0.05 M phosphate buffer, pH 8.0 containing 0.5 mg/ml EDTA with no SDS and the incubation was for 30 min.

2.15 Incorporation of TNBS into Mitochondrial Membranes.

A modified procedure of Godin and Ng (1972) was used by following the increase in adsorbance of the reaction mixture at 335 nm as a function of time. Experiments were carried out at 37°C in a volume of 3 ml containing 1.8 ml of 0.05 M tris buffer, pH 7.4 unless otherwise indicated, 0.1 ml of 6 mM TNBS which had been adjusted to pH 7.0 before use, the compound to be tested in aqueous solution and the volume made up to 2.9 ml with distilled water. The reaction was started by addition of 0.1 ml of 10 mg/ml mitochondrial protein which had been washed three times with 0.01 M tris buffer, pH 7.4 and resuspended in the same buffer. Incubations were for 10 min unless otherwise indicated, at which time 2.0 ml of 5% (w/v) SDS in 0.5 M HCl was added and the absorbance read at 335 nm against the appropriate blank without membranes. Results are expressed as absorbance at 335 nm per 10 min per mg protein.

2.16 Structural Protein Isolation.

Isolated mitochondria were washed twice in 0.25 M sucrose and 1000 mg of mitochondrial protein were suspended in 0.25 M sucrose + 0.025 M phosphate buffer, pH 7.4 to a protein concentration of 2.5 mg/ml. To the suspension was added 4.3 mg of pargyline and 2 mg of (^{14}C) pargyline (14.06 μCi) and the suspension was incubated for 20 min at room temperature followed by centrifugation at 10,000 x g for 20 min. The pellet was washed twice in 0.9% KCl suspended in 0.9% KCl and dialyzed overnight against 20 l of 0.9% KCl. The dialyzed suspension was then centrifuged at 20,000 x g for 20 min and the structural protein was isolated from the pellet by the method of Criddle, Bock, Green and Tisdale (1962) and by the method of Zahler, Saito and Fleischer(1968). Electrophoresis was performed as described in Section 2.11 in the presence of 1% SDS.

The (^{14}C) pargyline-enzyme complex was measured by dissolving 1 to 5 mg of protein in 1 ml of Protosol at 50°C for 12 h. After cooling to room temperature, 5 ml of scintillation fluor was added and the vial counted for 2 to 20 min. Electrophoresis gels were cut in 6 mm sections and incubated in 1 ml of Protosol at 50°C for 24 hours. After cooling to room temperature 5 ml of scintillation fluor was added and the vial counted. The counting efficiency for these two systems calculated from the internal standard method was about 80% for ^{14}C.

2.17 Treatment with Phospholipases.

Suspensions of mitochondria (10 mg protein/ml) and soluble preparations of MAO (10 mg protein/ml) in 0.05 M tris buffer, pH 7.8 were made 1 mM in $CaCl_2$ and 0.5 mg of phospholipase C (Cl.welchii) per 10 mg protein or 0.05 mg of phospholipase A (bee venom) per 10 mg protein was added. Bovine serum albumin (0.2 mg/ml) was also present when phospholipase A was used. The mixtures were incubated at 37°C for 20 min and with mitochondria, the suspension was centrifuged at 20,000 x g for 20 min. The pellet was resuspended in tris buffer for TNBS incorporation experiments or in 0.05 M phosphate buffer, pH 7.4 for enzyme assays and the phospholipid content was determined by the method described in Section 2.12. After incubation of the soluble enzyme preparation, EDTA was added to a concentration of 1 mM and enzyme assays or gel filtration experiments were performed.

2.18 Trypsin Digestion.

Isolated mitochondria were washed with 0.25 M sucrose once and resuspended in 0.25 M sucrose containing 0.05 M phosphate buffer, pH 7.5 to a protein concentration of 2.5 mg/ml. Varying amounts of trypsin (20 to 200 μg trypsin per mg of protein) were added to the

suspension and incubated at 37°C. At varying times samples were removed, a three fold excess of soya bean trypsin inhibitor was added to inhibit the trypsin and assayed for residual MAO activity. In some experiments the mitochondria were lysed by washing with 1 mM phosphate buffer, pH 7.5 before incubating with trypsin. Soluble MAO preparations were treated in a similar manner as described above except sucrose was omitted from the incubation mixture. In some mitochondrial experiments, the turbidity of the suspension was followed at 520 nm.

2.19 Resolution by Sonication.

This procedure was performed essentially as described by Racker and Proctor (1970). Mitochondrial MAO was labelled with (^{14}C) pargyline as described in Section 2.16 and washed 3 times with 0.06 M phosphate buffer, pH 8.0. Suspensions of labelled and unlabelled mitochondria (10 mg protein) in 20 ml of 0.25 M sucrose and 0.06 M phosphate buffer, pH 8.0, were adjusted to pH 9.6 with 1 N NaOH. The suspensions were sonicated 1 to 8 times for 30 seconds with careful cooling in an ice bath. After sonication, the pH was adjusted to 6.0 with 1 N HCl and the membranes isolated by centrifugation at 100,000 x g for 1 h. In some experiments after sonication the membrane suspension was adjusted to pH 7.5 before centrifugation.

3 RESULTS

One way to characterise and study the nature of MAO and its different forms is to use highly purified preparations of the enzyme. Because most purified preparations are tedious to prepare with conventional purification techniques and the recoveries are low (see introduction, Section 1.4) the following section shows the results of attempts to purify the enzyme by affinity chromatography. To use this method, the enzyme must first be prepared for use in column procedures and therefore a number of solubilization methods were utilized. The solubilized enzyme was also subjected to gel filtration and electrophoresis to determine if different fractions could be separated. Partially purified preparations were also prepared for other experiments, the results of which are described in other sections.

3.1 Phospholipid Calibration Curve.

A calibration curve for the determination of phospholipids is shown in Fig. 3. Similar absorbance readings were obtained using either phosphatidylcholine or phosphatidylethanolamine as standards and the colour produced obeys Beer's law through 300 µg of phospholipid.

The chromogenic solution reacts directly with the phospholipid phosphorus in chloroform forming a Prussian blue complex, therefore conversion of organic to inorganic phosphorus is not necessary. Because the coloured complex was dissolved and read in a chloroform-dichloroethane mixture, the presence of water soluble phosphorus (i.e. phosphate buffers) does not interfere with the lipid determination. This method is simple, reproducible and rapid to perform.

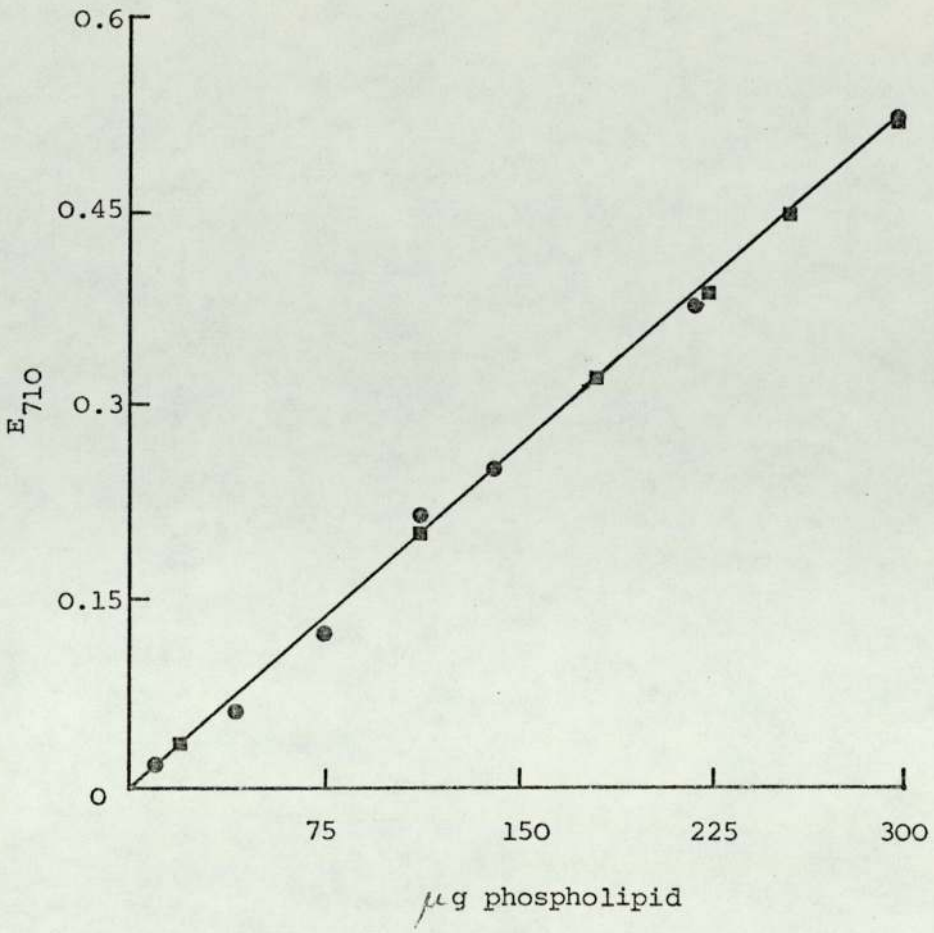


Fig. 3. Calibration curve of phospholipid determination. Assays were performed as described in Section 2.12 and phosphatidylcholine (●) and phosphatidylethanolamine (■) were used as standards. Each point on the graph is the mean of 3 determinations.

3.2 Solubilization of MAO.

3.2.1 Solubilization by Sonication.

Sonication of mitochondria in 0.25 M sucrose and 0.05 M phosphate buffer, pH 7.5 yields, after centrifugation at 100,000 x g, a soluble enzyme preparation and the result of a typical experiment is shown in Table 5. There was a slight decrease in specific activity after sonication and a yield of 54% of the total mitochondrial enzyme in the supernatant. Similar results were obtained using tyramine, benzylamine or serotonin as substrate. Sonication in the presence of glass beads to help break up the membrane or the addition of 1 mM benzylamine to protect the enzyme did not increase the specific activity or the recovery of the enzyme in the supernatant. This preparation could be stored frozen for periods of up to 1 month without loss of activity or precipitation. The enzyme was dialyzed against the affinity column equilibrating buffer before application to the column.

3.2.2 Solubilization by Digitonin.

Incubation of mitochondria with 1.5 mg digitonin per 10 mg of protein results in less than 16% of the enzyme in the supernatant (see Table 6). Increasing the digitonin concentration to 2.5 mg digitonin per 10 mg protein results in 59% of the tyramine, and 61% of the benzylamine activity and 73% of the serotonin activity in the supernatant. As shown in Table 6, the soluble serotonin activity has a slightly higher specific activity than the mitochondrial enzyme whereas the benzylamine activity has a similar specific activity. The enzyme was stable for periods up to 1.5 to 2 months frozen and was dialyzed against the affinity chromatography column equilibrating buffer before application to the adsorbent.

TABLE 5.

Solubilization by Sonication

Step	Total protein	Total Activity	Specific Activity	Yield
	mg	cpm	cpm/mg protein/ 20 min	%
Mitochondria	1450	28,473,650	19,637	100
Supernatant after sonication	1075	15,467,100	14,388	54

MAO was assayed with tyramine as substrate by the ion exchange method. The efficiency of counting in all ion exchange enzyme assays was found to be consistant for each individual experiment between 70 and 76%. Determinations of MAO activity differed by less than 5%.

TABLE 6.

Solubilization by Digitonin Extraction

Step	Assay Substrate	Total Protein	Total Activity	Specific Activity	Yield
		mg	cpm	cpm/mg protein/ 20 min	%
Mitochondria	tyramine	183	11,857,668	64,796	100
	serotonin	183	13,877,073	75,831	100
	benzylamine	183	36,857,481	201,407	100
1.5 mg/10 mg protein digitonin extraction	tyramine	91.8	1,161,363	12,640	10
	serotonin	91.8	2,158,904	23,497	16
	benzylamine	91.8	4,958,304	53,965	13
2.5 mg/10 mg protein digitonin extraction	tyramine	112.2	7,054,799	62,877	59
	serotonin	112.2	10,107,537	90,085	73
	benzylamine	112.2	22,565,103	201,115	61

The efficiency of counting in all toluene extraction enzyme assays was found to be consistant for each individual experiment between 78 and 83%. Determinations of MAO activity differed by less than 3%.

3.2.3 Solubilization by Triton X-100.

As shown in Table 7, treatment of water washed mitochondria with 10% ($^{W}/_{V}$) ammonium sulfate to remove loosely bound membrane proteins followed by extraction with 0.18% triton X-100 increases the specific activity of the soluble enzyme from 1.5 to 3 fold depending on substrate. The amounts of enzyme recovered were 43% of the tyramine activity and 47% and 38% of the benzylamine and serotonin activity respectively. The enzyme could be stored frozen for periods up to a month without loss of activity and was dialyzed against the column equilibrating buffer in affinity experiments.

3.2.4 Solubilization with Perchlorate.

Treatment of mitochondria suspended in distilled water with 0.5 M perchlorate results in removal of moderate amounts of protein but less than 0.4% of the MAO activity. These results are shown in the first two extraction steps in Table 8. The next 4 extractions with perchlorate, performed in 0.05 M tris buffer, pH 8.1 resulted in the release into the 100,000 x g supernatant of between 5 and 10% of the enzyme activity. As shown in Table 8, the specific activity of the released enzyme increases to the fifth extraction after which the specific activity decreases. The amount of enzyme released is fairly constant with the first three extractions in tris buffer, followed by a decrease in the fourth extraction. When the supernatants of the 4 extractions were pooled a 30% recovery of the total enzyme activity was obtained when tyramine was used as substrate.

The soluble enzyme prepared in this manner precipitates at pH values below 7.8 and this can be followed by observing the increase in E_{520} as turbidity develops with decreasing pH (see Fig. 4). The

TABLE 7.

Solubilization by Triton X-100.

Step	Assay Substrate	Total Protein	Total Activity	Specific Activity	Yield
		mg	cpm	cpm/mg protein/ 20 min	%
Mitochondria water washed	tyramine	1000	53,323,000	53,323	100
	serotonin	1000	75,414,000	75,414	100
	benzylamine	1000	130,909,000	130,909	100
$(NH4)_2SO_4$-10%($^{w}/_{v}$) treated mitochondria.	tyramine	532	46,945,808	88,244	88
	serotonin	532	66,280,816	124,588	87
	benylamine	532	116,509,010	219,000	89
0.18% triton X-100 Supernatant	tyramine	189	22,931,937	121,333	43
	serotonin	189	29,155,140	154,260	38
	benzylamine	189	61,527,230	325,540	47

TABLE 8.

Extraction of MAO by Perchlorate

Step	Total Protein	Total Activity	Specific Activity	Yield
	mg	cpm	cpm/mg protein / 20 min	%
Mitochondria	670	12,906,210	19,263	100
Perchlorate extraction in 1 Distilled Water	66.5	48,240	728	0.4
2 Distilled Water	37.62	26,860	714	0.2
3 in Tris	16.12	1,157,748	71,785	8.9
4 in Tris	11.6	1,078,997	93,017	8.3
5 in Tris	9.5	1,039,993	109,473	8.1
6 in Tris	7.5	681,712	90,895	5.2

MAO was assayed with tyramine as substrate by the ion exchange method.

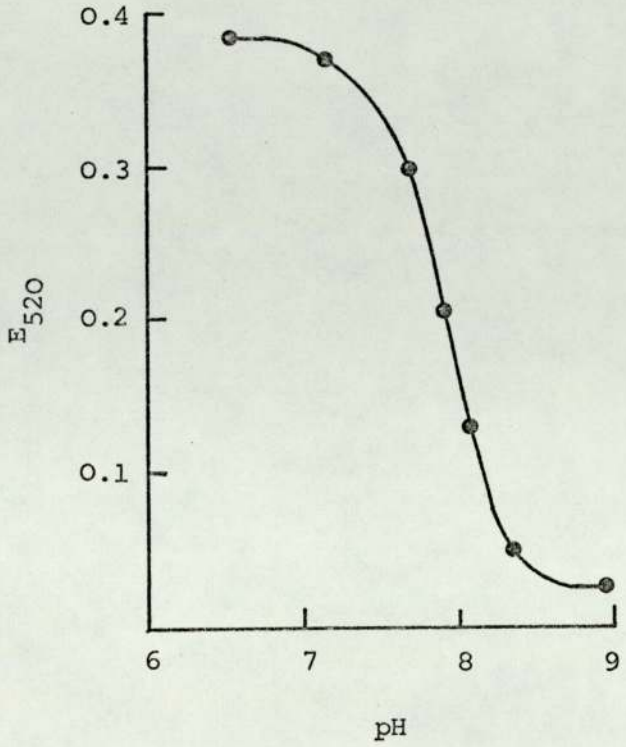


Fig. 4. Effect of pH on the turbidity of perchlorate soluble MAO. Enzyme samples (0.5 mg protein/ml) in 0.05M tris buffer were adjusted to the indicated pH with HCl and measured at 520nm (●).

precipitated enzyme in the pH range 6.8 to 7.5, is still active but could not be resolubilized by increasing the pH back above 7.8. This preparation could be stored at $4^{\circ}C$ for periods up to 1 week after which precipitation occurs. Also freezing of the enzyme solution induces precipitation.

3.2.5 Other solubilization Methods.

Other attempts to solubilize MAO without the aid of detergents are summarized in Table 9. Addition of phospholipase A (see Section 2.17) to a suspension of mitochondria in tris buffer, pH 7.8 in the presence of $CaCl_2$ results in a clearing of the suspension within 5 min. However, centrifugation at 40,000 x g brings all the MAO activity down in the pellet. Treatment of a lysed mitochondial suspension with 200 µg of trypsin per mg protein for 20 min resulted in loss of some activity but only 5 to 10% of the enzyme activity was solubilized.

Table 9 also shows the results of some attempts to solubilize MAO by solvent extraction. When mitochondria were extracted with the organic solvents ethanol, butanol or methyl ethyl betone (see Section 2.5.5) the enzyme lost all activity. In mitochondria suspensions extracted with 50% ($^{v}/_{v}$) ethylene glycol, a polarity reducing agent, the supernatant contained, after removal of ethylene glycol, 10 to 20% of the total MAO activity. However, results varied from preparation to preparation.

3.3 Electrophoresis of Solubilized Preparations.

Polyacrylamide gel electrophoresis of the soluble MAO preparations is shown in Fig. 5. When the gels were stained for protein a number of bands were seen migrating towards the anode with all preparations.

TABLE 9.

Other Solubilization Methods

Method	% of total solubilized
Trypsin digestion	5 to 10
Phospholipase treatment	0
Ethylene glycol (50% $^{v}/_{v}$) extraction	10 - 20
Ethanol extraction	loss of all activity
Butanol extraction	loss of all activity
Methyl ethyl ketone extraction	loss of all activity

MAO activity was measured with tyramine as substrate.

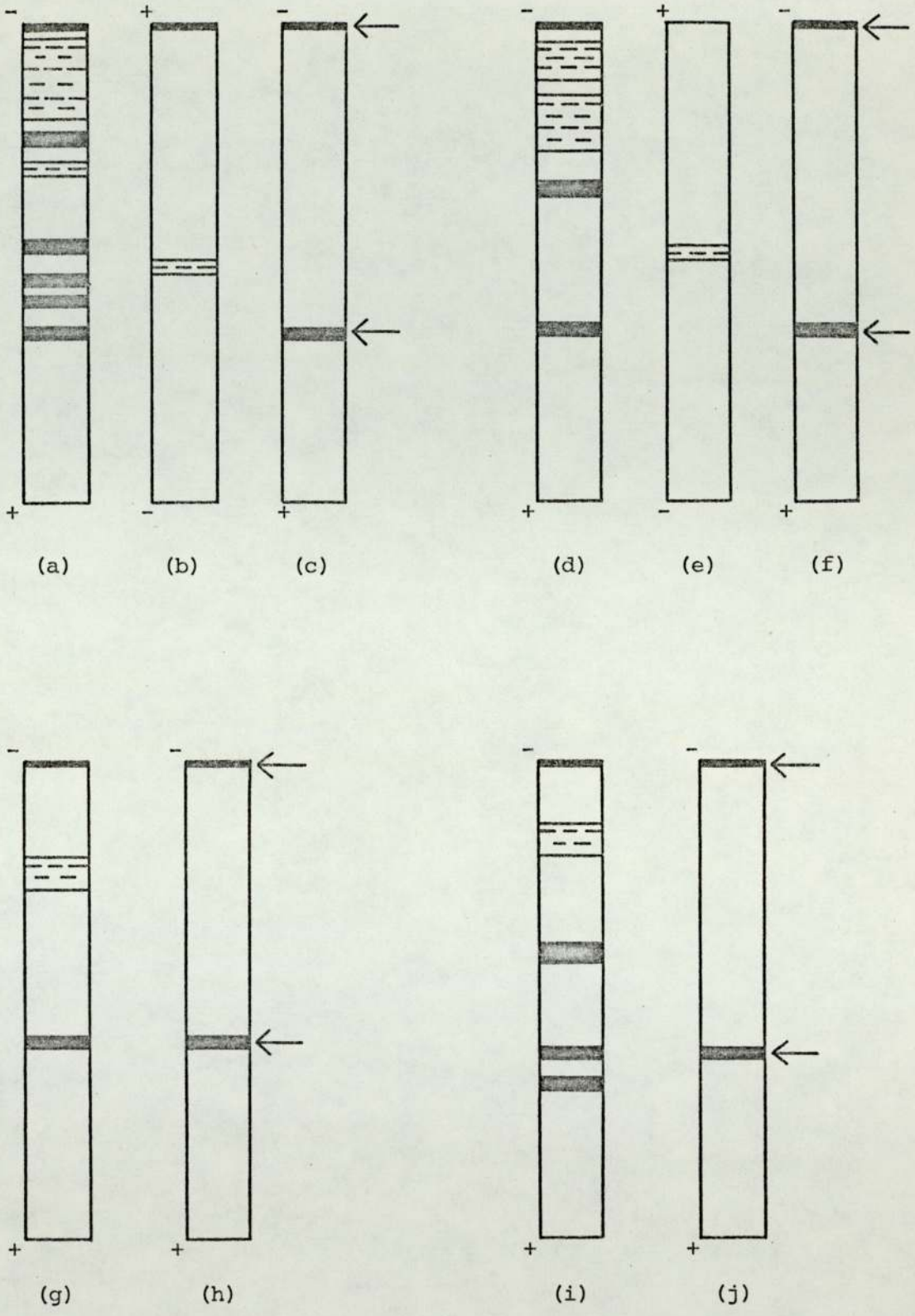


Fig. 5. Electrophoresis of soluble MAO preparations.

Continued/.....

Fig. 5. Electrophoresis of soluble MAO preparations described in Section 2.11. The gels were stained for protein by the method of Hartman and Udenfriend (1971) and for MAO activity by the method of Glenner et al. (1957). Similar results were obtained if either tyramine or tryptamine was the substrate used when staining for MAO activity. (a) Sonicated soluble protein migrating towards the anode. (b) Sonicated soluble protein migrating towards the cathode. (c) Sonicated soluble protein migrating towards the anode and stained for MAO activity. (d) Digitonin soluble protein migrating towards the anode. (e) Digitonin soluble protein migrating towards the cathode. (f) Digitonin soluble protein migrating towards the anode and stained for MAO activity. (g) Perchlorate soluble protein migrating towards the anode. (h) Perchlorate soluble protein migrating towards the anode and stained for MAO activity. (i) Triton X-100 soluble protein migrating towards the anode. (j) Triton X-100 soluble protein migrating towards the anode and stained for MAO activity.

Sonicated and digitonin soluble MAO were the only two preparations to show bands migrating towards the cathode and neither of these bands stained for MAO activity. When the gels were stained for MAO activity, each enzyme preparation showed two bands, one at the origin of the gel and one migrating towards the anode (see Fig. 5). Similar results when staining for MAO activity were obtained when either tyramine or tryptamine was used as substrate in the assay mixture.

3.4 Gel filtration of Solubilized Preparations

When sonicated soluble MAO was subjected to gel filtration on a column of Sepharose 4B that had been equilibrated with 0.05M phosphate buffer, pH 8.0, about 90% of the MAO activity applied was eluted in a position corresponding to the void volume (see Fig. 6). Two protein peaks were retarded but neither contained any MAO activity. When either digitonin or triton X-100 soluble MAO was subjected to gel filtration on Sepharose 4B, the column was equilibrated with 0.05M phosphate buffer, pH 8.0 containing 0.05% triton X-100. Similar results were obtained in that 70% of the digitonin soluble and about 85% of the triton X-100 soluble MAO activity were recovered in the void volume and no activity was detected in the retarded peaks. When perchlorate soluble MAO was applied to a column of Sepharose 4B equilibrated with 0.05M phosphate buffer, pH 8.0, no detectable enzyme activity eluted from the column.

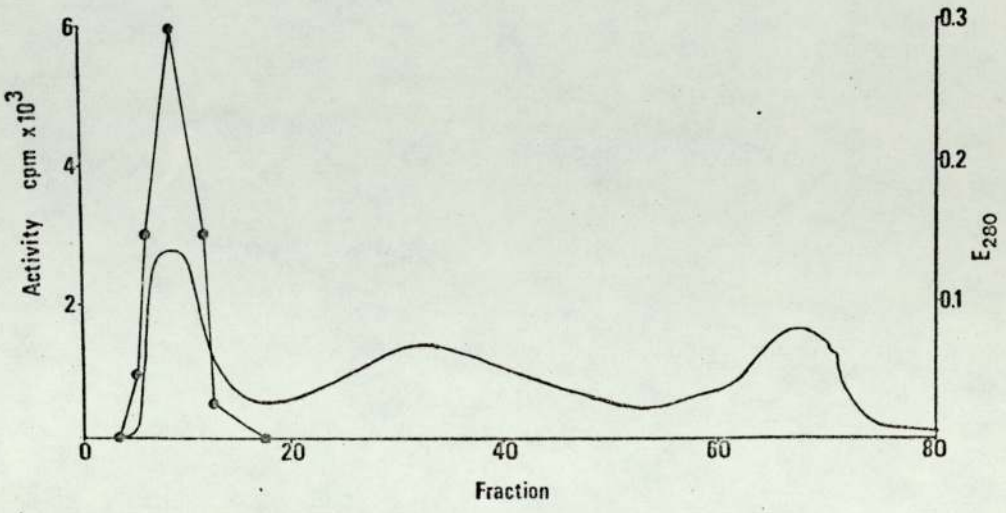


Fig. 6. Gel filtration of sonicated soluble MAO on Sepharose 4B. A 40 cm x 2.5 cm column was equilibrated with 0.05M phosphate buffer, pH 8.0. Enzyme samples were made 1% (w/v) in sucrose before application to the column and 3.2 ml fractions were collected. ——, Protein elution pattern measured at 280 nm and MAO activity measured with tyramine as substrate (●).

3.5 Affinity Chromatography.

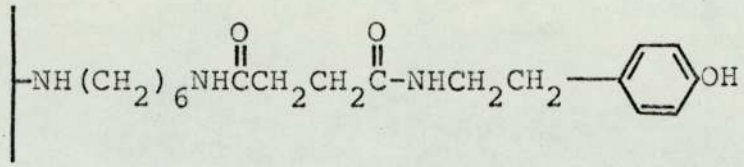
A variety of affinity adsorbents were prepared for use in attempts to purify MAO and the structures are shown in Fig. 7. Because of the possibility of the insoluble matrix interfering with the enzyme - ligand interaction, a 6 carbon chain group (diaminohexane) was chemically inserted between the matrix and the bound ligand of all prepared adsorbents. Also, with some adsorbents as shown in Fig. 7 a second spacer group was used to move the ligand even further from the matrix.

Unless otherwise indicated all affinity adsorbents were equilibrated with 0.005M phosphate buffer, pH 8.0 before application of protein, which was dialyzed against the same buffer, to the column. At the end of each experiment, all columns used were washed with 6M urea to remove any tightly bound protein before reuse.

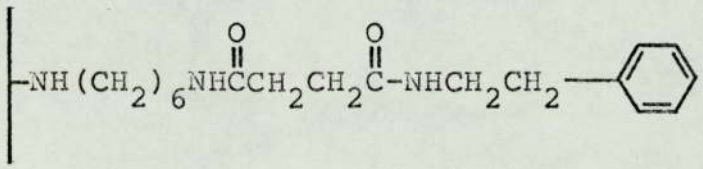
When the Mannich reaction (see Section 2.6.9) was used to prepare the tryptamine and serotonin-Sepharose, two possible bound ligand structures are possible because both the indol and side chain nitrogens contain hydrogen atoms that could react with the aldehyde groups. The structures of the two possible ligands are shown in Fig. 7.

3.5.1 Tyramine and Benzylamine-Sepharose.

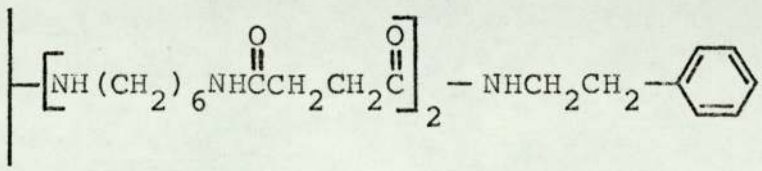
The results of application of sonicated soluble MAO to a column of tyramine-Sepharose are shown in Table 10. Less than 10% of the enzyme activity was bound to the adsorbent. However 34% of the total protein applied to the column was bound increasing the specific activity of the nonbound enzyme by about 1.4. The amount of enzyme bound to the adsorbent could not be increased by reducing the pH to



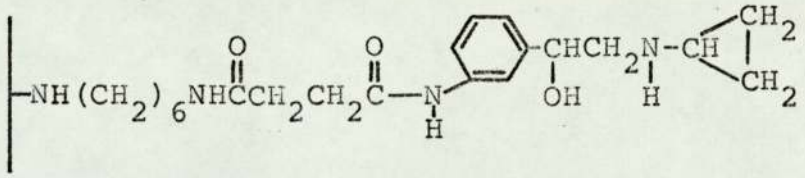
Tyramine-Sepharose, 7.8 μmoles/ml



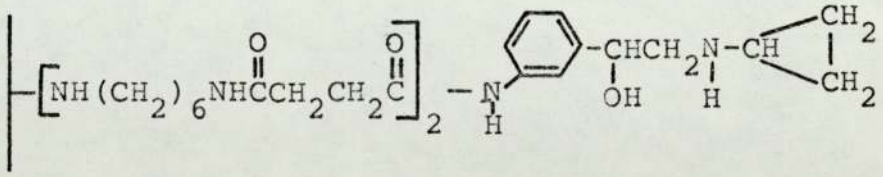
Benzylamine-Sepharose, 6.5 μmoles/ml



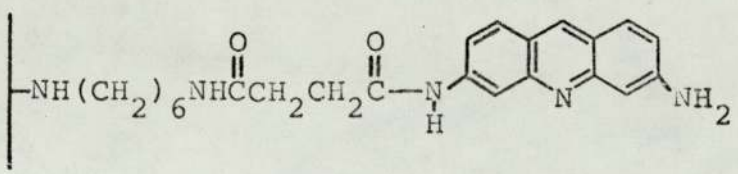
Benzylamine-Sepharose, 4.8 μmoles/ml



AB-15-Sepharose, 6.7 μmoles/ml



AB-15-Sepharose, 6.0 μmoles/ml



Proflavine-Sepharose, 7.1 μmoles/ml

Fig. 7. Affinity chromatography adsorbents.

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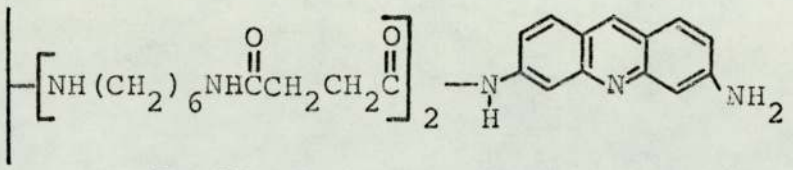
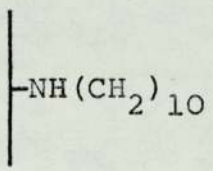
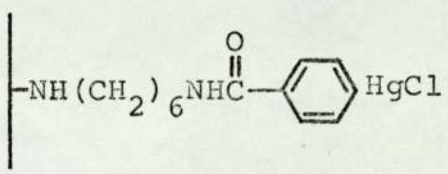


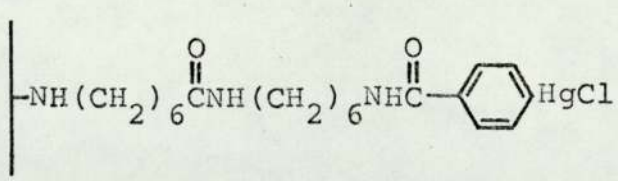
Proflavine-Sepharose, 5.3 μmoles/ml



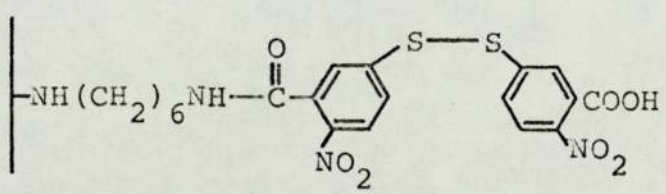
Dodecylamine-Sepharose,



Organomercurial-Sepharose.



Organomercurial-Sepharose.



DTNB-Sepharose, 7.4 μmoles/ml

Fig. 7. Affinity chromatography adsorbents.

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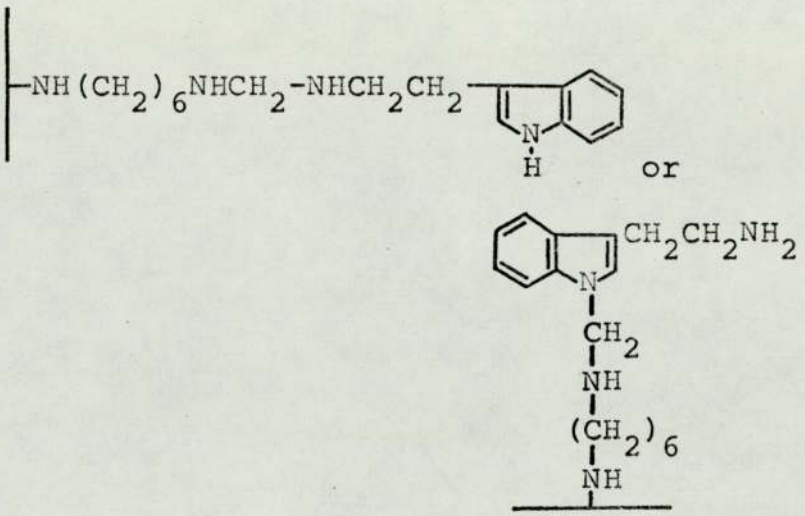
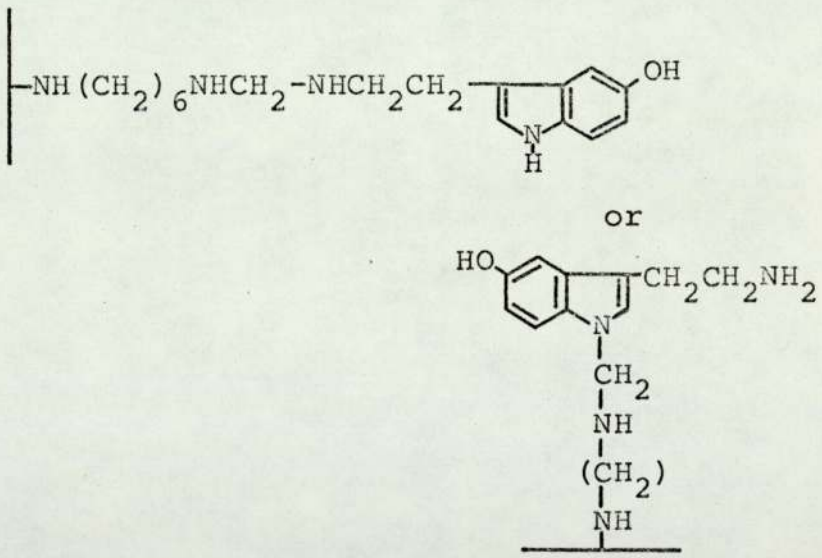


Tryptamine-Sepharose, 8.2 μmoles/ml



Serotonin-Sepharose, 5.9 μmoles/ml

Fig. 7. Affinity chromatography adsorbents.

TABLE 10.

Tyramine-Sepharose with Sonicated Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Sonicated soluble MAO	18.6	381,653	20,519	100	100	-
Nonbound eluate	12.2	346,297	28,385	90.74	66	1.4

Enzyme assayed with tyramine as substrate using the ion exchange method.

7.2 or increasing the pH to 8.8 (above pH 8.0 tris buffers were used). Similar results to those shown in Table 10 were obtained using digitonin, perchlorate and triton X-100 soluble MAO.

When digitonin soluble MAO was applied to a column of benzylamine-Sepharose, less than 5% of the enzyme was bound (see Table 11). Similar to experiments with tyramine-Sepharose, 22% of the protein did bind to the benzylamine-Sepharose slightly increasing the specific activity of the nonbound enzyme. The protein elution pattern of digitonin soluble MAO from a column of benzylamine-Sepharose is shown in Fig. 8 and the bound protein could be eluted with 1M KCl at pH 8.0. Increasing the pH to 8.5 or lowering the pH to 7.2 had no effect on the lack of MAO being bound and similar results were also obtained when benzylamine was attached 2 by 6 carbon side arm lengths from the matrix. When sonicated, triton X-100 or perchlorate soluble MAO were applied to benzylamine-Sepharose, results similar to those shown in Table 11 were obtained.

3.5.2 AB-15-Sepharose.

Sonicated soluble MAO when applied to AB-15-Sepharose as shown in Table 12, bound 10% of the MAO activity and 13.5% of the protein applied. Some of the bound protein could be eluted by increasing the phosphate buffer concentration to 0.2M and decreasing the pH to 7.6 (see Fig. 9). These eluting conditions, released 1.4% of the enzyme with a 5 fold increase in the specific activity (see Table 12). The amount of enzyme bound and released could not be increased by changes in pH or ionic strength or by increasing the distance between the insoluble matrix

TABLE 11.

Benzylamine-Sepharose with Digitonin Soluble MAO

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Digitonin Soluble MAO	9.3	359,008	38,728	100	100	-
Nonbound eluate	7.3	342,997	47,115	95	78	1.2

Enzyme assayed with tyramine as substrate.

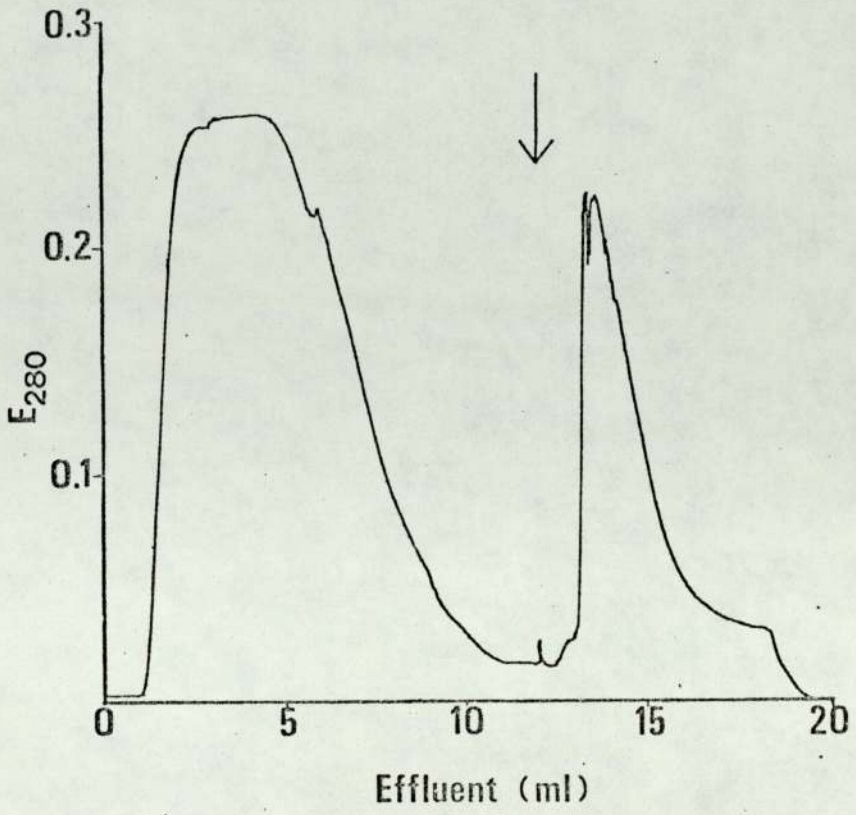


Fig. 8. The protein elution pattern of digitonin soluble MAO from a column of benzylamine-Sepharose. Protein samples in 0.005M phosphate buffer, pH 8.0 were applied to the column and eluted with the same buffer as described in Section 2.7. The arrow indicates when 1M KCl in 0.1M phosphate buffer, pH 8.0 was applied to the column to elute the bound protein. The protein elution pattern was measured at 280 nm (——).

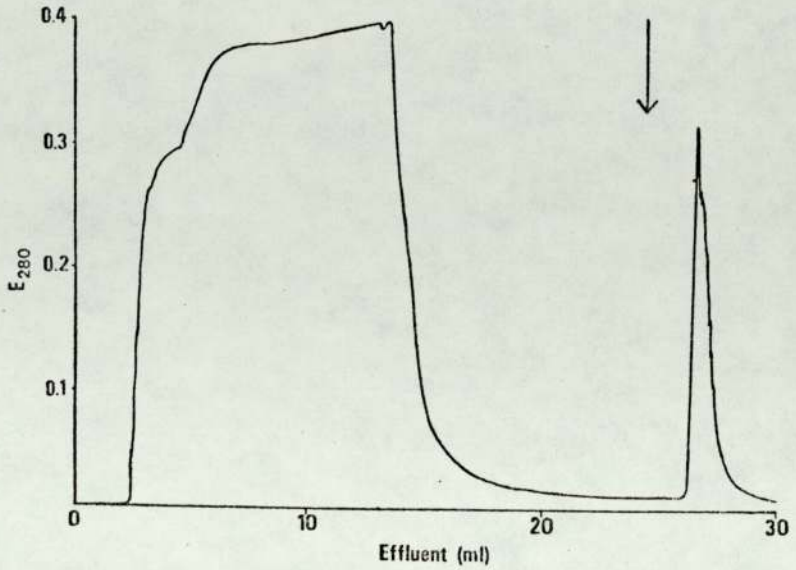


Fig. 9. The protein elution pattern of sonicated soluble MAO from a column of AB-15-Sepharose. Protein samples in 0.005M phosphate buffer, pH 8.0 were applied to the column and eluted with the same buffer as described in Section 2.7. The arrow indicates when 0.2M phosphate buffer, pH 7.6 was applied to the column to elute the bound protein. The protein elution pattern was measured at 280 nm (——).

TABLE 12.

AB-15-Sepharose with Sonicated Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Sonicated Soluble MAO	28.7	373,674	13,020	100	100	-
Nonbound eluate	24.84	338,072	13,610	90.5	86.5	1.0
0.2 M Phosphate buffer pH 7.6 eluate	.08	5,200	65,000	1.4	0.3	5

Enzyme assayed with tyramine as substrate by the ion exchange method.

and the AB-15 ligand by 2 x 6 carbon chain lengths. When digitonin, triton X-100 or perchlorate soluble MAO were applied to the column, no enzyme activity was bound and there was no increase in the specific activity of the nonbound fraction.

3.5.3 Proflavine-Sepharose.

The results of the application of sonicated soluble MAO to proflavine-Sepharose are shown in Table 13. Only 6.0% of the enzyme activity was bound to the adsorbent. However, 10% of the applied protein is bound and most of this can be eluted by passing 1M NaCl through the column. There was no increase in the specific activity of the nonbound MAO fraction and no enzyme is released when the column was washed with 1M NaCl. Similar results to that shown in Table 13 were obtained by varying the pH, increasing the spacer group length or with triton X-100 and perchlorate soluble MAO.

When digitonin soluble MAO was applied to a column of proflavine-Sepharose, 95% of the enzyme activity and 78% of the protein were bound to the column (see Table 14). This indicated a non-specific binding possibly between the ligand (proflavine) and MAO bound digitonin. Changes in pH and ionic strength or passage of 0.05M proflavine would not elute the bound protein. However, as shown in Table 14, 34% of the bound protein could be eluted with 6M urea and after dialysis to remove urea, 25% of the enzyme was found in this fraction with a decrease in specific activity.

TABLE 13.

Proflavine-Sepharose with Sonicated Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Sonicated soluble MAO	30	458,700	15,290	100	100	-
Nonbound eluate	27.1	432,691	15,984	94	90	-

Enzyme assayed with tyramine as substrate using the ion exchange method.

TABLE 14.

Proflavine-Sepharose with Digitonin Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	CPM	cpm/mg protein /20 min	%	%	
Digitonin soluble MAO	24.6	1,359,888	55,280	100	100	-
Nonbound eluate	5.2	72,074	13,967	5	22	-
Urea eluate	8.4	339,972	40,666	25	34	-

Enzyme assayed with tyramine as substrate.

3.5.4 Tryptamine and Serotonin-Sepharose.

When samples of perchlorate soluble MAO at pH 8.0 were applied to a column of tryptamine-Sepharose, 73% of the enzyme and 86% of the protein were bound to the adsorbent (see Table 15a). The non-bound enzyme had an increase in specific activity by a factor of 1.9. Some of the bound protein could be eluted with 1M NaCl in 0.1M phosphate buffer, pH 8.0 but this fraction contained no MAO activity. The enzyme could not be eluted in an active form by changes in pH, or ionic strength or by 6M urea. Similar results were obtained with the serotonin-Sepharose as shown in Table 15b.

The fact that MAO could not be eluted from the absorbents even under denaturing (6M urea) conditions, indicated a possible irreversible attachment to the matrix. Evidence supporting this is shown in Fig. 10 where samples of perchlorate soluble MAO are applied to electrophoresis gels in which the bottom half of the gel contains either tryptamine-Sepharose or unsubstituted Sepharose as control. After electrophoresis, the protein on the control gel migrated half way through the Sepharose layer whereas, protein applied to the tryptamine-Sepharose gel migrated and stopped at the beginning of the Sepharose layer. Staining the gels for MAO activity showed that the protein bound to the tryptamine-Sepharose had retained its MAO activity.

3.5.5 Dodecylamine-Sepharose.

Table 16a shows the results of sonicated soluble MAO on a column of dodecylamine-Sepharose. This absorbent bound 59% of the enzyme activity and 46% of the protein was bound to the adsorbent and

TABLE 15.

Tryptamine and Serotonin-Sepharose with Perchlorate Soluble MAO.

a. Tryptamine-Sepharose.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Perchlorate soluble MAO	11.64	721,435	61,979	100	100	-
Nonbound eluate	1.7	191,706	115,486	27	14	1.9

MAO assayed with tyramine as substrate.

b. Serotonin-Sepharose.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Perchlorate soluble MAO	27	1,684,476	62,388	100	100	-
Nonbound eluate	5.2	341,376	65,649	20	19	1.1

MAO assayed with tyramine as substrate.

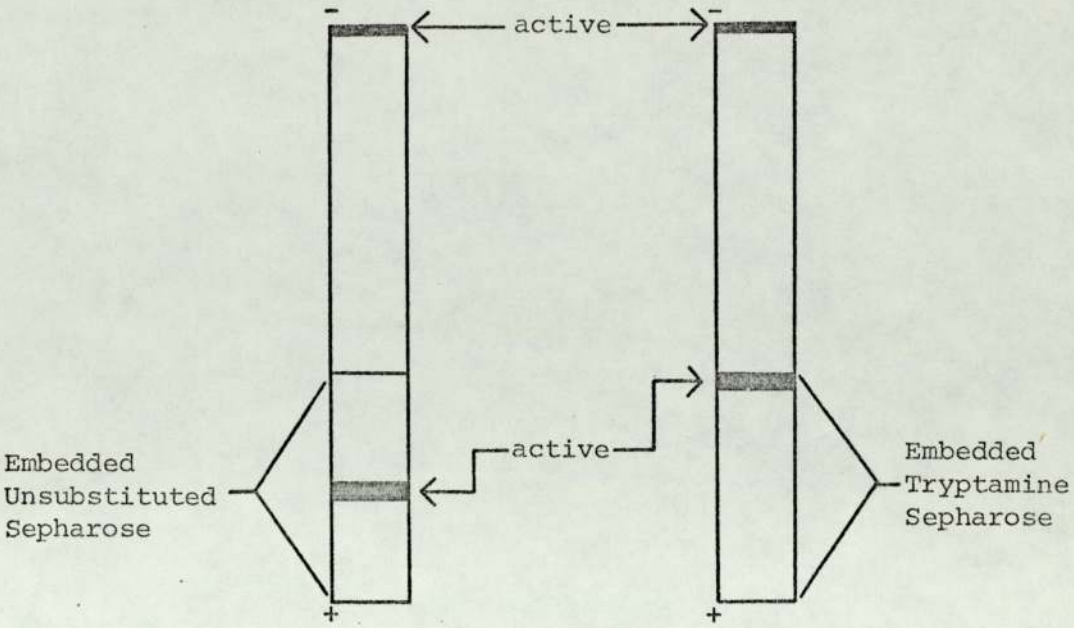


Fig. 10. Electrophoresis of perchlorate soluble MAO in polyacrylamide gels in which the bottom half of the gel contained either unsubstituted or tryptamine- Sepharose. The gels were prepared and electrophoresis was carried out from cathode to anode for 50 min as described in Section 2.11. The gels were stained for MAO activity by the method of Glenner et al. (1957).

TABLE 16.

Affinity Chromatography with Dodecylamine-Sepharose

a. Sonicated Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Sonicated soluble MAO	3.9	79,556	20,090	100	100	-
Nonbound eluate	2.1	32,118	15,150	41	54	-

Enzyme assayed with tyramine as substrate using the ion exchange method.

b. Triton X-100 Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Triton X-100 soluble MAO	5.0	510,955	101,380	100	100	-
Nonbound eluate	2.2	157,516	71,600	31	44	-

Enzyme assayed with tyramine as substrate.

most of the protein could be eluted by passing a solution of 50% (v/v) ethylene glycol in 0.1M phosphate buffer, pH 8.0 (see Fig. 11). However, this fraction contained no MAO activity after passage through a column of Sephadex G-50 to remove the ethylene glycol. The bound enzyme could not be eluted with changes in pH or ionic strength or by denaturing (6M urea) conditions. Similar results were obtained with triton X-100 soluble MAO (see Table 16b) and with digitonin and perchlorate soluble MAO.

3.5.6 Affinity Chromatography with SH Binding Adsorbents.

When sonicated soluble MAO was applied to a column of organo-mercurial - Sepharose 2% of the MAO activity and 72% of the protein was bound (see Table 17a). The specific activity of the nonbound MAO was increased by a factor of 3.5. Some of the bound protein could be eluted with 0.05M cysteine or 0.05 M mercaptoethanol in 0.1M phosphate buffer, pH 8.0 (see Fig. 12). However, it was found that some protein was bound irreversibly to the matrix reducing its capacity in subsequent experiments and reducing the overall usefulness of this adsorbent. Similar results were obtained with digitonin and triton X-100 soluble MAO and Table 17b shows that the specific activity of perchlorate soluble MAO is also increased 4 times by this method. Increasing the distance of the ligand from the insoluble matrix with 2 x 6 carbon chain lengths does not result in an increased of the amount of MAO bound.

Because of the irreversible binding of protein to the organo-mercurial - Sepharose, this column has a limited lifespan. Therefore use was made of the DTNB-Sepharose which binds SH containing proteins and elution of bound protein is easily accomplished by passage

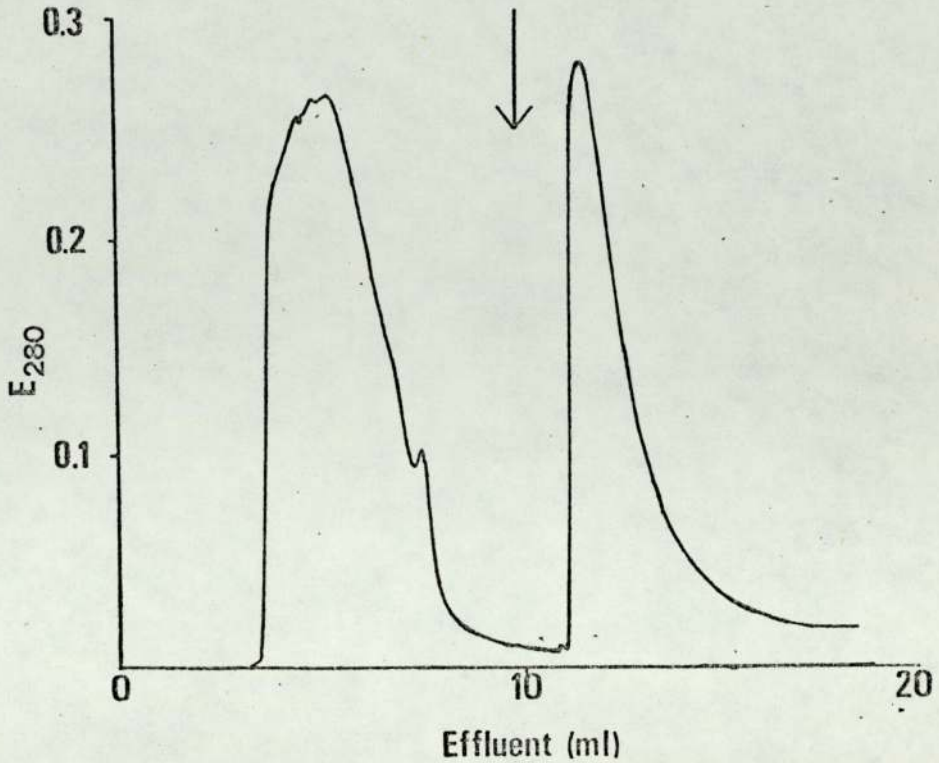


Fig. 11. The protein elution pattern of sonicated soluble MAO from a column of dodecylamine-Sepharose. Protein samples in 0.005M phosphate buffer, pH 8.0 were applied to the column and eluted with the same buffer as described in Section 2.7. The arrow indicates when a solution of 50% (v/v) ethylene glycol in 0.1M phosphate buffer, pH 8.0 was applied to the column to elute the bound protein. The protein elution pattern was measured at 280 nm (——).

TABLE 17.

Affinity Chromatography with Organomercurial-Sepharose.

A. Sonicated Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein/ 20 min	%	%	
Sonicated soluble MAO	35	551,845	15,767	100	100	-
Nonbound eluate	9.8	539,655	54,843	98	28	3.5

MAO assayed with tyramine as substrate using the ion exchange method.

B. Perchlorate Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein /20 min	%	%	
Perchlorate soluble MAO	10.5	935,046	89,222	100	100	-
Nonbound eluate	2.6	920,222	353,931	98	24	4

MAO assayed with tyramine as substrate.

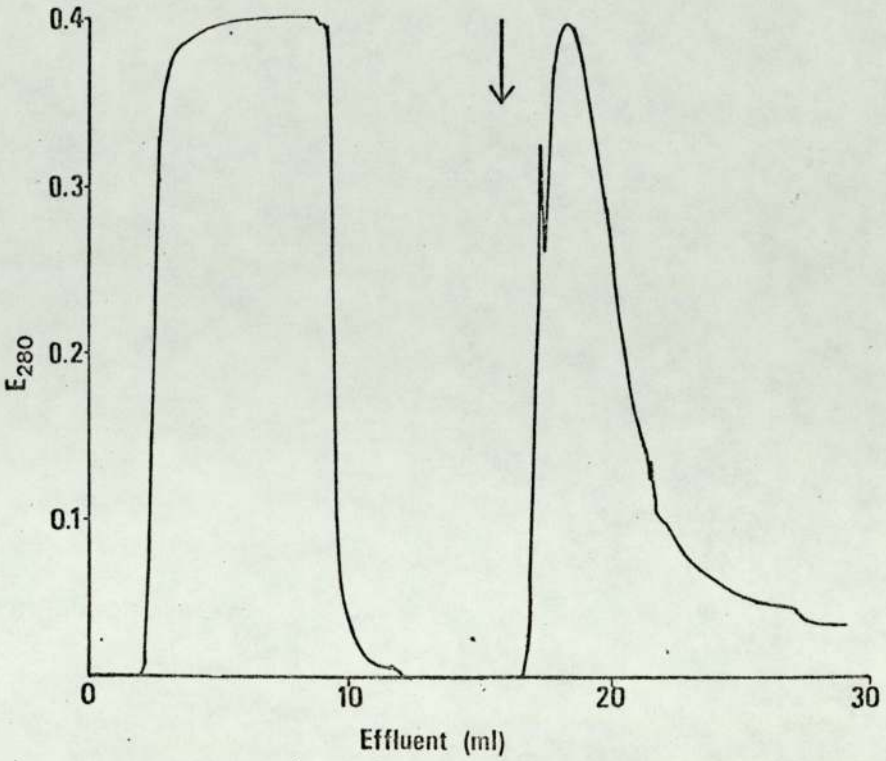


Fig. 12. The protein elution pattern of sonicated soluble MAO from a column of organomercurial-Sepharose. Protein samples in 0.005M phosphate buffer, pH 8.0 were applied to the column and eluted with the same buffer as described in Section 2.7. The arrow indicates when a solution of 0.05M cysteine in 0.1M phosphate buffer, pH 8.0 was applied to the column to elute the bound protein. The protein elution pattern was measured at 280 nm (——).

of 0.05M mercaptoethanol in 0.1M phosphate buffer, pH 8.0 through the column. When triton X-100 soluble MAO is applied to a column of DTNB-Sepharose 80% of the contaminating protein and 19% of the MAO activity is bound. After dialysis to remove the TNB anion released by the bound protein, the specific activity of MAO had increased 4 times (see Table 18).

3.5.7 Affinity Chromatography with Phospholipase A Treated MAO.

Treatment of triton X-100 and sonicated soluble MAO with phospholipase A to remove some of the protein bound lipids results in less than 15% loss of enzyme activity (see Section 2.17). Application of these preparations to DTNB-Sepharose results in 86% of the MAO activity and 95% of the protein being bound to the matrix (see Table 19). The specific activity of the unbound enzyme increased 3 times but was only 5% of the total activity applied to the column. Up to 80% of the bound protein and 50% of the bound MAO activity could be eluted with 0.05M mercaptoethanol in 0.1M phosphate buffer, pH 8.0. As Table 19 shows, there was no increase in the specific activity of bound MAO after elution.

When phospholipase A treated triton X-100 soluble MAO preparation was applied to a column of proflavine-Sepharose, 70% or more of the MAO activity and 19% of the protein did not bind to the column, increasing the specific activity of this fraction by about 4 (see Table 20). Figure 13 shows a protein trace of this experiment and most of the bound protein could be eluted with 1M KCl in 0.1M phosphate buffer, pH 8.0.

TABLE 18.

DTNB-Sepharose with Triton X-100 solubilized MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein / 20 min	%	%	
Triton X-100 soluble MAO	33.06	2,260,500	68,500	100	100	-
Nonbound eluate	6.36	1,742,640	274,000	77	19	4.0

TABLE 19

DTNB-Sepharose with Phospholipase A treated Triton X-100 Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein/ 20 min	%	%	
Phospholipase A treated triton X-100 soluble MAO.	22.6	1,078,020	47,700	100	100	-
Nonbound eluate	1.1	154,530	144,491	14	5	3
0.05 M eluate	17.6	490,045	27,811	46	78	-

TABLE 20.

Proflavine-Sepharose with Phospholipase A treated Triton X-100 Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein / 20 min	%	%	
Phospholipase A treated triton X-100 soluble MAO.	22.5	955,980	42,318	100	100	-
Nonbound eluate	4.2	669,480	159,400	70	19	4

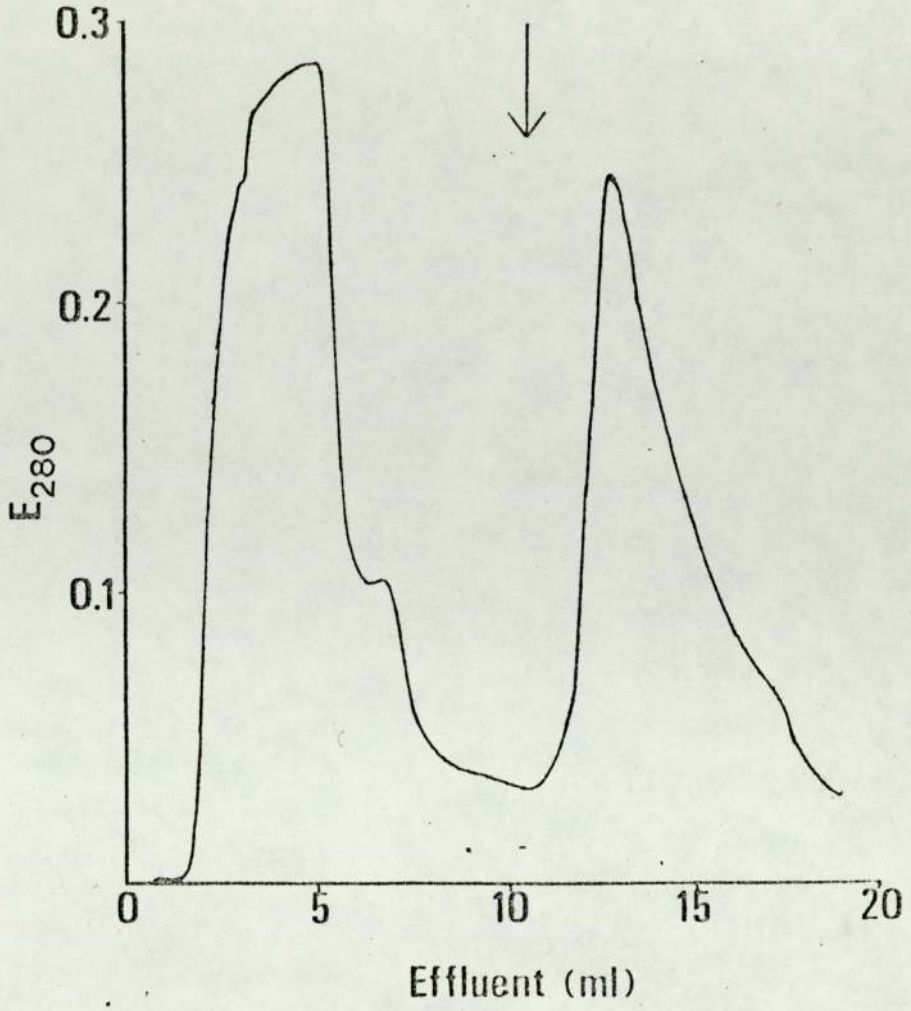


Fig. 13. The protein elution pattern of phospholipase A treated, triton X-100 soluble MAO from a column of proflavine-Sepharose. Protein samples in 0.005M phosphate buffer, pH 8.0 were applied to the column and eluted with the same buffer as described in Section 2.7. The arrow indicates when a solution of 1M KCl in 0.1M phosphate buffer, pH 8.0 was applied to the column to elute the bound protein. The protein elution pattern was measured at 280 nm (——).

Application of the phospholipase A treated MAO preparations to a column of AB-15-Sepharose provides results as shown in Table 21. The matrix bound 91% of the MAO activity and 95% of the applied protein. The unbound fraction of the enzyme had a specific activity increase of 1.9 times but this fraction accounts for only about 10% of the applied enzyme. Small amounts of bound protein could be eluted with 2M NaCl, 50% ($^{v}/_{v}$) ethylene glycol, 0.5M sodium perchlorate and 6M urea all in 0.1M phosphate buffer, pH 7.4. However, after dialysis no MAO activity could be detected indicating either the enzyme was not released or was irreversibly denatured by the eluting conditions.

The phospholipase A treated MAO preparation when applied to tyramine, benzylamine or dodecylamine-Sepharose did not vary significantly from the results shown in Tables 10, 11 and 16 respectively.

3.6 Partial Purification of MAO.

Although none of the affinity adsorbents utilized gave a single step purification, the DTNB-Sepharose column used in conjunction with the procedures shown in Table 22 yields an enzyme preparation 15 fold purer than the starting material. Mitochondria lysed with low ionic strength buffer and washed with 10% ammonium sulfate increased the specific activity of the insoluble MAO by 2.5 times over the liver homogenate. Solubilization of the enzyme with triton X-100 followed by precipitation with ammonium sulfate recovers 43% of the enzyme 5.8 times purer. Resuspension of the enzyme in 0.05M phosphate buffer, pH 8.0 and eluted from the DTNB-Sepharose^ This unbound fraction recovered 34% of the MAO activity. was applied to a column of DEAE-Sephadex which bound the TNB anion and some protein but less than 5% of the MAO activity. This preparation was about 15 fold purer than the starting material and yielded about 30% of the mitochondrial MAO activity (see Table 22).

TABLE 21.

AB-15-Sepharose with Phospholipase A treated triton X-100 Soluble MAO.

Step	Total protein	Total Activity	Specific Activity	Enzyme Yield	Protein Yield	Purification
	mg	cpm	cpm/mg protein/ 20 min	%	%	
Phospholipase A treated triton X-100 soluble MAO	22.5	955,980	42,318	100	100	-
Nonbound eluate	1.1	87,098	78,466	9	5	1.9

MAO assayed with tyramine as substrate.

TABLE 22.

Partial Purification of MAO.

Step	Total protein	Total Activity	Specific Activity	Yield	Purification
	mg	cpm	cpm/mg protein/ 20 min	%	
Mitochondria lysed and 10% $(NH_4)_2SO_4$ treated	1,000	53,323,000	53,323*	100	2.5
Triton X-100 (0.18%) extraction and 20% $(NH_4)_2SO_4$ ppt.	189	22,931,937	121,333	43	5.8
Elution from DTNB-Sepharose.	71.2	18,129,820	254,799	34	12
Elution from DEAE-Sephadex	52.3	15,996,900	305,758	30	15

MAO was assayed using tyramine as substrate.

* The lysed and $(NH_4)_2SO_4$ washed mitochondrial preparation represents approximately a 2.5 fold purification over the liver homogenate.

4 DISCUSSION

4.1 Solubilization of MAO.

In order to obtain highly purified preparations of membrane bound proteins, the usual first step is to isolate the membrane of interest followed by solubilization of the membrane proteins. MAO of rat liver mitochondria is tightly bound to the outer mitochondrial membrane and therefore solubilization of the enzyme has been accomplished with few exceptions only by rather harsh measures (see Sections 1.3 and 1.4). One of the main reasons for solubilizing rat liver mitochondrial MAO in the present work was for use in affinity chromatography experiments which in a majority of purification attempts requires the enzyme to be in a form acceptable for column chromatography procedures. Since the method of affinity chromatography depends on the interaction of a bound ligand and the protein of interest, obtaining highly purified preparations requires that nonspecific interactions on the column must be reduced as much as possible. For this reason, solubilization of MAO was attempted by a number of different techniques with and without the use of detergents.

4.1.1 MAO Solubilized by Detergents.

Two detergents were used to solubilize rat liver MAO, triton X-100 and digitonin. In the present work, after treating the mitochondria with low and high ionic strength solutions to remove matrix and adsorbed proteins, between 40 and 50% of the enzyme was in the 40,000 x g supernatant in the presence of 0.18% triton X-100 (see Table 7). At this detergent concentration, the enzyme sedimented when treated with ammonium sulfate whereas any increase in the triton X-100 concentration beyond

0.18% resulted in a floating enzyme preparation. A number of workers have used triton X-100 in the range of 0.5 to 2.0% obtaining preparations which contained from 14 to 90% of the MAO activity (Barbato and Abood, 1963; Yasunobu et al., 1968; Norstrand and Glantz, 1973; Shih and Eiduson, 1973).

It was found that the recovery of the soluble serotonin activity (see Table 7) was reduced as compared to benzylamine and tyramine activity. This was in agreement with the findings of Shih and Eiduson (1973) and Severina and Zhivotova (1973) who suggested the detergent causes conformational changes in the enzyme imparing the serotonin activity.

A modified method of Erwin and Hellerman (1967) was used to extract MAO with digitonin and up to 80% of the enzyme was released into the 40,000 x g supernatant. Two concentrations of digitonin were used for extraction, 1.5 and 2.5 mg digitonin per 10 mg of protein but only the higher value gave consistently good results. While this work was in progress, Abdul Matleb and O'Brian (1975) reported that MAO was released into the supernatant over the concentration range of 1.5 to 2.5 mg digitonin per 10 mg protein which is in agreement with the method reported here. In contrast to the triton X-100 treated enzyme, the digitonin soluble enzyme had a reduced recovery of benzylamine activity (MAO B) as compared to serotonin activity (see Table 6) suggesting that digitonin may induce conformational changes in the enzyme impairing the benzylamine oxidizing capacity.

4.1.2 Solubilization without Detergents.

A number of attempts were made to solubilize MAO without the aid of detergents. Sonication has been used by a number of workers to solubilize MAO (see Table 1) and in the present study a soluble MAO was obtained by sonicating mitochondria at pH 7.5 in the presence of sucrose. About 55% of the enzyme was recovered and unlike the detergent solubilized enzyme no preferential decrease in MAO A or MAO B was observed. The amount of MAO recovered in the 100,000 x g supernatant could not be increased by the use of glass beads to help break up the membrane or by the presence of substrate to protect the active site as used by Houslay and Tipton, (1973) and Youdim and Sandler, (1968). These workers used detergent in combination with sonication and since detergent inhibits the enzyme (Severina and Zhivotova, 1973) perhaps the presence of substrate protected the enzyme from detergent inhibition.

Recent evidence has indicated that chaotropic agents destabilize water structure and this correlates well with their ability to increase the water solubility of nonelectrolytes and to destabilize membranes and enzyme complexes (for a review see Hatefi and Hanstein, 1974). Mitochondrial membranes treated with the chaotropic agent sodium perchlorate at pH 8.1 releases in a series of extractions, up to 30% of the MAO into the 100,000 x g supernatant (see Table 8). Since the specific activity of the extracted enzyme increases from 3 to 5.5 times, the solubilization procedure is also an effective purification step. Weakening of the hydrophobic interaction between protein and lipid is the most likely reason for the release of MAO and this is supported by the release of pig liver MAO into buffer after extraction of mitochondrial lipid with methyl ethyl ketone (Hollunger and Oreland, 1970).

MAO solubilized with perchlorate is an unstable preparation. Similar to the perchlorate treated preparation described by Houslay and Tipton (1973), precipitation occurred at pH values between 7.5 and 7.8 (see Fig. 4). This preparation could not be stored frozen or left for more than a few days at 4°C without precipitation. This may be due to polymerization, incomplete solubilization or most likely a combination of both. The usefulness of this preparation was very limited but was suitable for affinity chromatography experiments involving short (5 cm) bed height columns as filtration on columns with longer beds resulted in no enzyme elution (see Section 3.4).

Other procedures used in attempts to solubilize MAO without the aid of detergents (see Table 9) included solvent extraction as described by Hollunger and Oreland (1970) for solubilizing MAO from pig liver. However, extraction of rat liver mitochondria with ethanol, butanol or methyl ethyl ketone inactivated the enzyme, this difference may reflect a species difference in the enzyme. Treatment of mitochondrial membranes with the polar reducing agent ethylene glycol released between 10 and 20% of the MAO activity but the results varied between preparations and was therefore of little value. Some membrane bound enzymes such as tyrosine hydroxylase can be solubilized by controlled proteolytic digestion (Petrack, Sheppy and Feltzer, 1968) however, treatment of mitochondrial membranes with trypsin, released only between 5 and 10% of the MAO activity. A number of enzymes can be solubilized by treatment of membranes with phospholipase A (see Section 6.5 for a detailed discussion) but MAO was not released by this procedure (see Table 9) which is in agreement with the findings of Barbato and Abood (1963).

4.2 Electrophoresis and Gel Filtration of Soluble MAO

Polyacrylamide-gel electrophoresis indicated the similarity of the sonicated, perchlorate, triton X-100 and digitonin soluble MAO preparations (see Fig. 5). Each preparation had two bands of MAO activity one migrating towards the anode and one at the origin. This differs from the large variation in bands seen with other rat liver preparations (Sandler and Youdim, 1972). Houslay and Tipton (1973) showed that the band of MAO activity at the origin of the gel obtained by Youdim and Collins (1971) was due to the gel loading procedure using dry Sephadex. Using sucrose to load the protein on the gel, it was found that the origin band no longer appeared. However, in the present work, the protein was also loaded on the gels in the presence of sucrose but an origin band still appeared. This may be due to a highly polymerized enzyme or to a partially soluble fraction that could not penetrate the gel during electrophoresis.

When each of the solubilized preparations of MAO were applied to a column of Sepharose 4B, all the detectable enzyme activity eluted in a single peak in the void volume except the perchlorate soluble enzyme which did not elute probably due to precipitation on the column. This is in contrast to the sonicated, triton X-100 soluble preparation of Houslay and Tipton (1973) which was retarded as a single peak on a Sepharose 4B column and is also in contrast with Ragland (1968) who partially separated 3 peaks of detergent soluble rat liver MAO on a Sephadex G-200 column. These differences most likely reflect the different solubilization procedures used. For example, Houslay and Tipton (1973) used a combination of detergent and sonication and Ragland (1968) solubilized his preparation by extracting mitochondria with two detergents; sodium lauryl sulfate and a non-ionic detergent.

4.3 Affinity Chromatography.

There are a large number of compounds that interact with MAO which indicates that the technique of affinity chromatography may be useful in purifying the enzyme. However, the number of potentially useful ligands is limited to those which contain reactive groups for coupling to an insoluble matrix.

Evidence has accumulated indicating that MAO contains hydrophobic regions either in or adjacent to the active site capable of interacting with hydrocarbon or phenyl groups and a polar region which interacts with some phenyl group substituents (Severina and Sheremet'evskya, 1967; McEwen et al., 1969; Severina, 1973). The attachment of tyramine and benzylamine through their side chain amino groups to succinylated amino hexane-Sepharose may allow an interaction involving the hydrophobic and polar region to take place (see Fig. 7). Also, covalent attachment through the amino group would probably eliminate the possibility of MAO oxidizing the bound substrate. Further attempts could be made to bind MAO through hydrophobic interactions by attachment of dodecylamine to Sepharose. This adsorbent has been used successfully to isolate plasma lipids (Deutsch et al., 1973). The substrates tryptamine and serotonin were also attached to Sepharose by the Mannich reaction (see Fig. 7). This method was used by Ranadive and Sehon (1967) to attach serotonin through the indole nitrogen to BSA for the preparation of antibodies to serotonin. However, because both substrates contain reactive hydrogens on both the indole and side chain nitrogens the bound ligand may be a mixture attached through both sites (see Fig. 7).

Competitive inhibitors can be useful ligands in purifying enzymes by affinity chromatography (Cuatrecasas and Anfinsen, 1971).

The substrate analog inhibitor AB-15 (Huszti, 1972) and proflavine (Gorkin et al., 1964) are both competitive inhibitors of MAO that contain free amino groups suitable for covalent attachment to Sepharose. In addition, rat liver MAO contains SH groups and some of them are freely reactive (Klyashtorin and Gridneva, 1966; Symes and Sourkes, 1975). These groups may therefore be useful in binding the enzyme to an adsorbent containing covalently bound p-chloromercuribenzoate or DTNB.

In general, elution of sonicated, perchlorate, digitonin or triton X-100 soluble MAO from the affinity adsorbents resulted in either little enzyme being bound or in some cases the bound enzyme could not be eluted in an active form. Enzyme preparations applied to tyramine or benzylamine-Sepharose did not bind, but the eluate had a slightly increased specific activity (see Tables 10 and 11). However, the increase in purity was not sufficient enough to justify using these two adsorbents in a purification procedure. Similar results were obtained when triton X-100, sonicated or perchlorate soluble MAO was applied to proflavine-Sepharose (see Table 13). When digitonin soluble MAO was applied to the proflavine column 95% of the MAO activity and 80% of the protein was bound and this could be eluted with urea but with no increase in specific activity (see Fig. 14). This indicated an interaction between the protein bound digitonin and the ligand. Dodecylamine-Sepharose was unhelpful in purifying the enzyme as 50% was bound and could not be eluted. The nonbound fraction did not have an increase in specific activity (see Table 16). The AB-15 adsorbent was ineffective in both binding and purifying most soluble MAO preparations, however, about 10% of the sonicated soluble MAO was bound and 1.4% could be eluted 5 fold pure (see Table 12). The amount bound could not be increased by varying the buffer conditions (ionic strength or pH).

When the soluble MAO preparations were applied to either tryptamine or serotonin-Sepharose most (80%) of the enzyme and protein were bound and little could be eluted (see Table 15). The protein seemed to be bound in an irreversible manner and a possibility may be that the presence of formaldehyde during the Mannich reaction activated the Sepharose in some way so that the protein became bound in a covalent way. An interesting aspect of the tryptamine-Sepharose was that the bound MAO was still enzymatically active. This was indicated by applying a sample of MAO to an acrylamide gel in which the bottom half contained either tryptamine or unsubstituted Sepharose. After electrophoresis, MAO stained for activity at the top of the tryptamine-Sepharose and in the middle of the control gel (see Fig. 10). This suggests that this method may have a future use for attaching MAO to an insoluble matrix for studying membrane effects on the enzyme. For purification purposes however, these adsorbents were not helpful.

The SH binding adsorbents were found to be useful in purifying MAO. Organomercurial-Sepharose did not bind any of the soluble MAO but did bind large amounts of contaminating protein, increasing the specific activity of the nonbound fraction 4 times (see Table 17). This adsorbent had a limited lifespan, however, as with each experiment, some protein was bound irreversibly reducing the capacity for further binding of contaminating protein. Therefore, use was made of DTNB-Sepharose which purified the nonbound MAO fraction 4 fold and with triton X-100 soluble MAO, bound about 20% of the MAO (see Table 18). This adsorbent did not bind protein in an irreversible manner.

There may be several reasons why in most experiments MAO did not bind to the adsorbents. The insoluble matrix may have

interfered with the ligand-protein interaction. However, all adsorbents were prepared with a 6 carbon spacer group and in some cases a 12 carbon spacer group but this did not affect the binding (see Fig. 7). From gel filtration experiments it was found that with the exception of perchlorate soluble MAO, all the enzyme preparations eluted in the void volume of a Sepharose 4B column (see Fig. 6). The enzyme was therefore not penetrating the gel pores which would effectively reduce the amount of bound ligand available for interaction. Another reason could be that covalent attachment of the MAO substrate and inhibitor molecules to the Sepharose adsorbent may result in a ligand that either has no affinity for the enzyme or possibly cannot bind to enzyme groups that are masked by the folding of the protein molecule. The results from the tyramine, benzylamine and proflavine-Sepharose experiments indicate that one of the above reasons may be valid. However, the results of the AB-15 and DTNB-Sepharose columns suggest another reason; that the enzyme preparation itself was preventing the binding. The solubilized MAO preparations used probably all contained lipid material (the triton X-100 soluble MAO contained 221 μg phospholipid/mg protein) and some preparations were probably bound to membrane fragments. Other workers have shown that their soluble enzyme preparations contain lipid material (Erwin and Hellerman, 1967; Houslay and Tipton, 1973).

To test the possibility of bound lipid material masking the groups for ligand attachment, triton X-100 and sonicated soluble MAO preparations were treated with phospholipase A before application to affinity columns. The enzyme still did not bind to the tyramine or benzylamine adsorbents and the results were the same as for the

untreated preparation on the dodecylamine-Sepharose . However, when the phospholipase A treated MAO was applied to a column of DTNB-Sepharose, 86% of the enzyme was bound and 46% could be eluted still active with mercaptoethanol, but there was no increase in the specific activity (see Table 19). Similar to the untreated preparation, phospholipase A treated MAO did not bind to proflavine-Sepharose but the enzyme eluted 4 fold purer (see Table 20). This indicated that the phospholipase A treatment exposed groups that allowed non-enzymatic protein, to interact with the bound proflavine. However, these results also indicated that attachment of proflavine to an insoluble matrix abolished its affinity for MAO. When the treated preparation was applied to a column of AB-15-Sepharose, 91% of the MAO and non-enzymatic protein were bound (Table 21) and the enzyme could not be eluted in an active form. This suggested that again phospholipase A treatment exposed groups common to 91% of the protein which could interact with AB-15-Sepharose. However, because the enzyme could not be eluted, this adsorbent is not helpful for purifying the enzyme.

The use of affinity chromatography in purifying MAO from the present results shows that a single step purification was unobtainable. However, from the variety of adsorbents used, the DTNB and proflavine-Sepharose can be used to obtain 4 fold increases in specific activities and may therefore be useful in combination with conventional techniques for purifying the enzyme (see Table 22).

A single step highly purified MAO preparation may still be possible, however, this will depend on the synthesis of some novel compounds that combine both good affinity for the enzyme plus a suitable group attachment to an insoluble matrix. It would be interesting to speculate that with the development of suitable substrate selective

inhibitors, affinity chromatography would be the ideal method to separate the different MAO forms. In addition, as the present results indicates, MAO preparations are needed in which they are largely free of bound lipid and other membrane material so they do not mask ligand binding sites.

4.4 Partial Purification of MAO.

A partially purified preparation of MAO was prepared employing DTNB-Sepharose. Using triton X-100 to solubilize the enzyme and fractionation with ammonium sulfate followed by elution from DTNB-Sepharose and DEAE-Sephadex, a 15 fold purification of the enzyme is obtained with a 30% recovery (see Table 22). This procedure is useful for preparing MAO not only because of the high recovery of the solubilized enzyme (70%) but also because preparation is complete within 3 hours.

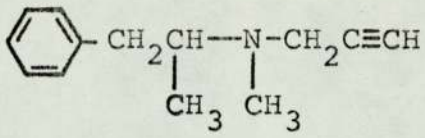
5. RESULTS

Recent evidence indicates that the mitochondrial membrane environment may influence some of the properties of MAO (Oreland and Ekstedt, 1972) and may be responsible for the formation of the multiple forms of the enzyme (Houslay and Tipton, 1973). The following sections show the results of experiments designed to investigate this hypothesis by comparing properties of membrane bound MAO to the properties of MAO after the membrane environment had been altered by various means. In addition, the results from these experiments plus some others were used to help determine the relationship of the enzyme to the structure of the outer mitochondrial membrane.

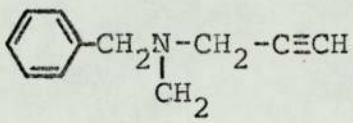
5.1 Inhibitors of MAO

Substrate selective inhibitors of MAO have been used not only to help classify the multiple MAO forms but also to help determine the nature of the multiple forms (see Sections 1.7 and 1.8). The following experiments were performed with the above reasons in mind not only with some widely used inhibitors but also with some derivatives of these compounds. These experiments were also used to further elucidate the mode of action of these inhibitors and their selectivity.

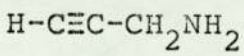
A number of acetylene compounds were used to inhibit MAO and the structures are shown in Fig. 14. Using the nomenclature proposed by Johnston (1968), Deprenil is a selective inhibitor of the B type enzyme (see Fig. 15). A similar inhibition pattern is shown in Fig. 16 for pargyline inhibition. Benzylamine oxidation is preferentially inhibited over serotonin oxidation with the inhibition of tyramine oxidation showing a double sigmoid curve. Propargylamine (Fig.17) selectively inhibits benzylamine and tyramine oxidation before serotonin



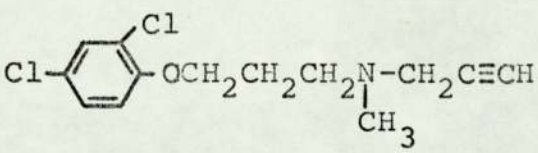
Deprenil



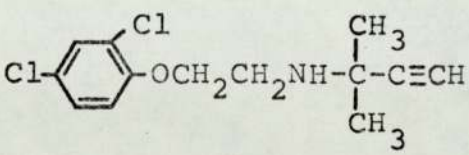
Pargyline



Propargylamine



Clorgyline



Lilly 51618

Fig. 14. Structural formulas of MAO inhibitors.

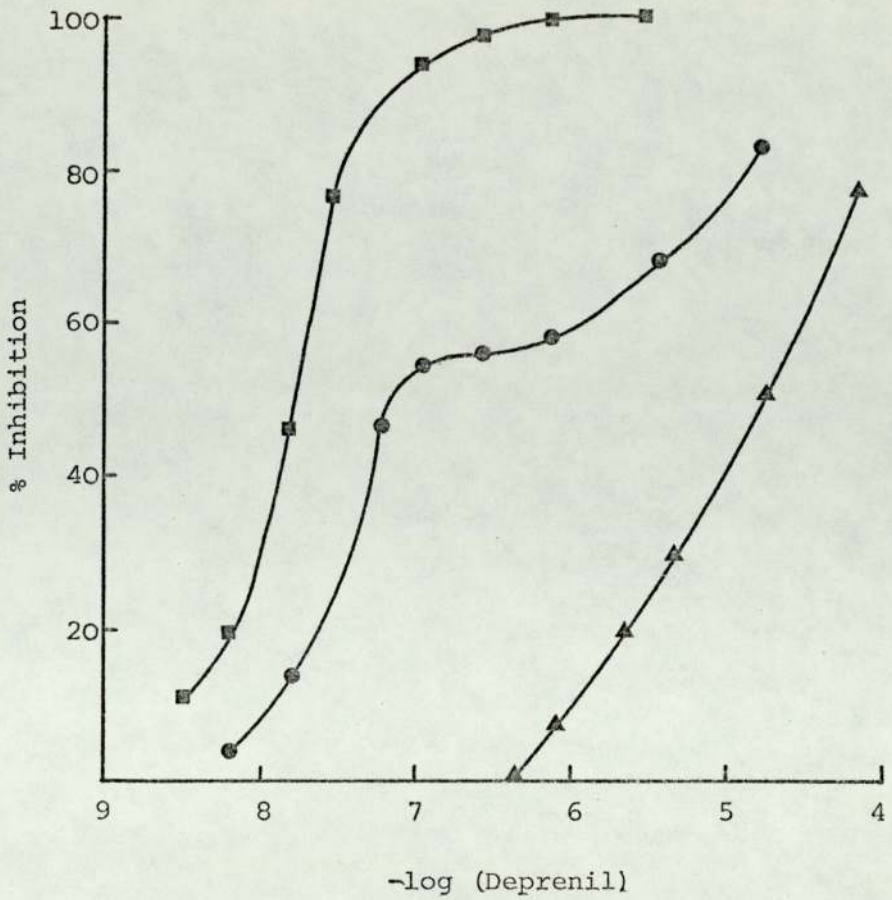


Fig. 15. Inhibition of membrane bound MAO with Deprenil. Enzyme samples (50μl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 10 min with 50 μl of Deprenil in water to give the final concentration indicated. After this incubation, no further time-dependent loss of MAO activity was observed with any substrate. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph is the mean of at least 3 determinations.

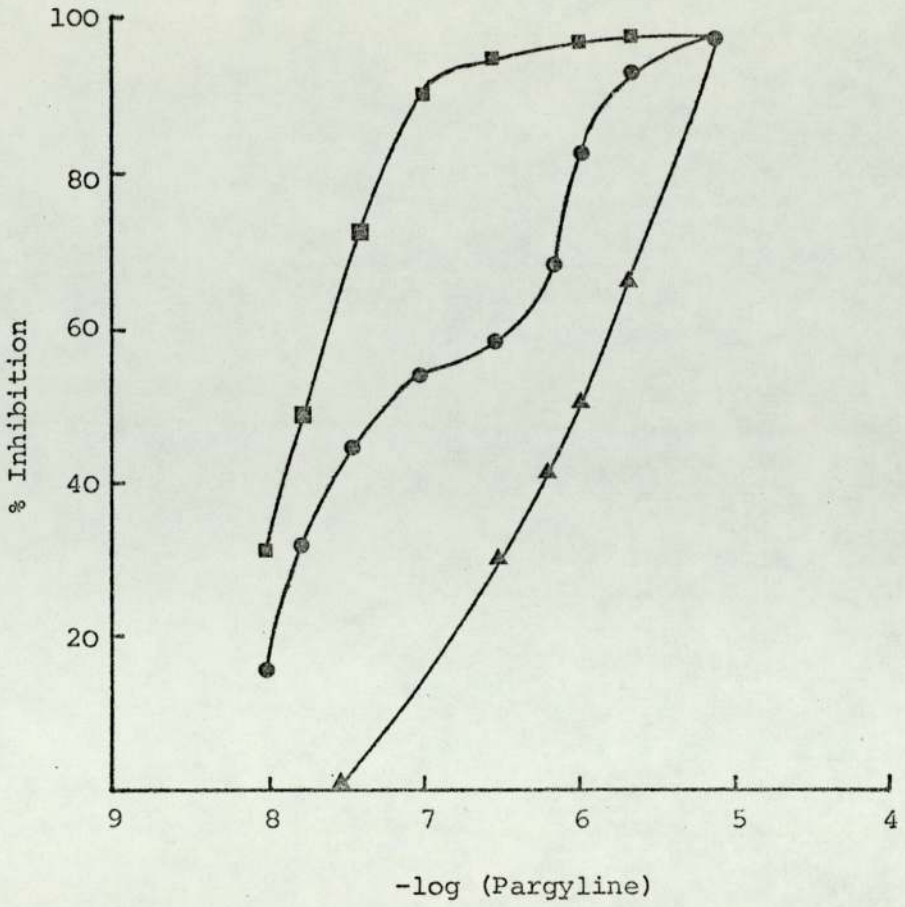


Fig. 16. Inhibition of membrane bound MAO with pargyline. Enzyme samples (50μl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 10 min with 50 μl of pargyline in water to give the final concentration indicated. After this incubation, no further time-dependent loss of MAO activity was observed with any substrate used. MAO was assayed with tyramine (●), serotonin (▲) and benzylamine (■) as substrates. Each point on the graph represents the mean of at least 3 determinations.

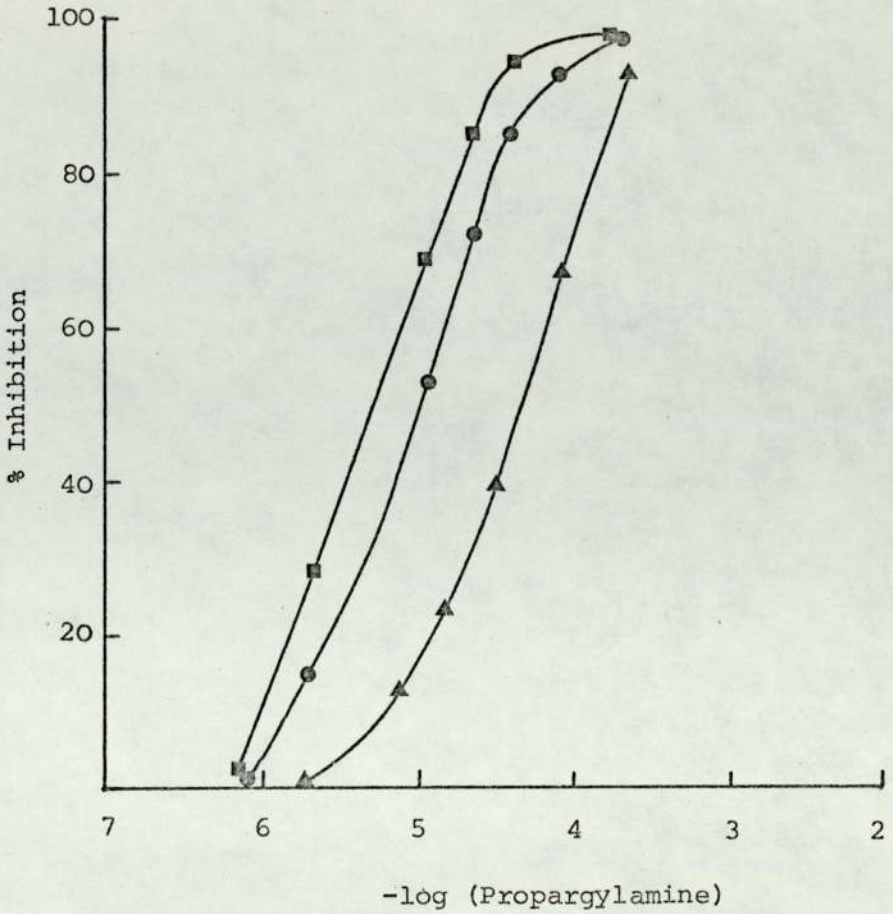


Fig. 17. Inhibition of membrane bound MAO with propargylamine. Enzyme samples (50µl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 30 min with 50µl of propargylamine in water to give the final concentration indicated. After this incubation, no further time-dependent loss of MAO activity was observed with any of the substrates used. Control experiments in the absence of inhibitor showed no loss of enzyme activity over the preincubation period. The enzyme was assayed with tyramine (●), serotonin (▲) and benzylamine (■) as substrates. Each point on the graph is the mean of at least 3 determinations.

oxidation but has no sigmoid curve. Inhibition of rat liver MAO by Clorgyline (Fig. 18) shows a selective inhibition for the A enzyme (serotonin oxidation) with the inhibition of tyramine oxidation showing a double sigmoid curve. The inhibition of MAO by Lilly 51618 is shown in Fig. 19. Little selectivity between the A and B forms of the enzyme was observed and no time-dependent inhibition was detected. The inhibition produced by Lilly 51618 could be easily reversed by dialysis whereas all the other inhibitors used were irreversible.

5.2 Effect of Clorgyline on Mitochondrial Membranes.

It has been shown that the acetylenic inhibitors pargyline and Clorgyline are highly specific for partially purified MAO preparations (Hellerman and Erwin, 1968; Williams and Lawson, 1975). In addition, the results of Erwin and Dietrich (1971) using (^{14}C) pargyline suggest that this compound is a specific inhibitor for MAO in vivo. However, Rando and De Mairena (1974) and Houslay and Tipton (1975c) showed that some of the acetylenic compounds that inhibit MAO also inhibit other enzymes. Furthermore, it has been shown that Deprenil strongly inhibits the uptake of noradrenaline into brain slices and the depletion of noradrenaline from microsomes of rat heart indicating other possible binding sites for the compound besides MAO. (Knoll and Magyar, 1972). Since MAO is tightly bound to the outer mitochondrial membrane (see Section 1.3), these acetylenic inhibitors may interact with components of the membrane other than MAO.

Godin and Ng (1972) have used the rate of the TNBS reaction with amino groups to study the lipid-protein interactions of the erythrocyte membrane. In addition, the rate of TNBS incorporation was used to study the perturbation of the membrane by ions and drugs.

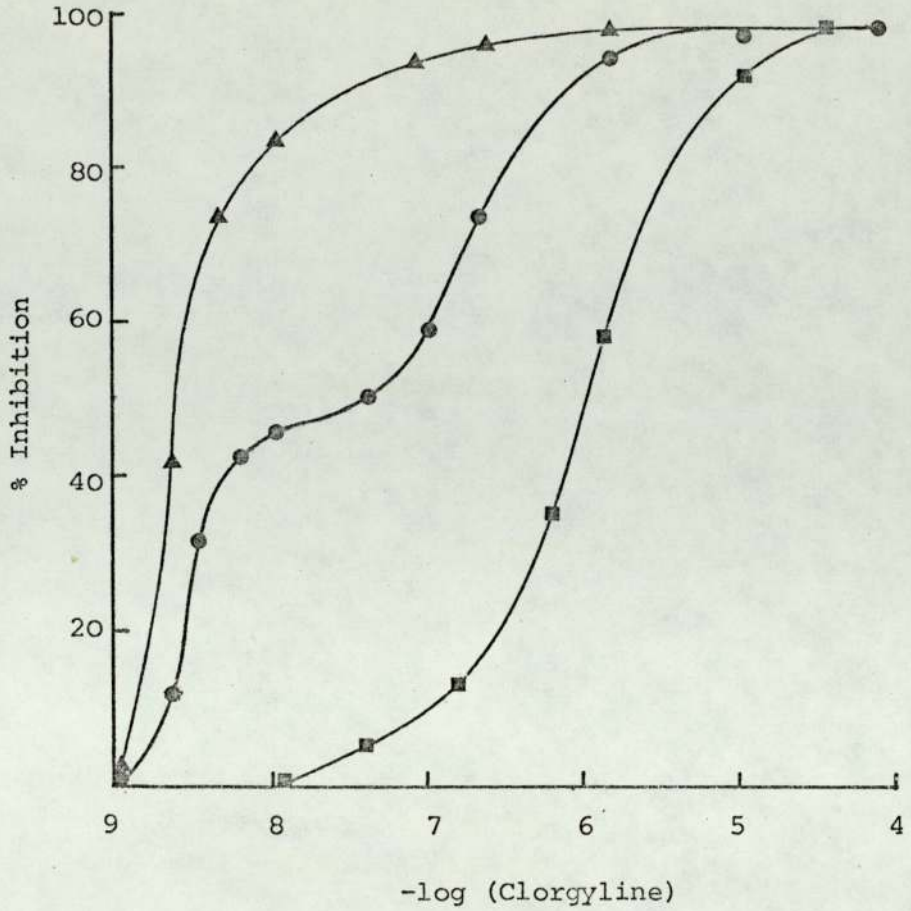


Fig. 18. Inhibition of membrane bound MAO with Clorgyline. Enzyme samples (50µl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 10 min with 50 µl of Clorgyline in water to give the final concentration indicated. After this incubation, no further time-dependent loss of MAO activity was observed with the substrates used. MAO activity was measured with tyramine (●), serotonin (▲) and benzylamine (■) as substrates. Each point on the graph represents the mean of at least 3 determinations.

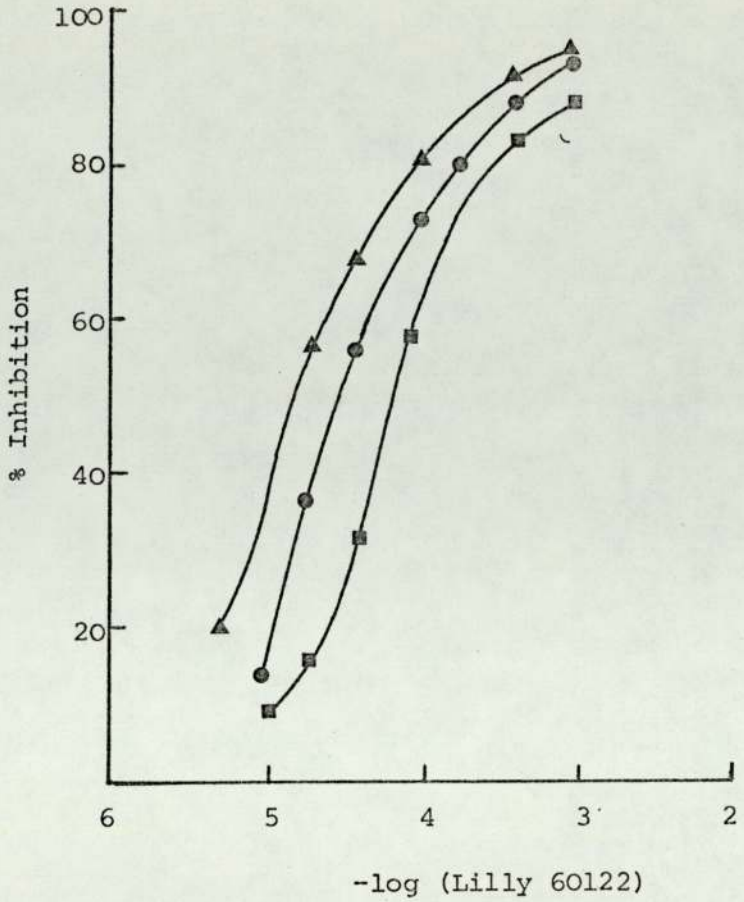


Fig. 19. Inhibition of membrane bound MAO with Lilly 51618. Enzyme samples (50 µl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 15 min with 50 µl of Lilly 51618 in water before the addition of substrate. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Control experiments in the absence of inhibitor, showed no loss of enzyme activity with the substrates used. Each point on the graph represents the mean of at least 3 determinations.

The following shows the results of experiments where the rate of TNBS incorporation into mitochondrial membranes was used to study the effect of Clorgyline on the membrane and its relationship to MAO inhibition.

Reaction of 0.2 mM TNBS with mitochondrial protein (1 mg) and lipid amino groups was linear with time up to 20 min. The rate of incorporation of 0.2 mM TNBS into mitochondrial membranes was linear over the range 0.5-10 mg of protein per 3.0 ml of reaction mixture. The stimulation of the rate of TNBS incorporation into mitochondrial membranes by the MAO inhibitor Clorgyline at pH 7.5 is shown in Fig. 20. The same concentration dependent rate increase of TNBS incorporation by Clorgyline is observed for both intact or lysed mitochondria. The incorporation of TNBS increases linearly over the pH range 7.0 to 8.0, but the stimulation of TNBS incorporation by Clorgyline decreases over the same pH range (see Fig. 21).

Several explanations are possible for the stimulation of TNBS incorporation by Clorgyline. No direct interaction between the TNBS and Clorgyline molecules could be detected and as shown in Fig. 22 the apparent K_{diss} of Clorgyline for stimulation of TNBS into mitochondrial membranes is unchanged over a 6 fold range of TNBS concentrations. This indicates that the stimulatory effects of Clorgyline do not depend on complex formation with TNBS. Pargyline or propargylamine over the same concentration ranges do not affect the incorporation of TNBS into mitochondrial membranes indicating a change in ionic strength was not responsible for the stimulation of TNBS incorporation. It would seem therefore that Clorgyline may be perturbing the membrane structure in a way that increases the rate of TNBS incorporation.

Table 23 shows the effect of mitochondrial membrane treatment with phospholipase C on the stimulation of TNBS by chlorpromazine and Clorgyline. Treatment of mitochondrial membranes decreased

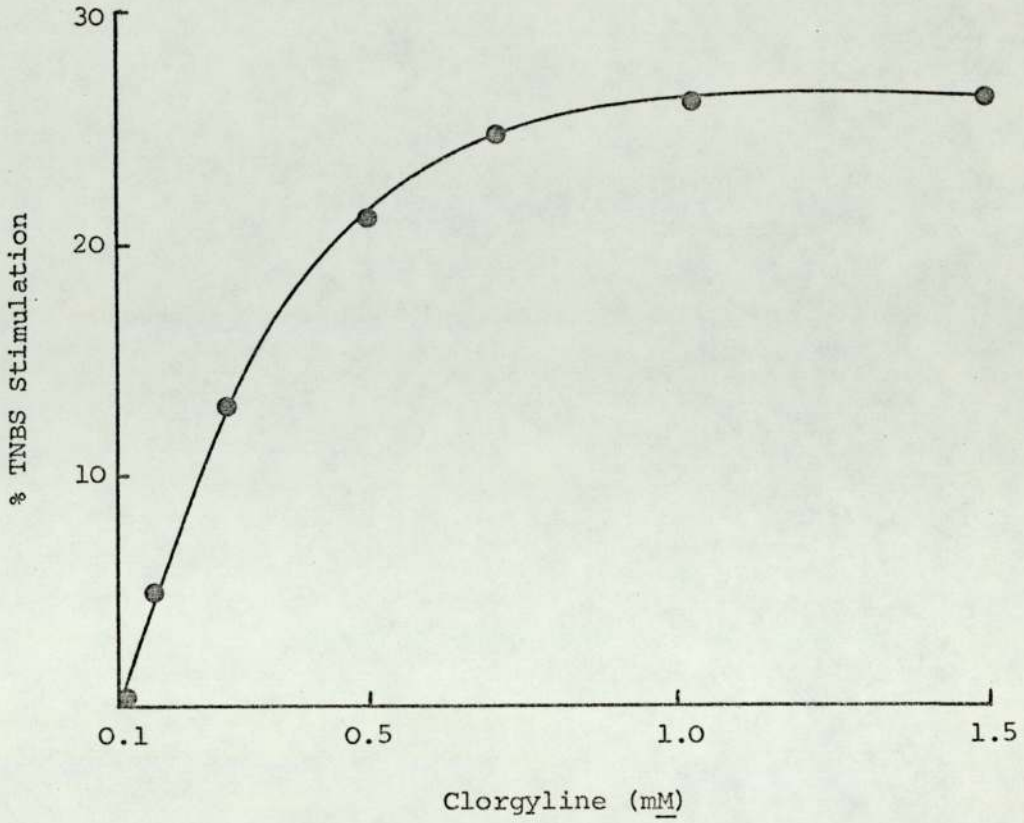


Fig. 20. Effect of Clorgyline on the rate of TNBS incorporation into mitochondrial membranes. Assays were performed in 0.05M tris buffer, pH 7.5 as described in Section 2.15. Each point on the graph represents the mean of 4 determinations.

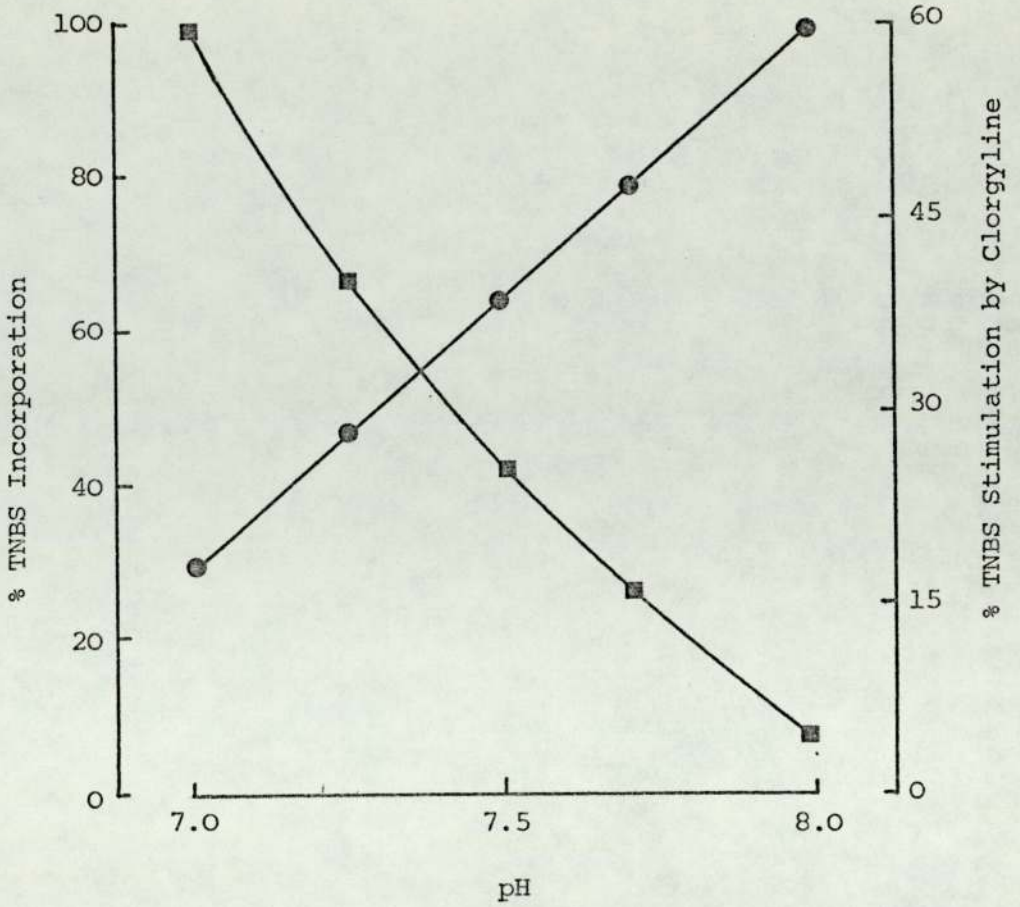


Fig. 21. Effect of pH on TNBS incorporation and Clorgyline stimulation. All assays were performed with 0.2mM TNBS final concentration in 0.05M tris buffer at the pH indicated. In experiments with Clorgyline present a final concentration of 0.5mM was used. Each point on the graph represents the mean of at least 4 determinations. TNBS incorporation expressed as a % of the control value at pH 8.0 (●) and Clorgyline stimulation of TNBS incorporation (■).

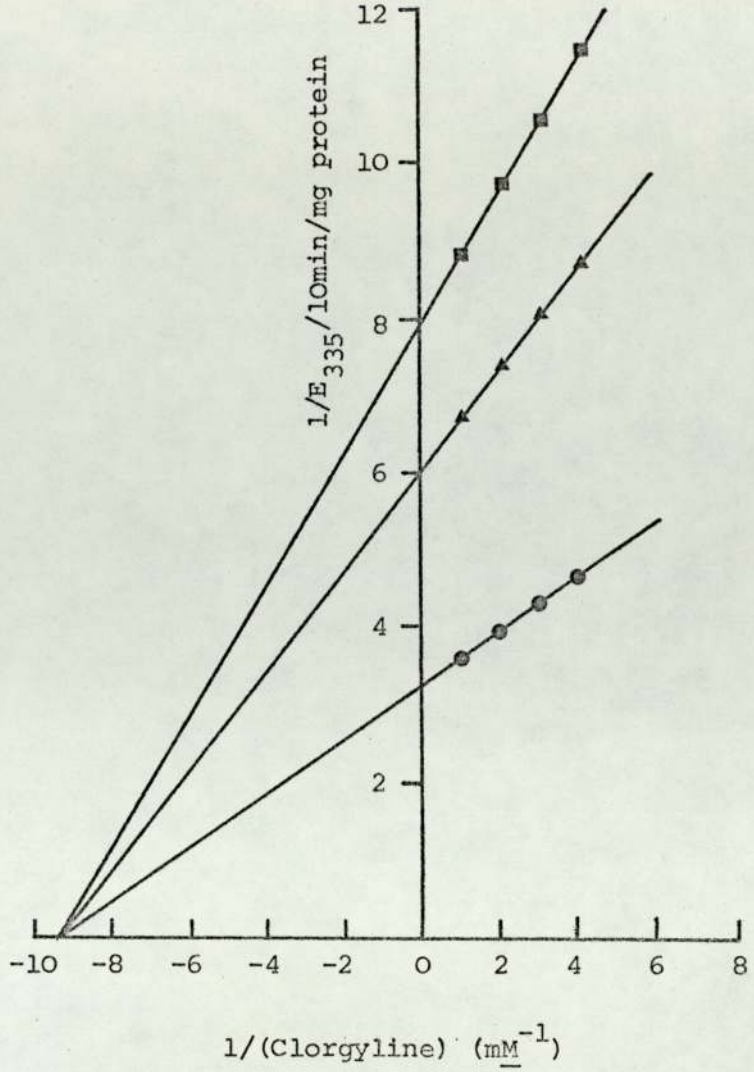


Fig. 22. Effect of increasing TNBS concentrations on the apparent $K_{diss.}$ of Clorgyline for the stimulation of TNBS incorporation into mitochondrial membranes. Assays were performed as described in section 2.15 except that the final TNBS concentrations were 0.2 mM (■), 0.33mM (▲) and 1.2mM (●).

TABLE 23.

Effect of Phospholipase C on the Stimulation of TNBS Incorporation by Chlorpromazine and Clorgyline.

	Control membranes	Phospholipase C treated membranes.
	ε_{335}/ 10 min/ mg protein	
Control	0.14 (100)	0.12 (100)
Chlorpromazine (0.1 mM)	0.23 (171)	0.12 (100)
Clorgyline (0.5 mM)	0.17 (129)	0.15 (126)

Assays were performed as described in Section 2.15 and the numbers in parenthesis are percentages. Each number represents the mean of at least 4 determinations.

the lipid phosphoryl groups by 66% (144 µg phospholipid/mg protein to 49.2 µg phospholipid/mg protein) but had no effect on the Clorgyline stimulation of TNBS. Chlorpromazine, at a concentration of 0.1 mM stimulated TNBS incorporation by 71% but after membrane treatment this was abolished (see Table 23). Chlorpromazine as shown in Fig. 23 is also an inhibitor of MAO but phospholipase C treatment of the membrane has no effect on its MAO inhibition. Table 24 shows the effects of some compounds on Clorgyline stimulation of TNBS. Arsenite (5 mM) reduces the increased incorporation of TNBS due to Clorgyline by half and mercuric chloride (5 mM) which itself causes an increase of TNBS incorporation, does not allow Clorgyline to produce any further stimulation. EDTA (1 mM) has no effect on either TNBS incorporation or Clorgyline stimulation and both 4-hydroxyquinone and ANS reduce the rate of TNBS incorporation but also reduce the Clorgyline stimulation almost to the control value.

Mitochondrial membranes incubated for 10 min with 0.1 mM Clorgyline or pargyline and then dialyzed to remove free inhibitor showed no detectable MAO activity. These preparations had the same SH content measured by their reaction with DTNB (see Section 2.13), as control preparations. Both control and treated preparations showed the same concentration dependent Clorgyline stimulation of TNBS incorporation.

5.3 Properties of Membrane Bound MAO.

Several methods have been used to help elucidate the effect of membrane constituents and the molecular nature of MAO. Oreland and Ekstedt (1972) found that lipids conferred heat stability and resistance to tryptic digestion to pig liver MAO. Houslay and Tipton (1973) found that reduction of enzyme bound lipids abolished the differential heat

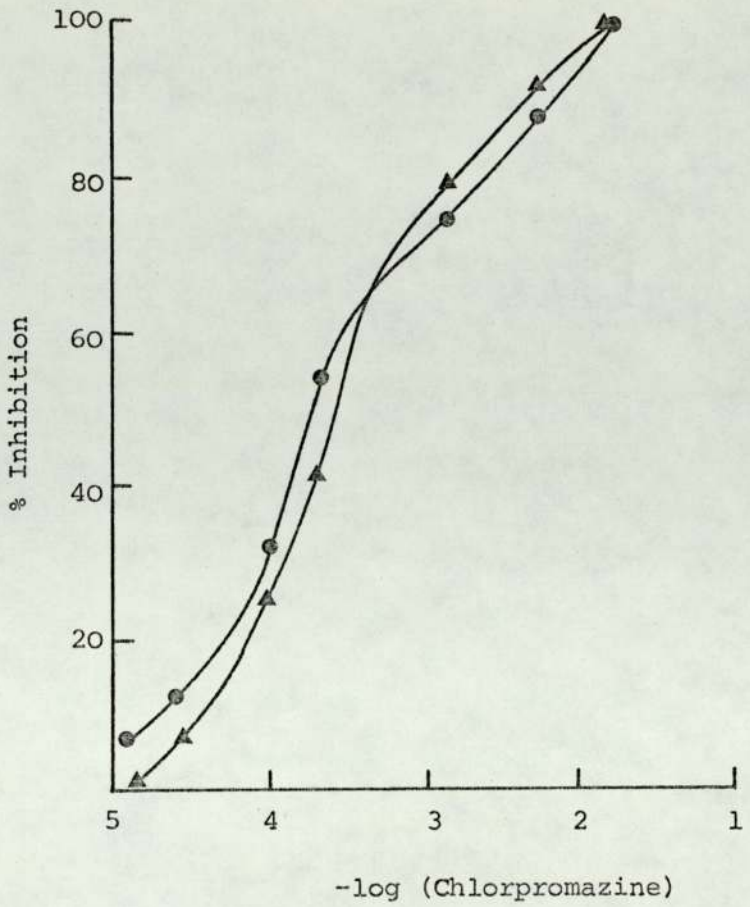


Fig. 23. Inhibition of membrane bound MAO by chlorpromazine before and after phospholipase C treatment. Enzyme samples (50μl, 2.5 mg protein/ml in 0.05M tris buffer, pH8.0) were incubated at 37°C for 5 min with 50μl of chlorpromazine in water before the addition of substrate. Enzyme assays were performed with tyramine as substrate by the ion exchange method. Phospholipase C treatment had no effect on on the MAO activity. Control (●) and phospholipase treated membranes (▲).

TABLE 24.

Effect of some Compounds on the stimulation of TNBS Incorporation by Clorgyline.

	Control membranes	Clorgyline (0.5 mM) treated membranes
	ε_{335}/ 10 min/ mg protein	
Control	0.15	0.2 (131)
As^{+++} (50 mM)	0.15	0.17 (116)
Hg^{++} (5 mM)	0.2	0.2 -
EDTA (1 mM)	0.16	0.21 (128)
4-hydroxyquinone (1 mM)	0.12	0.12 (104)
ANS (1 mM)	0.13	0.14 (106)

Assays were performed as described in Section 2.15 and the numbers in parenthesis are percentages. Each number represents the mean of at least 4 determinations.

stability and the Clorgyline inhibition pattern of rat liver MAO A and MAO B. This suggested that the membrane lipids were responsible for the formation of the multiple MAO forms. Another method that has been extensively used to indicate that lipids affect membrane bound enzymes is Arrhenius plots (for a review see Sechi, Bertoli, Landi, Parenti-Castelli, Lenaz and Curatola, 1973). The following results show the effects of the heat stability, tryptic digestibility and Arrhenius plots of membrane bound rat liver mitochondrial MAO which in addition to the Deprenil inhibition pattern (Fig. 15) are compared to the results in further Sections (see Sections 5.4, 5.5 and 5.6) after the membrane environment had been altered. In addition, the tryptic digestion of MAO can be used to help determine the relationship of MAO to the outer mitochondrial membrane.

The time course of loss in intact mitochondrial membrane bound MAO activity towards serotonin, tyramine and benzylamine during incubation of the enzyme at 50°C is shown in Fig. 24. The same time course of loss in MAO activity is seen also with mitochondria lysed by washing with 1 mM phosphate buffer, pH 7.5.

The effect of trypsin on the turbidity of mitochondrial suspensions is shown in Fig. 25. Incubation of mitochondria with 200 µg trypsin per mg of mitochondrial protein at 37°C results in about 95% loss of turbidity over a 60 min period compared with a 40% loss in control suspensions. Over a 40 min time period (see Fig. 26) trypsin inactivated 65% of the serotonin activity and 43% and 23% of the tyramine and benzylamine activity respectively as compared to control enzyme samples without trypsin. Similar results were obtained for trypsin inactivation of MAO for both the intact and lysed mitochondria. Table 25a shows the percent of MAO activity remaining towards tyramine, serotonin and benzylamine in both intact and lysed mitochondria

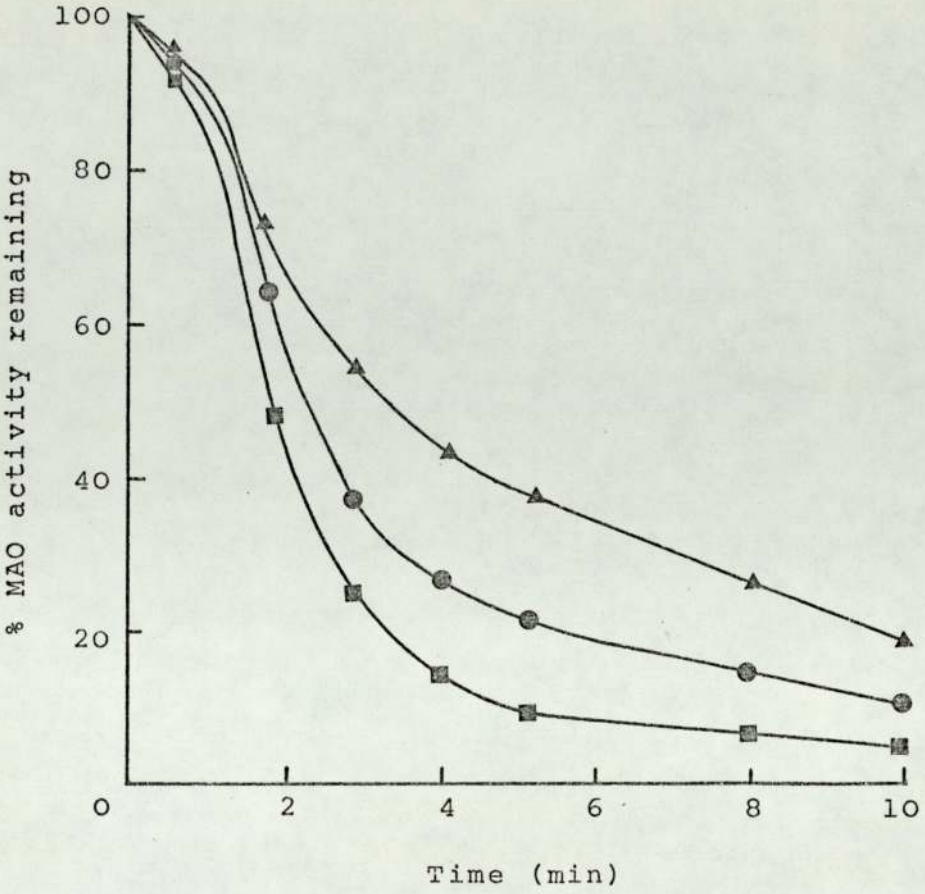


Fig. 24. Effect of heat treatment on the activity of membrane bound MAO. Enzyme samples (2.5 mg protein/ml) were incubated at 50°C in 0.05M phosphate buffer, pH 8.0. At the times indicated, samples were removed and assayed for MAO activity at 37°C. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph is the mean of at least 3 determinations.

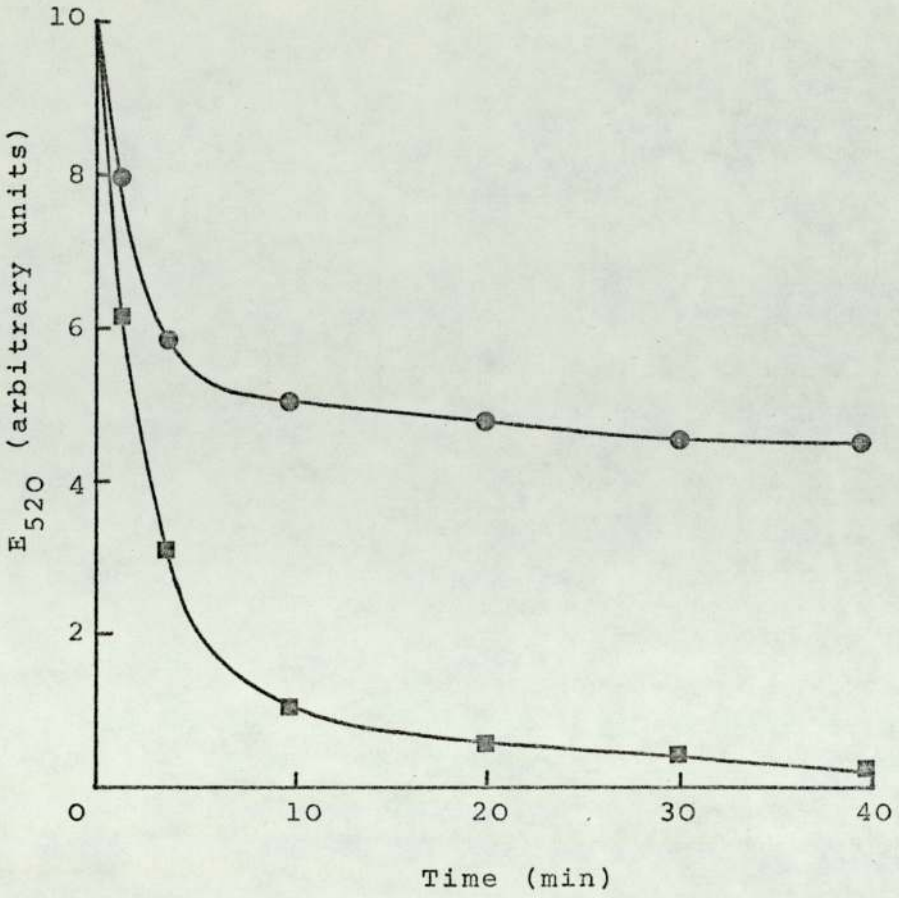


Fig. 25. Turbidity changes in mitochondrial suspensions induced by trypsin. Mitochondria were suspended in 0.25 M sucrose containing 0.01M phosphate buffer, pH 7.5 to a final concentration of 0.5 mg protein/ml and incubated at 37°C. At the times indicated, samples were removed and read at 520nm. Control suspensions (●) and suspensions that contained 200µg trypsin per mg of mitochondrial protein (■).

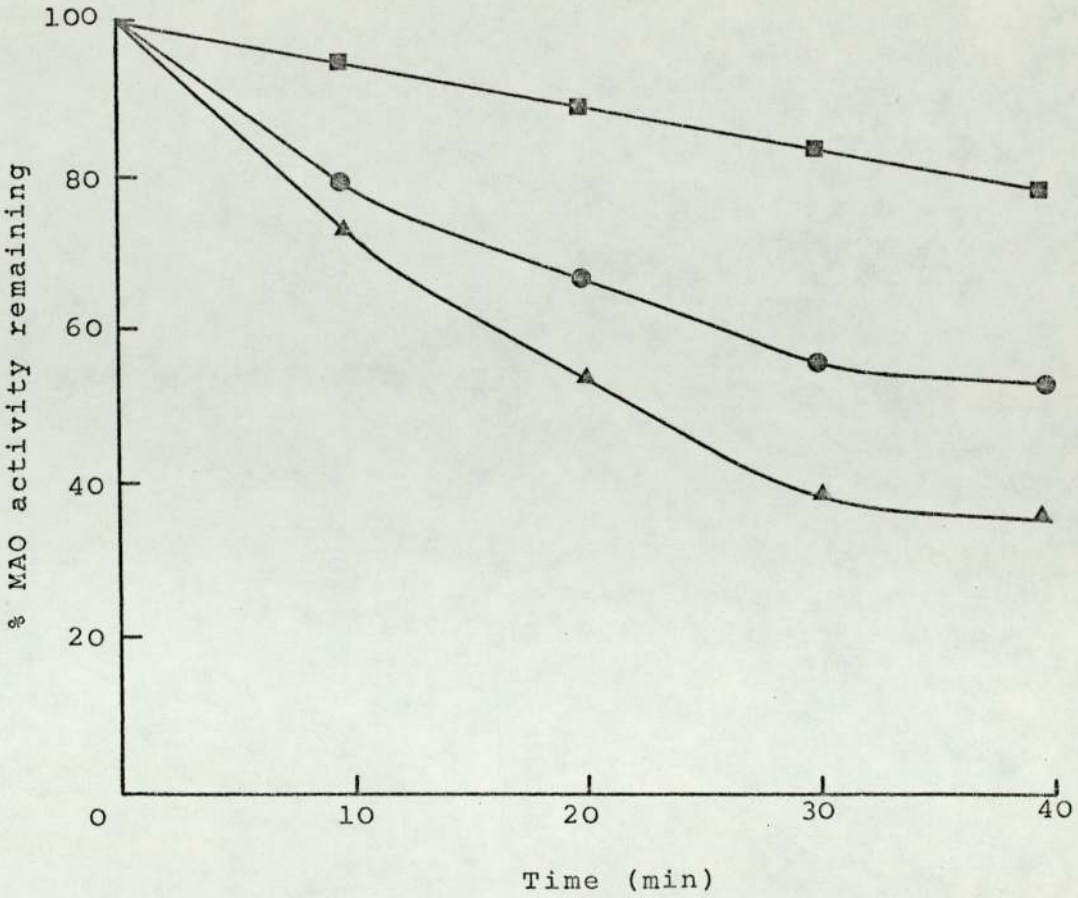


Fig. 26. Inactivation of membrane bound MAO by trypsin. The enzyme was incubated at 37°C in the presence of 200μg trypsin/mg of mitochondrial protein. At each time indicated, a 3 fold weight excess of soya bean trypsin inhibitor was added and the residual MAO activity was estimated. Control incubations without trypsin lost no enzyme activity over the 40 min. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph is the mean of at least 3 determinations.

TABLE 25.

Effect of Trypsin on Intact and Lysed Mitochondria.

a. 50 µg trypsin per mg mitochondrial protein.

Substrate	Control	Lysed mitochondria	Intact mitochondria
Tyramine	100	94	91
Serotonin	100	94	92
Benzylamine	100	89	86

b. 200 µg trypsin per mg mitochondrial protein.

Substrate	Control	Lysed mitochondria	Intact mitochondria
Tyramine	100	54	57
Serotonin	100	34	35
Benzylamine	100	84	77

Mitochondria were lysed by washing with 1 mM phosphate buffer, pH 7.5. Figures show percent activity of MAO as compared to control of 100%.

after a 40 min incubation with 50 µg trypsin per mg protein and Table 25b shows the percent activity remaining after treatment with 200 µg trypsin per mg protein as compared to control values. In both intact and lysed mitochondria the percent of enzyme lost at the lower trypsin concentration (50 µg) was similar with all substrates used.

An Arrhenius plot of membrane bound MAO (see Fig. 27) shows that for tyramine, serotonin and benzylamine as substrates, breaks in the plots occur at 26.8°C for each substrate. Below the transition temperature there was a 1.5-3.0 fold increase in the activation energy of the enzyme. The inhibition of MAO by Deprenil below the transition temperature was also investigated. The enzyme was preincubated with the inhibitor for 30 min and 60 min at 16° and 10°C respectively before the addition of substrate, however at both temperatures the percent inhibition at each inhibitor concentration was similar to those for the enzyme at 37°C as shown in Fig. 15.

5.4 Mitochondrial Lipid Peroxidation.

There are a number of ways to alter the environment of a membrane to study its effect on enzymes including the use of proteolytic enzymes, phospholipases, detergents and chaotropic agents. Another method which may be useful involves the oxidation and cleavage of polyunsaturated fatty acids which is commonly referred to as lipid peroxidation (for a review see Mengel, 1968). The process of lipid peroxidation not only disrupts membranes but also results in the production of compounds which can react with proteins and inhibit some enzymes (Desai and Tappel, 1963; McKnight and Hunter, 1966). The following shows the results of experiments on ferrous ion induced lipid peroxidation in rat liver mitochondria and its effects on MAO activity and on the properties of MAO A and MAO B.

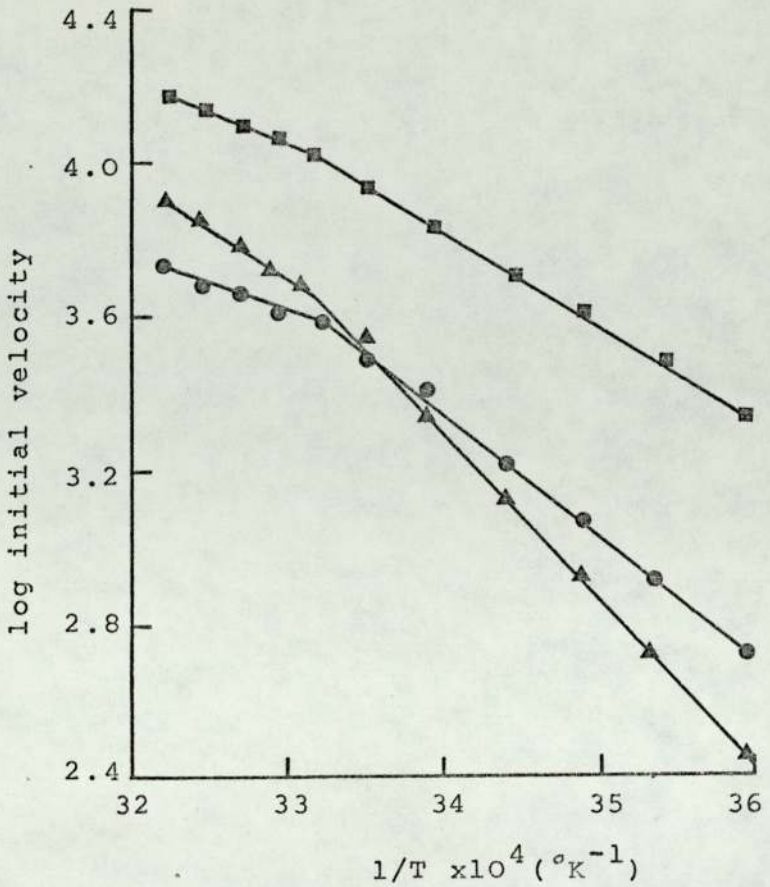


Fig. 27. Arrhenius plots of membrane bound MAO. Enzyme samples were incubated for 5 min at the indicated temperature before addition of substrate. Activity was measured with tyramine (●), serotonin (▲) and benzylamine (■) as substrates. Each point on the graph is the mean of 3 determinations.

As shown in Fig. 28 over the period of 60 min ferrous ion induced lipid peroxidation reduces the turbidity of mitochondrial suspensions by 90% whereas control suspensions were reduced by 30%. No decrease in turbidity was seen in suspensions that contained 20 μM EDTA. Over the same period as the turbidity decreased, there was an increase in the formation of lipid peroxides as measured by the thiobarbituric acid method (see Section 2.14) and a decrease in the total number of reactive SH groups by 41% (see Fig. 29).

At the end of the incubation in the presence of ferrous ions, the suspensions were centrifuged at 10,000 x g for 10 min and washed twice with 0.05 M phosphate buffer, pH 7.4. Table 26 shows the protein, lipid and SH content of the pellet. Control incubations lost 35% of the protein but little if any phospholipid or reactive SH groups as compared to stock suspensions of mitochondria. Lipid peroxidation was performed in the presence of 0.01 M mercaptoethanol or 0.2 mg of BSA. It is interesting to note that similar results were obtained when lipid peroxidation was performed at room temperature.

Table 27 shows the effect of lipid peroxidation for 60 min on the recovery of MAO activity in the pellet. No MAO activity was observed in the supernatant after the period of lipid peroxidation. The pellet from control incubations when assayed with tyramine, serotonin and benzylamine as substrates lost 38%, 50% and 38% of their activity respectively as compared to stock solutions. If the protein concentration is raised 10 fold to 2.0 mg/ml, no MAO activity with any substrate is lost at either 4°C or room temperature. The pellet from ferrous ion treated suspensions lost 66% of the tyramine and benzylamine activity and 73% of the serotonin activity. Thus lipid peroxidation induces the inhibition of 28% of the tyramine and benzylamine activity and 23% of the serotonin activity. Similar results were

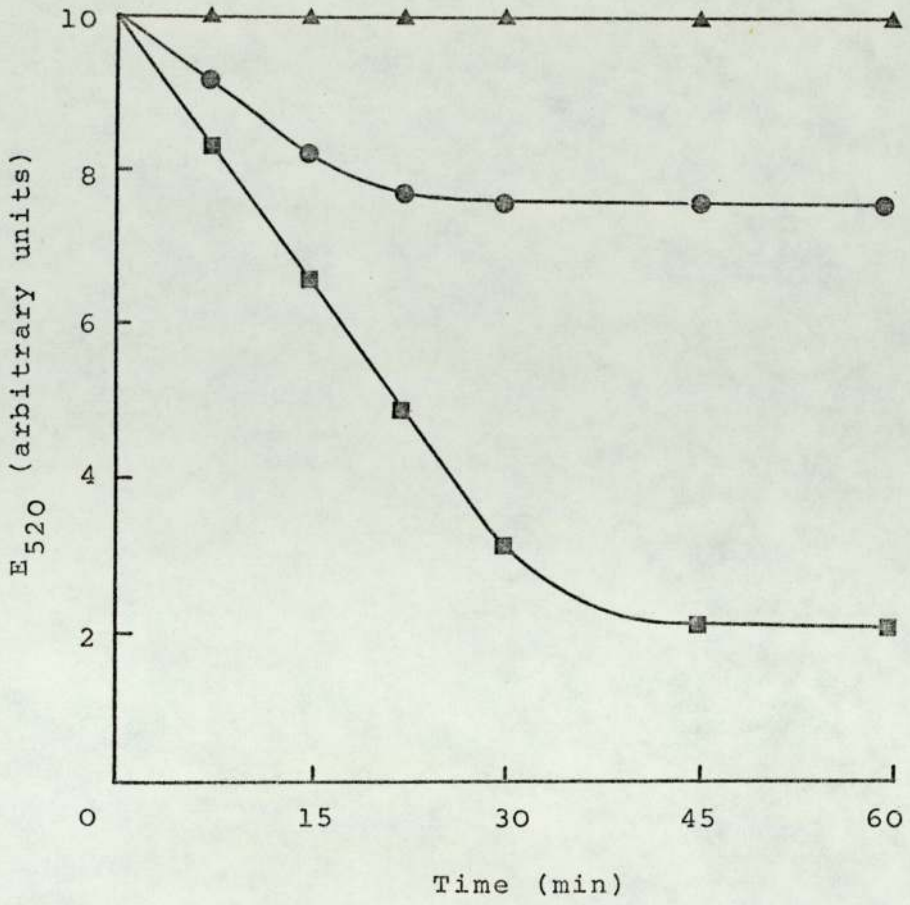


Fig. 28. Turbidity of mitochondrial suspensions during lipid peroxidation. Mitochondrial suspensions were incubated at 4°C as described in Section 2.14 with 20μ M ferrous ammonium sulfate. At the times indicated, samples were measured at 520 nm. Control suspensions (●) and suspensions that contained 20μM EDTA (▲) and 20μM ferrous ammonium sulfate (■).

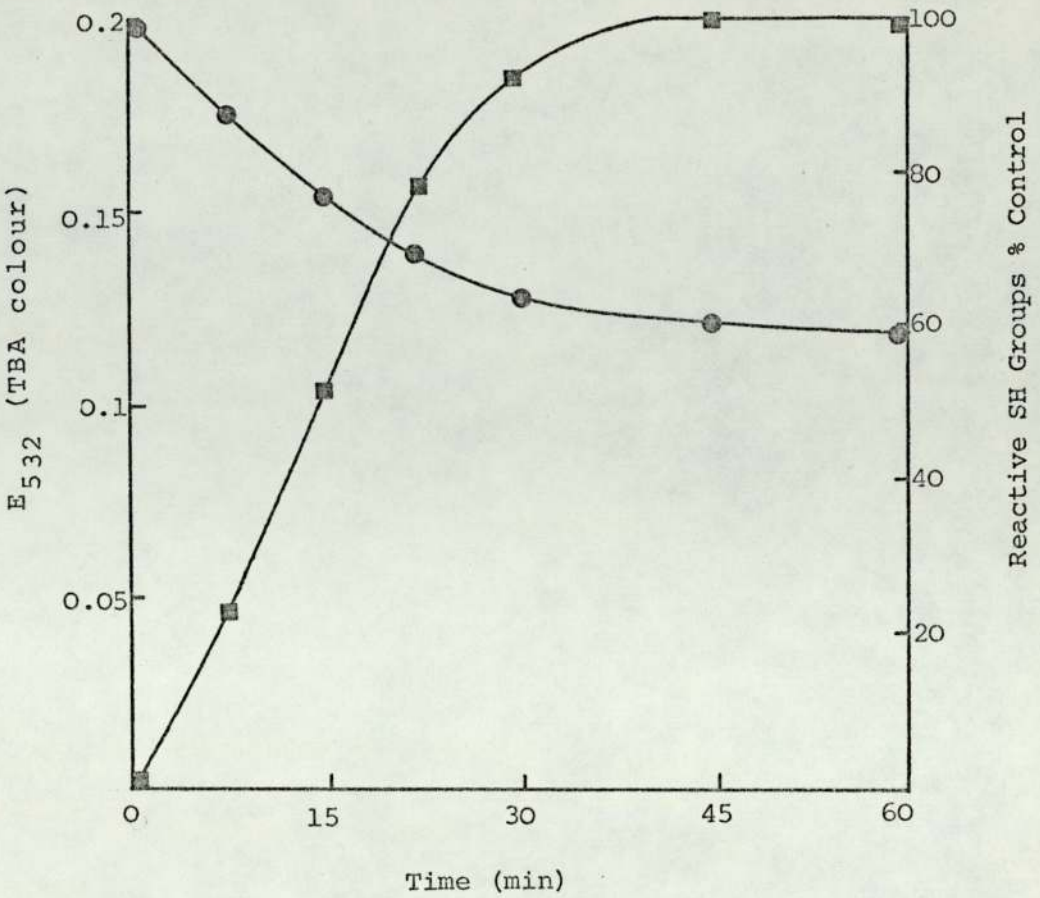


Fig. 29. Production of lipid peroxides and decrease in protein SH groups during ferrous ion induced lipid peroxidation. Mitochondrial suspensions were incubated at 4°C as described in Section 2.14 with 20 μM ferrous ammonium sulfate. At the times indicated, samples were assayed for lipid peroxide (■) and SH content (●). Each point on the graph represents the mean of at least 3 determinations.

TABLE 26.

Effect of Lipid Peroxidation on Rat Liver Mitochondria (P_2)

	Protein	Phospholipid	SH content
	mg	µg/mg protein	µg/mg protein
Stock P_2	120	192	16.5
Control P_2	77.1	187	16.1
Fe^{2+} treated P_2	25.1	102	8.6
Fe^{2+}/mercapto-ethanol treated P_2.	25.2	115	7.7
Fe^{2+}/BSA treated P_2.	30.7	118	10.1

Incubations were performed as described in Section 2.14 at 4°C. All determinations were performed on the pellet obtained after centrifugation at 10,000 x g for 10 min. Each number represents the mean of at least 3 determinations.

TABLE 27.

Effect of Mitochondrial Lipid Peroxidation on MAO activity.

	Substrates		
	Tyramine activity (%)	Serotonin activity (%)	Benzylamine activity (%)
Stock P_2	100	100	100
Control P_2	62	50	62
Fe^{2+} treated P_2	34	27	34
Fe^{2+}/mercapto-ethanol treated P_2	34	27	31
Fe^{2+}/BSA treated P_2	63	45	47

Lipid peroxidation was performed at 4°C as described in Section 2.14. All enzyme activity determinations were performed on the pellet obtained after centrifugation at 10,000 x g for 10 min. Each number represents the mean from at least 3 determinations. Figures represent % of MAO activity.

obtained when mercaptoethanol was present during lipid peroxidation. The loss of tyramine and serotonin activity was prevented by the presence of BSA but the benzylamine activity was only partially protected.

Some properties of MAO were determined after lipid peroxidation in the presence of BSA and the heat stability of the enzyme is shown in Fig. 30. The enzyme lost activity with all substrates at a greater rate than that of the untreated membrane bound enzyme (see Fig. 24). No change was observed in the inhibition pattern using Deprenil (see Fig. 15).

5.5 Effect of Phospholipases on Membrane Bound MAO.

The disposition of membrane components and the effect of lipids on membrane bound enzymes can be investigated by the use of pure phospholipases (for reviews see Coleman, 1973; Zwaal, Roelofsen and Colley, 1973). Therefore, the following results are from experiments on the effect of the phospholipases A and C on the activity and properties of membrane bound MAO. Phospholipase A from bee venom was used in the present study because the commercial preparations have a very high specific activity and this preparation has a similar substrate specificity to that of the snake venom enzyme (Zwaal et al., 1973).

The treatment of mitochondrial suspensions with phospholipase C removes up to 66% of the phospholipid phosphoryl groups without affecting the MAO activity towards tyramine, serotonin and benzylamine. Furthermore, the heat stability, Deprenil inhibition pattern, trypsin sensitivity and Arrhenius plots for this preparation did not differ significantly from control preparations (see Figs. 15, 24, 26 and 27).

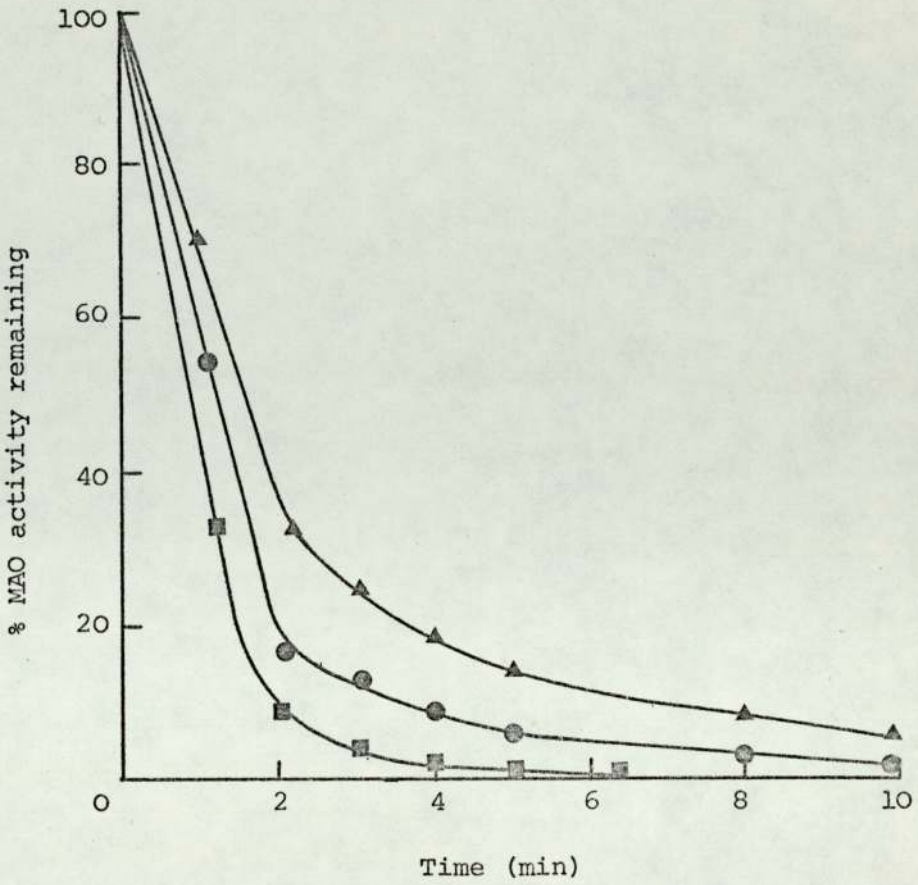


Fig. 30. Effect of heat treatment on the activity of membrane bound MAO after lipid peroxidation. Enzyme samples (2.5 mg protein/ml) were incubated at 50°C in 0.05M phosphate buffer, pH 8.0. At the times indicated, samples were removed and assayed for MAO activity at 37°C. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph is the mean of at least 3 determinations.

Treatment of mitochondrial suspensions with 0.05 mg of phospholipase A per 10 mg of protein and in the presence of 0.2 mg/ml of BSA results in clearing of the suspension within 3 min. After further incubation for 17 min , all the MAO activity sedimented at 20,000 x g after 20 min. As shown in Fig. 31 over a 40 min incubation with phospholipase A, 22% of the MAO activity was lost with tyramine, serotonin and benzylamine as substrates compared to control incubations. The pellet after centrifugation at 20,000 x g contained only 10% of the original suspended protein and had a phospholipid content of 110 µg/mg of mitochondrial protein as compared to 143 µg/mg for the mitochondrial protein in control suspensions. The heat stability of membrane bound MAO after phospholipase A treatment is shown in Fig. 32. Compared to untreated membranes (see Fig. 24), the rate of loss of MAO activity is greater in the treated preparations. The effect of trypsin on MAO activity after treatment with phospholipase A (Fig. 33) shows a similar time course loss of activity as the untreated preparation (Fig. 26) and after treatment of the mitochondrial membranes with phospholipase A, the Deprenil inhibition pattern (Fig. 34) was unchanged from that of untreated preparations (Fig. 15).

5.6 Effect of Phospholipases on Partially Purified MAO.

In the preceding section it was found that after treatment of the mitochondrial membrane with phospholipase A and C, considerable amounts of lipid material were still protein bound and the properties of MAO were similar to the untreated preparation. This may be due to the phospholipases being unable to penetrate the membranes to further degrade the lipids. Therefore, the following results are from experiments in which MAO was first solubilized and partially purified and then treated with phospholipase A and C. The solubilization of

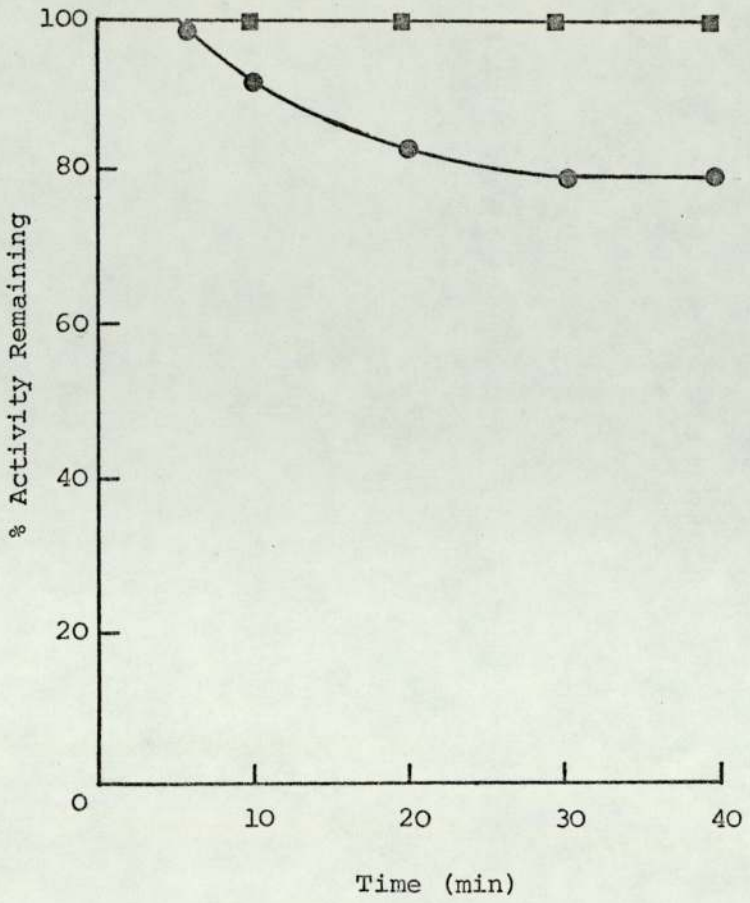


Fig. 31. Effect of phospholipase A treatment on membrane bound MAO activity. Phospholipase A treatment of mitochondrial suspensions was performed as described in Section 2.14. At each time indicated, 5μl of the suspension was added to 45μl of 0.05M phosphate buffer, pH 8.0 containing 1.0mM EDTA and assayed for MAO activity with tyramine, serotonin and benzylamine as substrates. Each point on the graph is common to all substrates and represents the mean of 2 determinations with each substrate. Control suspensions (■) and phospholipase A treated suspensions (●).

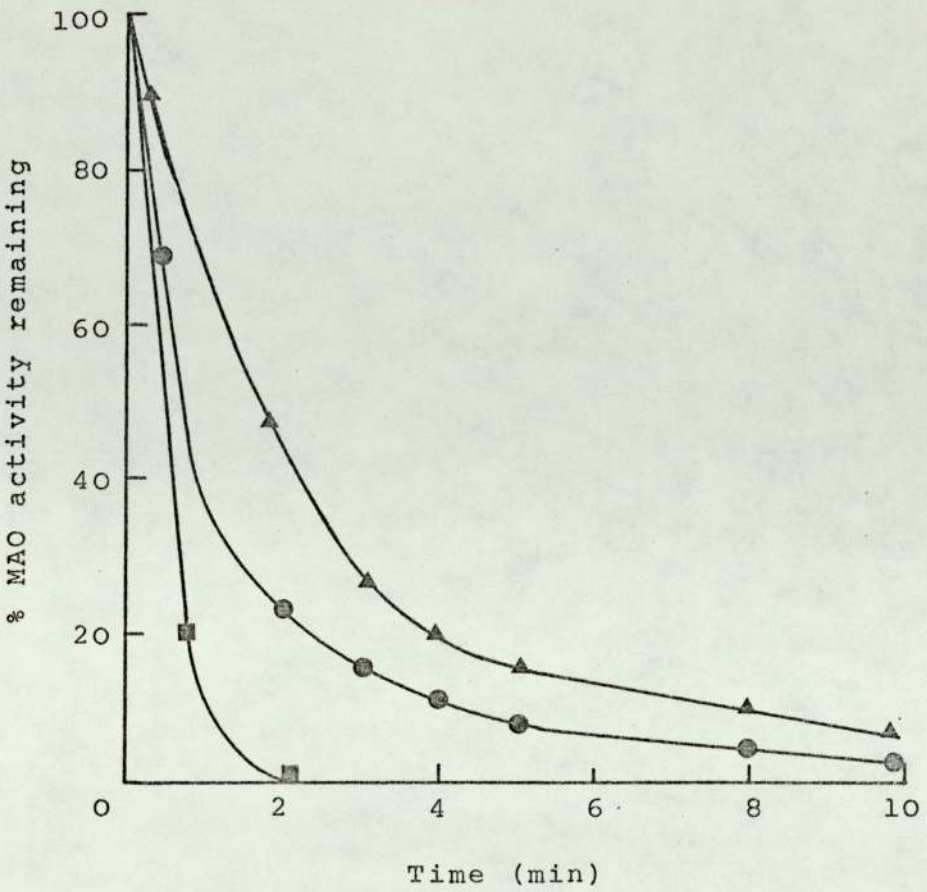


Fig. 32. Effect of heat treatment on the activity of membrane bound MAO after treatment with phospholipase A. Enzyme samples (2.5 mg protein/ml) were incubated at 50°C in 0.05M phosphate buffer, pH 8.0. At the times indicated, samples were assayed for MAO activity at 37°C. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph represents the mean of 3 determinations.

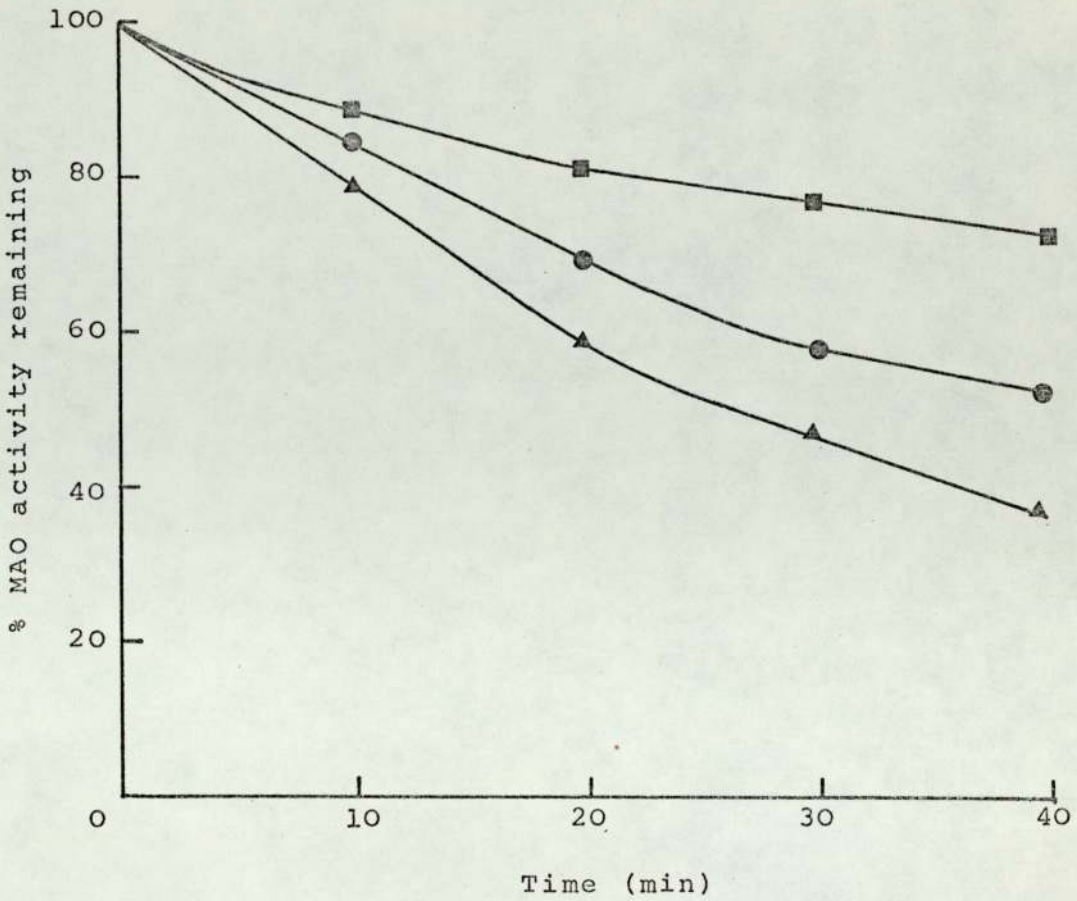


Fig. 33. Effect of trypsin on membrane bound MAO after treatment with phospholipase A. After treatment with phospholipase A, the enzyme was sedimented at 20,000x g and then resuspended to 2.5 mg protein/ml in 0.05M phosphate buffer, pH 7.5. The enzyme was treated with 200μg trypsin/mg protein at 37°C. At each time indicated, a 3 fold weight excess of soya bean trypsin inhibitor was added and the residual MAO activity estimated. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Control incubations without trypsin lost no enzyme activity over the period indicated. Each point on the graph represents the mean of 3 determinations.

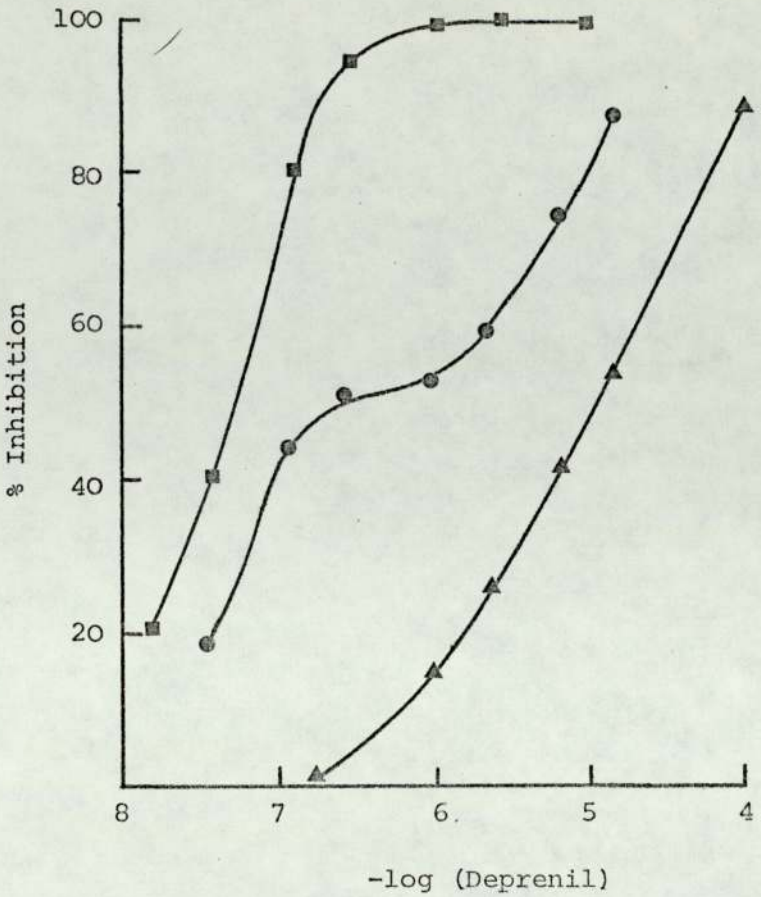


Fig. 34. Inhibition of membrane bound MAO with Deprenil after treatment with phospholipase A. Enzyme samples (50µl, 2.5 mg protein/ml in 0.1 M phosphate buffer, pH 8.0) were incubated at 37°C for 10 min with 50µl of Deprenil in water to give the final concentration indicated. After this incubation, no further time-dependent loss of MAO activity was observed with any substrate. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph represents the mean of at least 3 determinations.

the enzyme should allow the phospholipases further access to the enzyme bound lipids. The effect of lipid degradation on the MAO activity and properties were determined.

The solubilization and partial purification of MAO is shown in Table 22. The phospholipid content of the mitochondria was 181 µg/mg protein and the purified MAO preparation contained 221 ug/mg of protein. Gel filtration of the partially purified preparation on a column of Biogel A-150 m in the presence of 0.05% triton X-100 resulted in the elution of an initial large protein peak which contained 83% of the applied MAO activity and a broad second protein peak which contained no MAO activity (see Fig. 35). The eluted MAO activity had a lipid content of 162 µg/mg of protein. Gel filtration in the presence of 0.05% deoxycholate had no effect on the protein or MAO elution patterns but the total MAO activity recovered was reduced to 60%. The heat stability of the triton X-100 soluble, partially purified preparation of MAO is shown in Fig. 36 and there was no observed change in the Deprenil inhibition pattern, tryptic digestibility and Arrhenius plots of this preparation from the membrane bound enzyme. Treatment of the partially purified preparation with phospholipase C had no effect on the MAO activity and gel filtration of this preparation, in which 95% of the phosphoryl groups had been removed showed a similar pattern to that of the untreated preparation (see Fig. 35). The heat stability tryptic digestibility, Deprenil inhibition pattern and Arrhenius plots were unchanged compared to the untreated preparation.

Treatment of sonicated soluble (see Section 2.5.1) or triton X-100 soluble MAO with phospholipase A resulted in the loss of less than 10% of the MAO activity. Gel filtration of the phospholipase A treated triton X-100 soluble preparation on a column of Biogel A-150 m

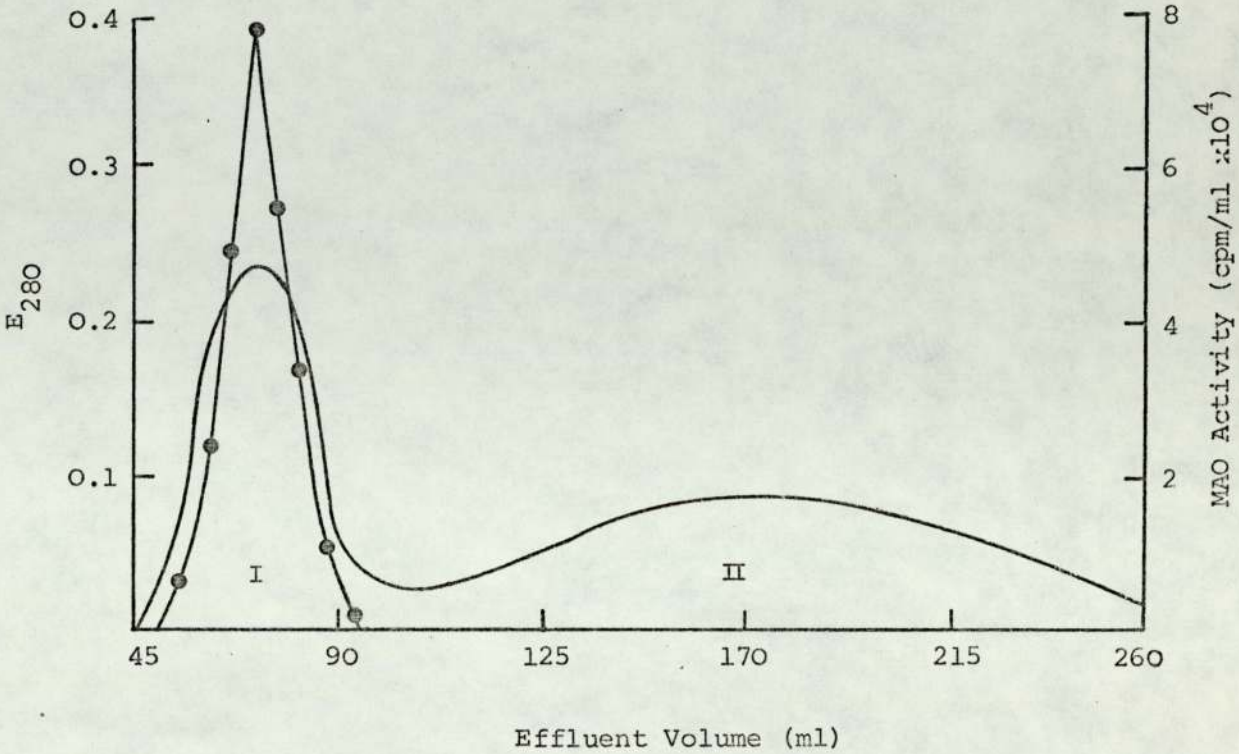


Fig. 35. Gel filtration of partially purified, triton X-100 soluble MAO on Biogel A-150m. A 40 cm x 2.5 cm column was equilibrated with 0.05M phosphate buffer, pH 8.0 containing 0.05% triton X-100. Enzyme samples were applied to the column in the presence of 1% (w/v) sucrose and 3.2 ml fractions were collected. ——, Protein elution pattern measured at 280nm and MAO activity measured with tyramine as substrate (●).

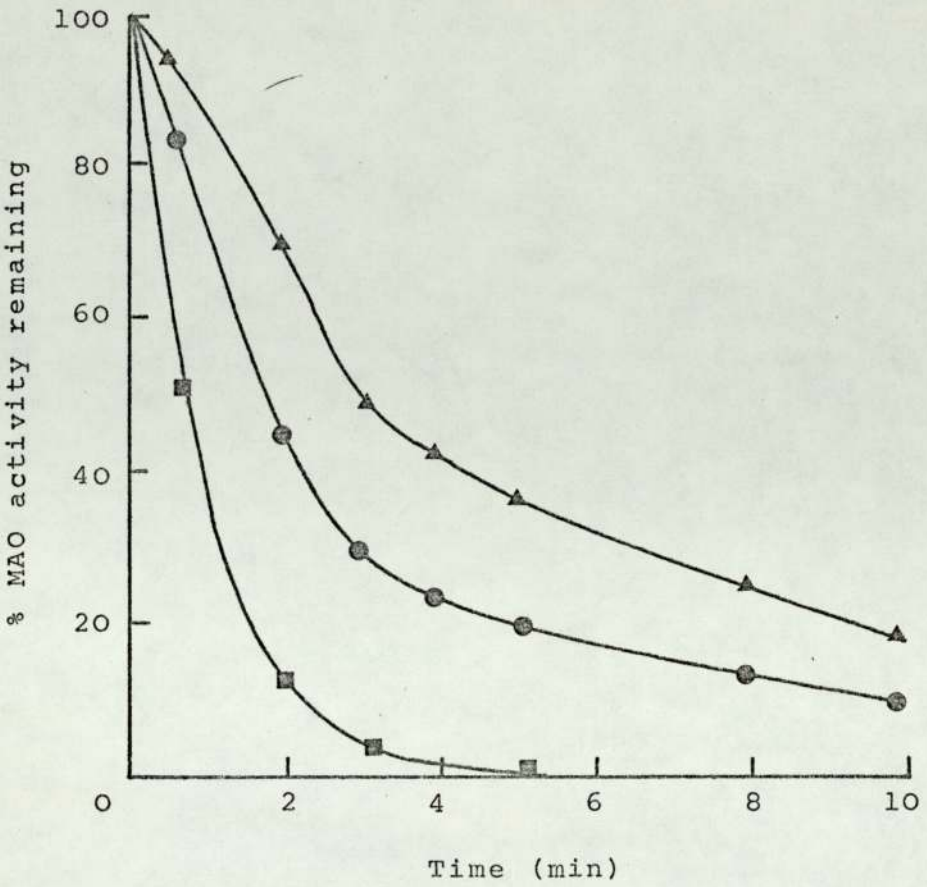


Fig. 36. Effect of heat treatment on the activity of partially purified triton X-100 soluble MAO. Enzyme samples were incubated at 50°C in 0.05M phosphate buffer, pH 8.0 at a protein concentration of 2.5 mg/ml. At the times indicated, samples were removed and assayed for MAO activity at 37°C. The substrates used were serotonin (▲), tyramine (●) and benzylamine (■). Each point on the graph represents the mean of at least 3 determinations.

in the presence of 0.05% triton X-100 is shown in Fig. 37. Similar to the pattern shown in Fig. 35, two protein peaks eluted, however, the second peak had increased considerably at the expense of the first peak. About 15% of the MAO activity towards tyramine eluted in the first (I) peak and 40% eluted in the second (II) peak. Similar protein elution patterns were observed if gel filtration was performed in the presence of 0.05% deoxycholate except the recovery of MAO was about 10% for peak I and about 25% for peak II. The lipid content of peaks I and II were found to be 47 and 14 µg/mg of protein respectively.

The K_m values of MAO for tyramine, serotonin, tryptamine and benzylamine are given in Table 28. There was no significant difference between the values of the membrane bound enzyme and the phospholipase A treated enzyme and these values are similar to those obtained by Weetman and Sweetman (1971). The heat stability of fraction II eluted from the Biogel A-150 m column is shown in Fig. 38 and is not affected by the presence of mitochondrial protein (1mg/ ml) in which the MAO activity had been abolished by pargyline. An Arrhenius plot of the B fraction is shown in Fig. 39 and transitions in the graph are seen at 25°C. The tryptic digestibility of MAO in fraction II is shown in Fig. 40. At a protein concentration of 100µg of trypsin/µg of protein, MAO activity decreased rapidly but differences in substrate sensitivity are seen. The inhibition of fraction II by Deprenil is shown in Fig. 41. Substrate selectivity is still observed and the inhibition pattern is similar to that of the membrane bound enzyme. Attempts to reduce the phospholipid content of fraction II were performed by solvent extraction, perchlorate treatment and further gel filtration but without success.

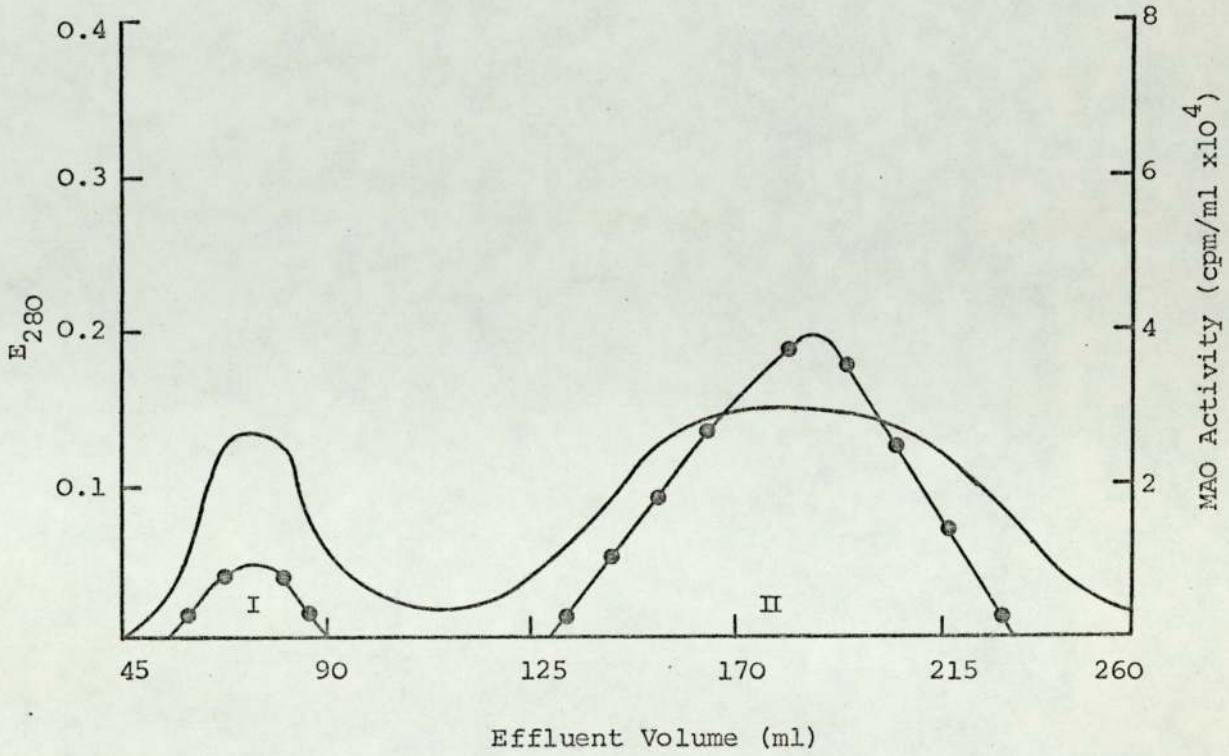


Fig. 37. Gel filtration of partially purified, triton X-100 soluble MAO after treatment with phospholipase A on Biogel A-150m. A 40 cm x 2.5 cm column was equilibrated with 0.05M phosphate buffer, pH 8.0 containing 0.05% triton X-100. Enzyme samples were made 1% (w/v) in sucrose before application to the column and 3.2 ml fractions were collected. ——, Protein elution pattern measured at 280nm and MAO activity measured with tyramine as substrate (●).

TABLE 28.

Km Values for Rat Liver MAO.

Substrate	Mitochondrial MAO Km (μM)	Triton X-100 soluble MAO, phospholipase A treated Km (μM).	Data from Weetman and Sweetman (1971) Km (μM)
Tyramine	67	69	45.6
Serotonin	98	87	57.2
Tryptamine	40	41	17.7
Benzylamine	133	115	-

Km values for the 4 substrates were determined from reciprocal plots covering a substrate concentration range of 10 μM to 100 μM for tyramine and tryptamine, 10 μM to 200 μM for serotonin and from 50 μM to 1 mM for benzylamine. All enzyme assays were performed at 37°C and at pH 8.0.

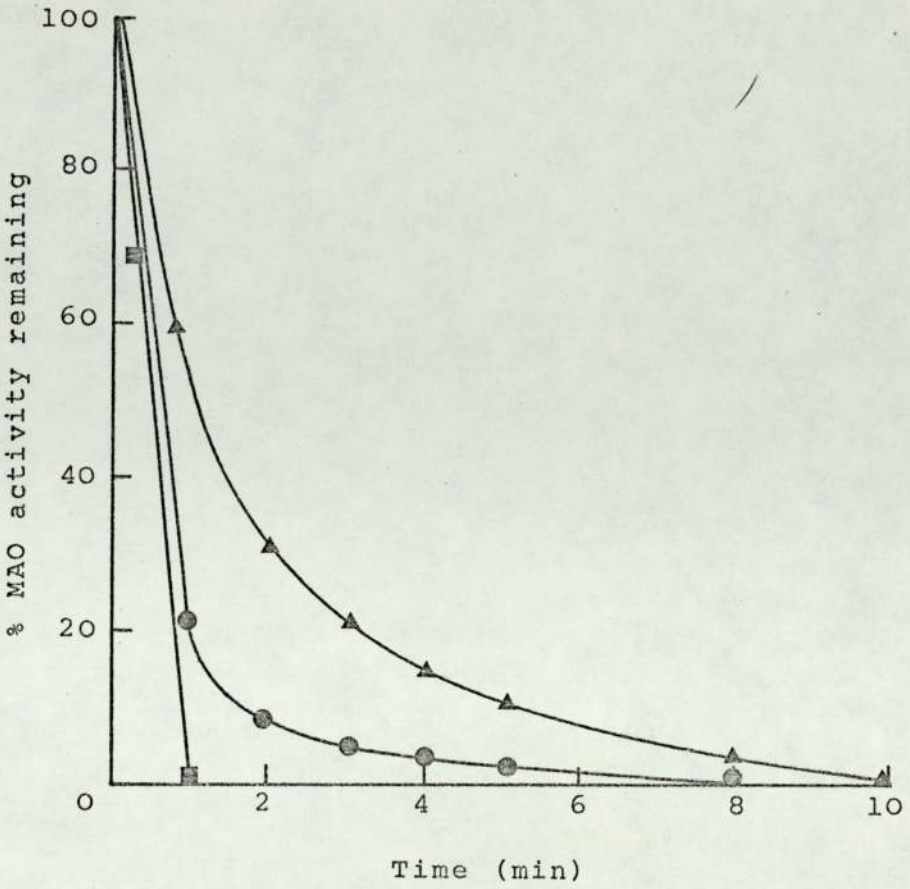


Fig. 38. Effect of heat treatment on the activity of partially purified, triton X-100 soluble MAO after treatment with phospholipase A. Fraction II eluted from a column of Biogel A-150m was used as the enzyme source. Enzyme samples (2.5 mg protein/ml) were incubated at 50°C in 0.05M phosphate buffer, pH 8.0. At the times indicated samples were assayed for MAO activity at 37°C. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph is the mean of 3 determinations.

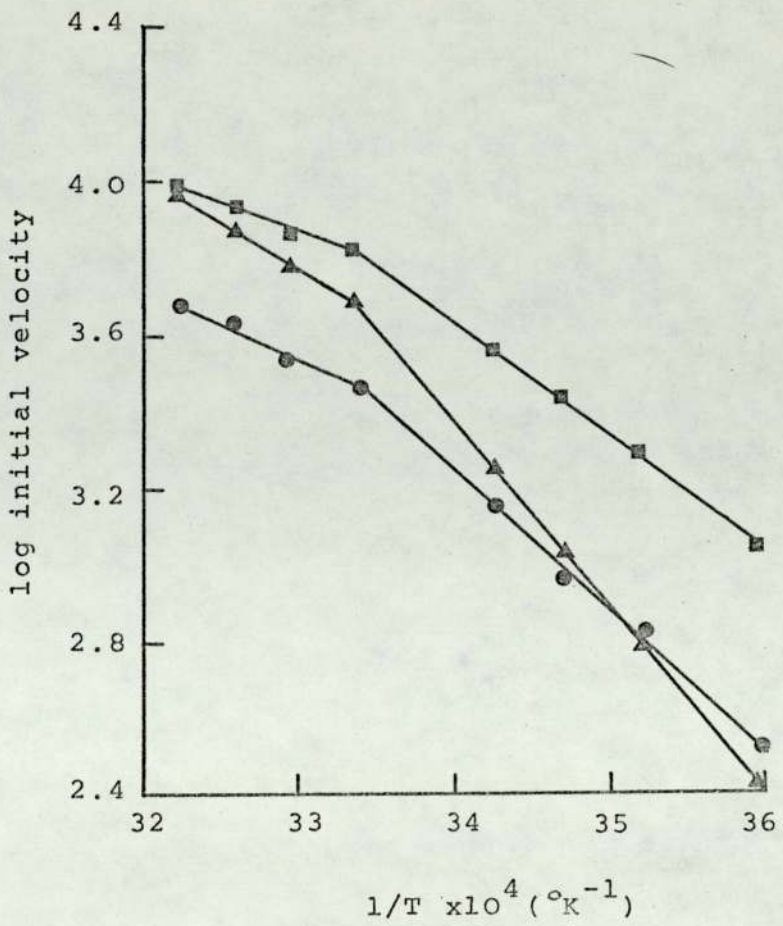


Fig. 39. Arrhenius plots of partially purified, triton X-100 soluble, phospholipase A treated MAO. Fraction II eluted from a column of Biogel A-150m was used as the enzyme source. Enzyme samples were preincubated for 5 min at the indicated temperature before the addition of substrate. Activity was measured with tyramine (●), serotonin (▲) and benzylamine (■) as substrates. Each point on the graph represents the mean of 3 determinations.

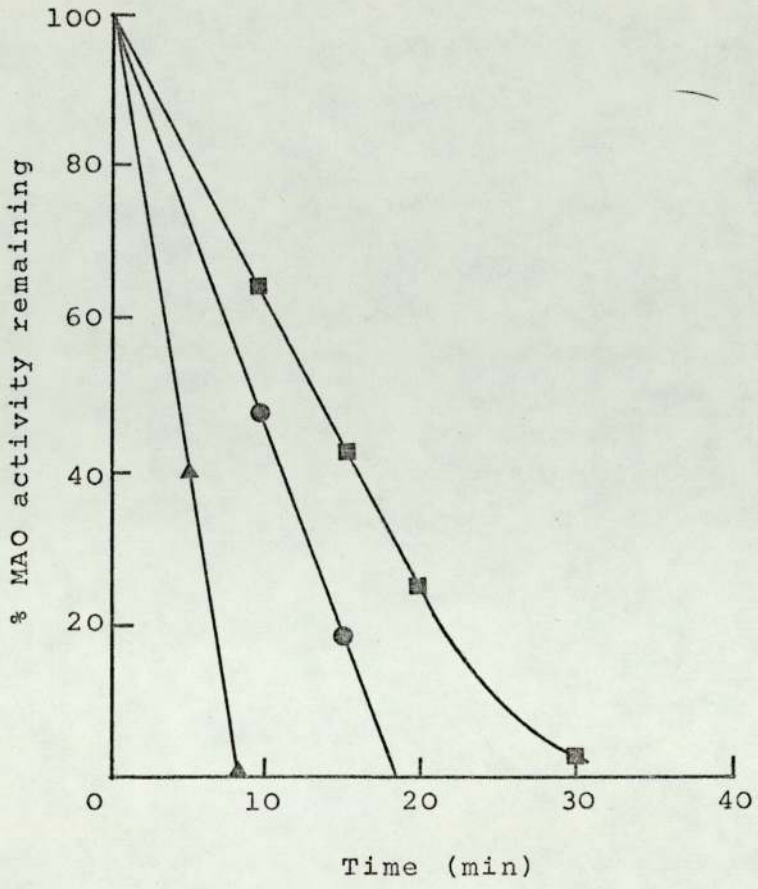


Fig.40. Effect of trypsin on partially purified, triton X-100 soluble MAO after treatment with phospholipase A. Fraction II eluted from a column of Biogel A-150m was used as the enzyme source. Enzyme samples were treated with 100µg of trypsin per mg of protein. At each time indicated, a 3 fold weight excess of soya bean trypsin inhibitor was added and the residual MAO activity estimated. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Control incubations without trypsin, lost no MAO activity over the time period indicated. Each point on the graph represents the mean of 3 determinations.

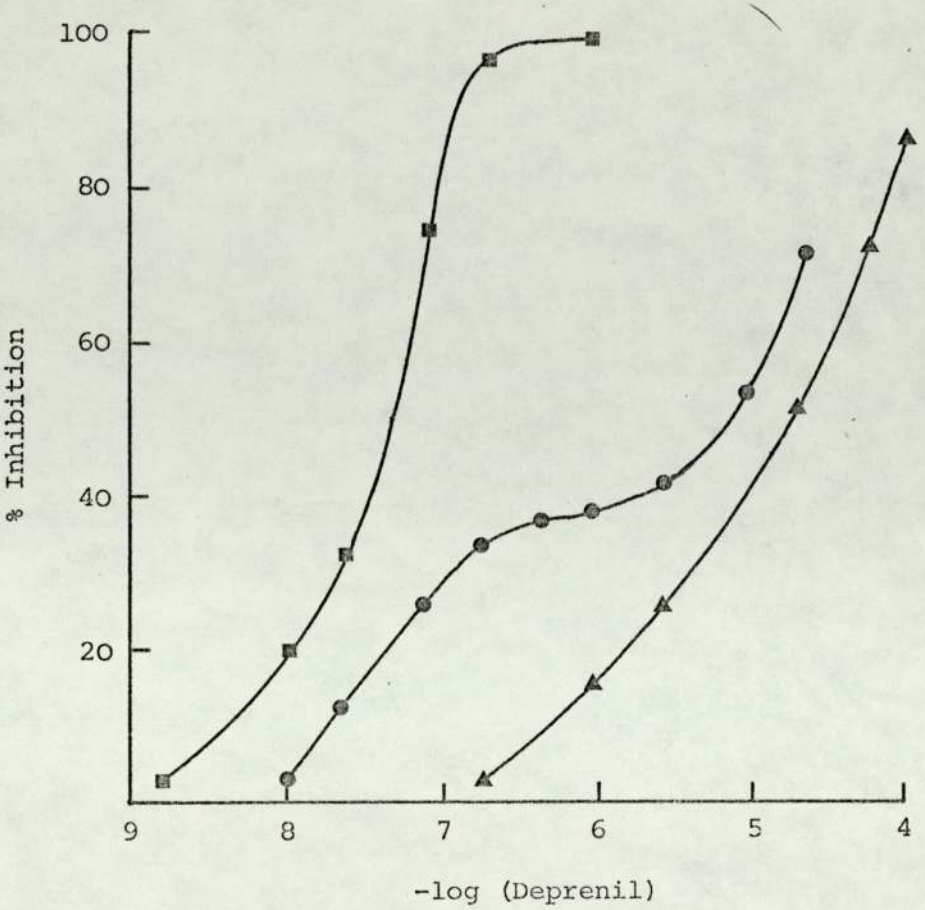


Fig. 41. Inhibition of partially purified, triton X-100 soluble MAO treated with phospholipase A. Fraction II eluted from a column of Biogel A-150m was used as the enzyme source. Enzyme samples (50µl, 2.0 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37° C for 10 min with 50µl of Deprenil in water to give the final concentration indicated. After this incubation, no further time-dependent loss of MAO activity was observed with any substrate. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph represents the mean of at least 3 determinations.

Table 29 shows the effect of DTNB-Sepharose on the MAO activity of different preparations. The enzyme was inhibited about 4.2% with intact mitochondria and no difference was observed if the mitochondria were lysed. With triton X-100 soluble MAO, inhibition was increased to 28.7% and the phospholipase A treated soluble preparation was inhibited 93.4%. Control incubation with mitochondrial protein in the presence of 1 mM mecaptoethanol, showed that MAO was not inhibited by the TNB anion. That MAO is readily inhibited by free DTNB is shown in Fig. 42.

5.7 Effect of Perchlorate on some Soluble MAO Preparations.

In the preceding sections, it was found that altering or removal of the membrane environment of MAO by lipid peroxidation or treatment with phospholipases had little effect in abolishing the differential properties of MAO A and MAO B towards tryptic digestibility, heat stability and the Depenil inhibition pattern. In addition, there was no change in the Arrhenius plots of the enzyme after lipid removal. However, Houslay and Tipton (1973) found that after treatment of MAO with the chaotropic agent sodium perchlorate, which reduced the lipid content of the enzyme, the differential heat stability and Clorgyline inhibition patterns of MAO A and MAO B were abolished. Therefore, it was decided to investigate the effect of perchlorate on a number of soluble MAO preparations in an attempt to repeat the results of Houslay and Tipton (1973).

The effect of perchlorate treatment on some solubilized preparations of MAO is shown in Table 30. Comparison of the amount of MAO activity recovered after treatment with 0.5 M perchlorate shows that with all preparations, benzylamine oxidation was affected the least and serotonin oxidation was most affected. With the triton X-100 solubilized MAO preparation and the enzyme prepared by the method of

TABLE 29.

Inhibition of some MAO Preparations with DTNB-Sepharose.

Preparation	% MAO inhibition with DTNB-Sepharose.
Intact mitochondria	4.2
Lysed mitochondria	4.5
Triton X-100 soluble MAO	28.7
Triton X-100 soluble MAO, phospholipase A treated.	93.4

Settled DTNB-Sepharose (100 µl) was added to 50 µl of enzyme preparation (2.5 mg of protein/ml in 0.25 M sucrose containing 0.05 M phosphate buffer, pH 8.0) and incubated for 30 min at 30°C before the addition of substrate. Tyramine was used as substrate.

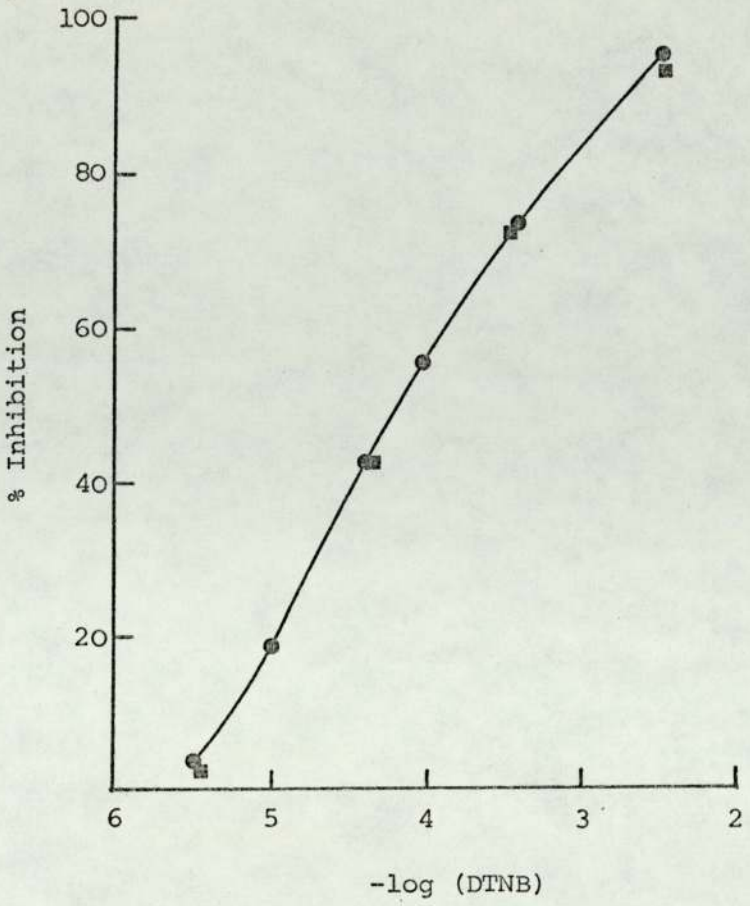


Fig. 42. Inhibition of membrane bound MAO by DTNB. Enzyme samples (50µl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 20 min with 50µl of DTNB in the same buffer to give the final concentration indicated before the addition of substrate. The substrates used were tyramine (●) and benzylamine (■). Each point on the graph is the mean of at least 2 determinations.

TABLE 30.

Effect of Perchlorate Treatment on Solubilized MAO.

	Assay Substrates		
Perchlorate (0.5M) treated preparations	Tyramine %	Serotonin %	Benzylamine %
Control	100	100	100
Sonicated soluble MAO	52.87	34	83
Partially Purified Triton X-100 soluble MAO.	50.7	12.0	87
Solubilization by Youdim and Sandler (1968).	53.2	13	93

Solubilized preparations were treated with perchlorate as described in Section 2.8 and compared to preparations not treated. Each percentage is a mean value from at least 3 determinations. Figures are % of MAO activity remaining after perchlorate treatment.

Youdim and Sandler (1968), treatment with perchlorate reduces the serotonin activity by 87 to 88% and with the sonicated soluble MAO preparation, serotonin activity is reduced 66%. Similar results were obtained when either triton X-100 (0.05%) or deoxycholate (0.05%) were used to equilibrate the Sephadex G-50 column (see Section 2.8) with all the preparations that required detergent for solubilization. The effect of perchlorate on the Deprenil inhibition pattern of some preparations is shown in Fig. 43. As the serotonin oxidizing ability is impared, the plateau region of tyramine inhibition is abolished.

The recovery of perchlorate soluble MAO from the total enzyme activity in the mitochondria with tyramine, serotonin and benzylamine as substrates is shown in Table 31. The soluble fraction contains 30% of the benzylamine activity, 22% of the tyramine activity and only 14% of the serotonin activity. The inhibition of this preparation by Deprenil is shown in Fig. 44. Substrate selectivity is still observed but the plateau of tyramine inhibition was increased to the 70% inhibition level.

5.8 Mitochondrial Structural Protein.

In 1961 Criddle, Bock, Green and Tisdale (1961) and Green, Tisdale, Criddle, Chen and Bock (1961) isolated a protein from beef heart mitochondria which accounted for 30 to 45% of the total mitochondrial protein and lacked enzymatic activity. They called this protein "structural protein" (SP) and suggested that its role in the mitochondria was to provide a backbone upon which mitochondrial enzymes, cytochromes and phospholipids were organized. Structural protein has now been isolated from a number of membranes and by a number of different techniques (see Rothfield and Finkelstein, 1968 ; Munn, 1974). The following results show the isolation of SP from rat liver mitochondria in which the MAO had been labelled with (^{14}C) pargyline to determine

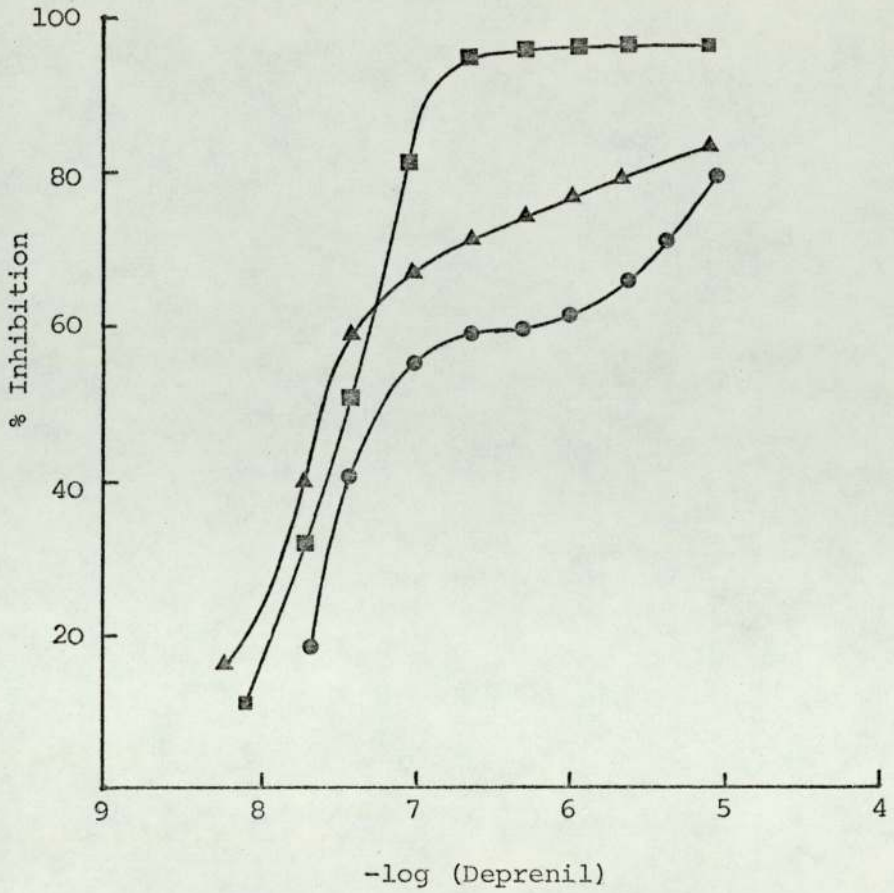


Fig. 43. Effect of perchlorate treatment on the Deprenil inhibition pattern of MAO. Soluble MAO preparations were treated with perchlorate as described in Section 2.8. Enzyme samples (50µl, 2.5 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 10 min with 50µl of Deprenil in water to give the final concentration indicated. After this incubation no further time-dependent loss of MAO activity was observed. Tyramine was used as substrate for all assays. Control inhibition patterns with sonicated and triton X-100 soluble MAO were similar (●), perchlorate treated sonicated soluble MAO (▲) and perchlorate treated triton X-100 soluble MAO (■). Each point on the graph is the mean of at least 3 determinations.

TABLE 31

Recovery of Perchlorate Soluble MAO from Mitochondria

	Substrate		
Fraction	Tyramine %	Serotonin %	Benzylamine %
Mitochondria	100	100	100
Perchlorate Soluble MAO.	22	14	30

The enzyme was prepared as described in Section 2.5.3. Figures are % of MAO activity as compared to control (100%).

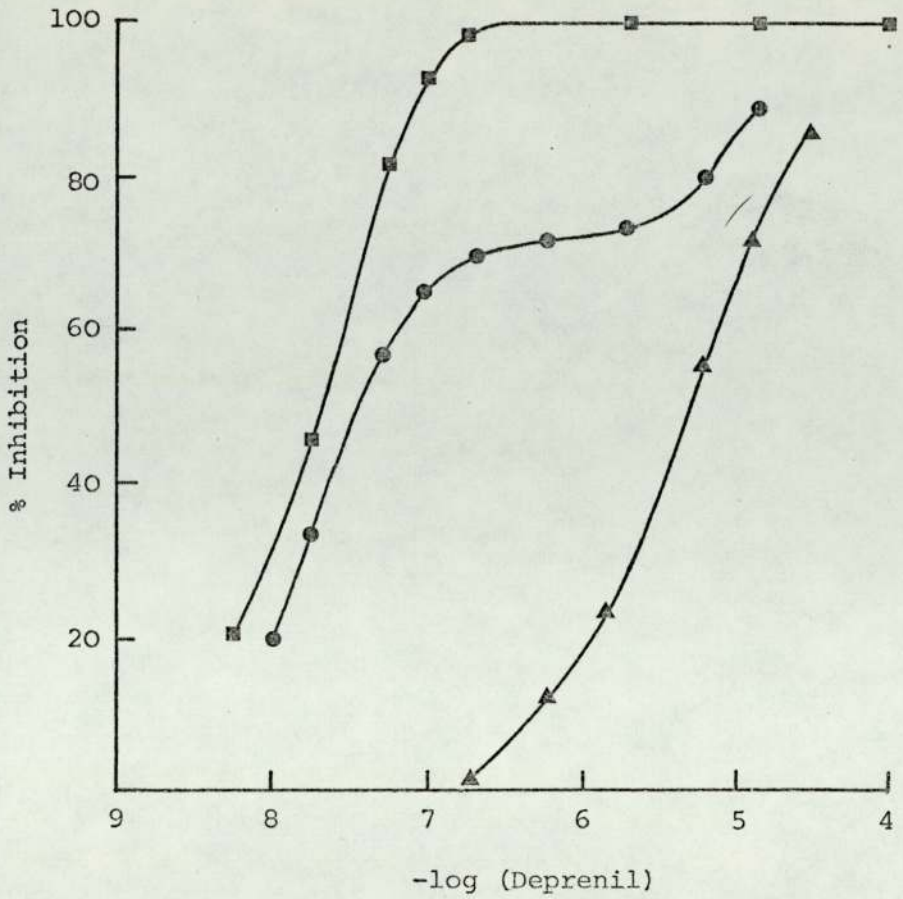


Fig. 44. Inhibition of perchlorate soluble MAO by Deprenil. Enzyme samples (50 µl, 2.0 mg protein/ml in 0.1M phosphate buffer, pH 8.0) were incubated at 37°C for 10 min with 50 µl of Deprenil in water to give the final concentration indicated. After this incubation no further time-dependent loss of MAO activity was observed with any substrate. The substrates used were tyramine (●), serotonin (▲) and benzylamine (■). Each point on the graph is the mean of at least 2 determinations.

if the enzyme constitutes part of this membrane protein. These results would be helpful in indicating the relationship of the enzyme to the structure of the outer mitochondrial membrane.

Mitochondria treated with (^{14}C) pargyline to label the membrane bound MAO and then treated with detergent as described by Criddle et al. (1962) results in 97% of the label in the supernatant after centrifugation (see Table 32). After bringing the supernatant to 12% saturation with ammonium sulfate and centrifugation, the pellet which is the structural protein is washed with sucrose and contains 29% of the label. Electrophoresis of the detergent soluble fraction at pH 8.5 is shown in Fig. 45. A number of protein bands were visible but the labelled enzyme is present only at the origin and the fastest moving anodic band. Electrophoresis of the structural protein fraction at pH 8.5 is shown in Fig. 46. Only two protein bands are present one at the origin and one migrating towards the anode, both of which contained labelled enzyme. The structural protein could not be washed with warm methanol as described by Criddle et al. (1962) as this procedure removed the ^{14}C label. Also, electrophoresis could not be performed at acidic pH as this also releases the ^{14}C label from the protein. Isolation of mitochondrial structural protein by acid extraction as described by Zahler et al. (1968) extracts 41% of the (^{14}C) pargyline from the mitochondria (see Table 33). Adjustment of the acid extraction to pH 6.5 results in precipitation of the structural protein which contained 8% of the original ^{14}C label.

5.9 Resolution of Mitochondrial Membranes by Sonication.

It has been suggested that MAO is a minor constituent of the outer mitochondrial membrane and is not necessary for the integrity

TABLE 32.

Isolation of Structural Protein from Mitochondria Labelled with (^{14}C) Pargyline.

Fraction	mg protein	Total cpm	cpm/mg protein	% of labelled enzyme
Labelled mitochondria.	382	709,200	1,070	100
Supernatant after centrifugation in presence of detergent.	372	395,278	1,062	97
Supernatant after 12% $(NH_4)_2SO_4$	229.7	211,758	922	52
Structural protein fraction	141.6	116,640	823	29
Sucrose washing of structural Protein	-	30,380	-	7

Mitochondria were labelled with (^{14}C) pargyline as described in Section 2.16 and the structural proteins were isolated by the method of Criddle et al. (1962), except that the final removal of bile salt with methanol was omitted.

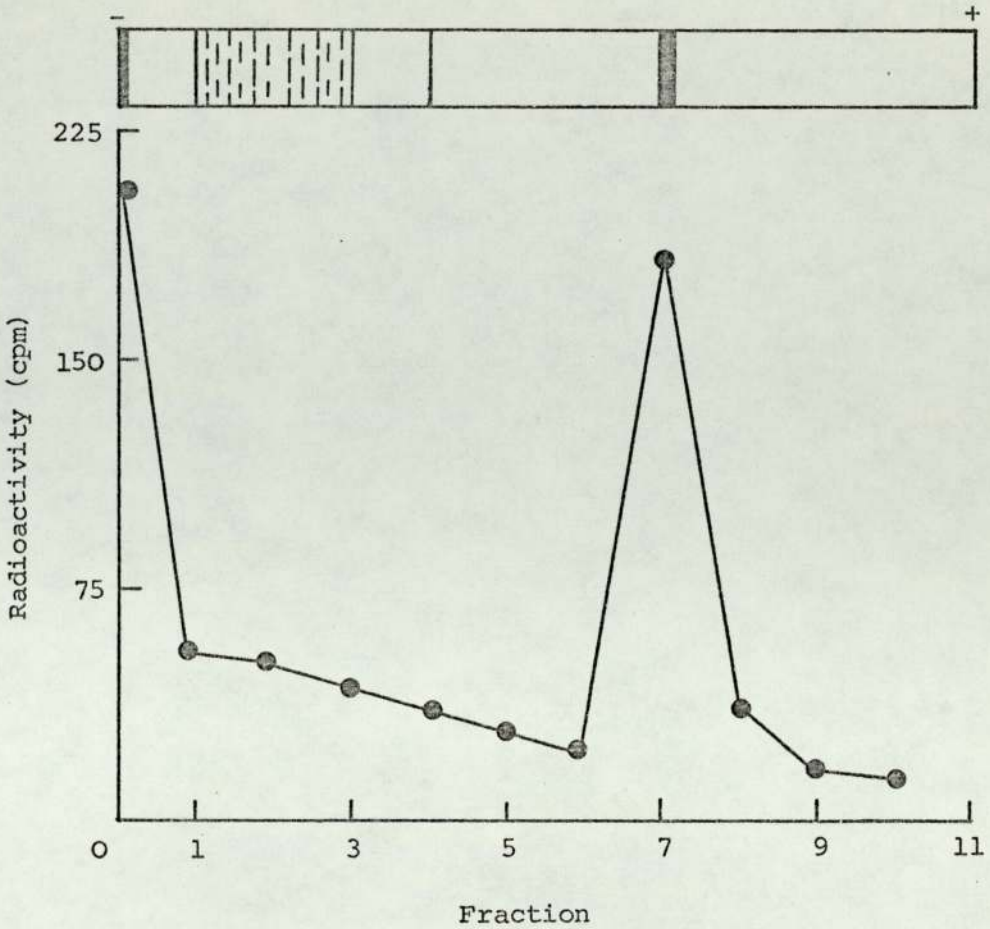


Fig. 45. Polyacrylamide gel electrophoresis of (^{14}C) pargyline labelled mitochondria treated with detergents by the method of Criddle et al. (1962). Electrophoresis was carried out from cathode to anode for 50 min in the presence of 1% SDS and the gels were stained for protein with Amido Black as described in Section 2.11. Gels were counted for ^{14}C as described in Section 2.16.

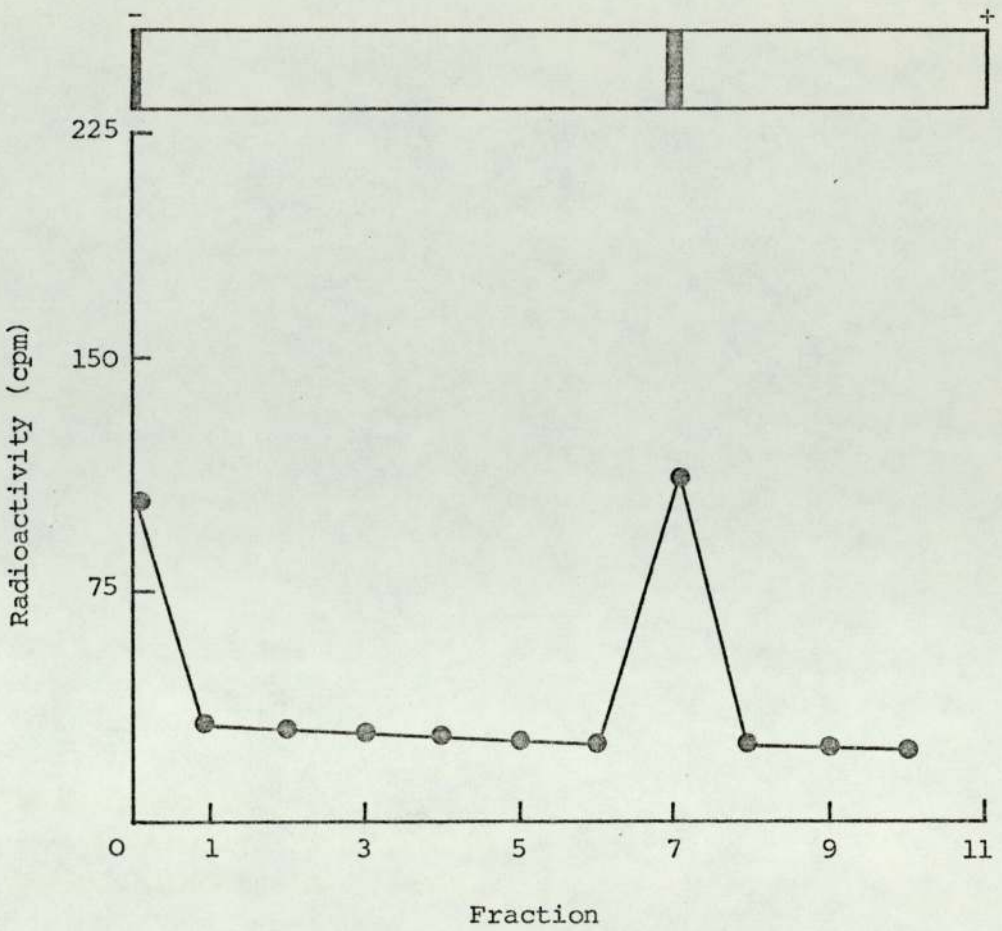


Fig. 46. Polyacrylamide gel electrophoresis of (^{14}C) pargyline labelled structural protein. Isolation of structural protein was performed as described by Criddle et al. (1962) except that the final removal of bile salt with methanol was omitted. Electrophoresis was carried out from cathode to anode for 50 min in the presence of 1% SDS and the gels were stained for protein with Amido Black as described in Section 2.11. Gels were counted for ^{14}C as described in section 2.16.

TABLE 33.

Isolation of Structural Protein from (^{14}C) labelled Mitochondria by Acid Extraction.

Fraction	mg protein	Total cpm	cpm/mg protein	% label recovered
Labelled mitochondria	242.6	406,767	1,677	100
Supernatant after acid extraction	-	165,540	-	41
Protein from acid extraction after dialysis	46.8	33,500	716	8

Mitochondria were labelled with (^{14}C) pargyline as described in Section 2.16 and the structural proteins were isolated by the method of Zahler et al., (1968).

of the membrane (see Section 1.3). These suggestions were made from experiments in which sonication was used to strip the enzyme from the membrane of bovine kidney cortex (Racker and Proctor, 1970). These workers used the loss of MAO activity to indicate that the membrane was depleted of the enzyme. However, their results could also be explained by a loss of MAO activity during sonication and membrane isolation while still being attached to the membrane. Therefore, the above hypothesis was investigated with rat liver mitochondrial MAO in which the enzyme had been labelled with (^{14}C) pargyline and using the sonication procedure of Racker and Proctor (1970).

Membrane samples (10.2 mg) sonicated at pH 9.6 for up to 8 x 30 second periods and then adjusted to pH 6.0, had a similar amount of ^{14}C in the supernatant after centrifugation as control suspensions with no sonication (see Table 34). The same table shows the results when after sonication the preparation was centrifuged at pH 7.5. Less than 5% of the ^{14}C label was released into the supernatant.

TABLE 34.

Resolution of Mitochondrial Membranes labelled with (^{14}C) Pargyline by Sonication.

Fraction	Total cpm/10.2 mg	cpm in pH 6.0 supernatant	% Recovered	cpm in pH 7.5 supernatant
Control	24,125	13,422	56	1,187
1 x 30s Sonication	24,125	13,294	55	1,266
4 x 30s Sonication	24,125	13,055	54	1,285
8 x 30s Sonication	24,125	13,698	57	1,307

Mitochondria were labelled with (^{14}C) pargyline as described in Section 2.16. Membranes were resolved as described in Section 2.19.

6. DISCUSSION.

6.1 Inhibitor Specificity of MAO.

The inhibitor specificity of Deprenil and Clorgyline is in agreement with the findings of Hall et al. (1969) and Houslay and Tipton (1974) that rat liver mitochondrial MAO contains at least two forms of the enzyme. Using the nomenclature of Johnston (1968) and Clorgyline as inhibitor, serotonin is a specific substrate for MAO A, benzylamine is a specific substrate for MAO B and tyramine shows a biphasic inhibition curve indicating it is a substrate for both enzymes (Fig. 18). Similar to the results of Knoll and Magyar (1972) and Houslay and Tipton (1974), Deprenil was shown to be a selective inhibitor of MAO B and Clorgyline a selective inhibitor of MAO A (see Figs. 15 and 18). In a recent review Neff and Yang (1974) classified pargyline as a non-specific inhibitor but suggested that it may preferentially inhibit the type B enzyme. In agreement with their suggestion, Fig. 16 shows that pargyline is a selective MAO B inhibitor which shows a similar selectively to Deprinil. These acetylenic inhibitors have been suggested to be acted upon by the enzyme in a similar fashion as substrates resulting in the transfer of electrons from the carbon atom vicinal to the amino nitrogen to the enzyme's flavin moiety. The resultant active principle then reacts to irreversibly inhibit the enzyme (Hellerman and Erwin, 1968). The inhibition of MAO by Lilly 51618, a Clorgyline derivative supports this hypothesis. The observation that the inhibition produced was fully reversible and that no time dependent inhibition was detected indicates that the two methyl groups on the carbon atom vicinal to the amino nitrogen prevents the enzyme from transferring the necessary electrons to form an active principle. A similar result was reported by Sweet et al. (1963) who with various analogues of pargyline suggested that

the intact propynyl grouping are essential for inhibitory action. It was also observed that the selectivity of this compound was greatly reduced compared to Clorgyline suggesting that the methyl groups also prevent a correct fit at the binding sites for full selectivity to be observed (Fig. 19).

In a recent review, Neff and Yang (1974) suggested that the hydrophobic regions on MAO that binds the aromatic moiety of inhibitor drugs and substrates are responsible for the formation of the various forms of the enzyme. Therefore, one could expect an irreversible inhibitor that does not contain an aromatic ring to have a greatly reduced or no selectivity. Propargylamine has previously been shown to be an irreversible MAO inhibitor (Ables and Tashjian, 1975) and when used with tyramine, serotonin and benzylamine as substrate, the selectivity is greatly reduced (Fig. 17). This would in part support the hypothesis of Neff and Yang (1974), however, this compound does show definite selectivity towards MAO B over MAO A indicating other binding sites may be involved in the observed selectivity. Severina and Sheremet'evskaya (1969) showed that aromatic hydroxyl groups bind to polar regions on the enzyme and suggested that the hydrogen atom from the hydroxyl group may also be involved in the formation of a hydrogen bond. Perhaps the lack of groups on the propargylamine molecule which could interact with the polar region or form hydrogen bonds with MAO A is the reason for its selectivity for MAO B.

6.2 Interaction of Clorgyline with Mitochondrial Membranes.

Trinitrobenzene sulfonic acid (TNBS) has been used for the study of amines (Means, Congdon and Bender, 1972), lipids (Siakotos, 1966) and as a probe for the erythrocyte membrane (Godin and Ng, 1972).

In the present work, the rate and extent of trinitrophenylation of mitochondrial membrane protein and lipid amino groups was used to monitor alterations in membrane structure induced by the MAO inhibitor Clorgyline. There was a concentration dependent increase in the rate of TNBS incorporation produced by Clorgyline (see Fig. 20) and this effect could not be attributed to a direct interaction of Clorgyline with the TNBS molecule or changes in ionic strength. In addition, the K_{diss} of Clorgyline was unchanged (Fig. 22) over a 6 fold range of TNBS concentrations suggesting that a complex formation between Clorgyline and the TNBS molecule does not enhance the reactivity of TNBS or increase its accessibility to any binding sites. Therefore, the stimulation of TNBS incorporation by Clorgyline was probably a reflection of the way Clorgyline perturbs the membrane structure.

The rate of TNBS incorporation into mitochondrial membranes increased with increasing pH (Fig. 21) which as suggested by Gordesky, Marinetti and Love (1975) may be due to a greater chemical reactivity of the nonprotonated amino groups at higher pH values. However, it is possible that the conformation of the membrane may be altered in such a way at higher pH values to expose more amino groups for reaction. The stimulation of TNBS incorporation induced by Clorgyline decreases with increasing pH (Fig. 21) which may reflect the possibility that the concentration of solubilized Clorgyline is higher at lower pH values.

It has been shown that chlorpromazine greatly increases the TNBS incorporation in erythrocyte membranes (Godin and Ng, 1972) and that this effect could be reduced by pretreatment of the membranes with phospholipase C. This indicated that chlorpromazine interacted with the phospholipid phosphoryl groups. A similar effect was seen with chlorpromazine on mitochondrial membranes (see Table 23)

which again could be greatly reduced after 60% of the phosphoryl groups had been removed by phospholipase C. It is interesting to observe however, that after mitochondrial treatment with phospholipase C, which had no effect on the MAO activity, the inhibition of MAO produced by chlorpromazine was not affected (see Fig. 23) indicating that the phospholipid phosphoryl group - chlorpromazine interaction does not reduce the concentration of the drug available for enzyme inhibition. In contrast, phospholipase C treatment had no effect on the Clorgyline stimulation of TNBS indicating that a Clorgyline-phosphoryl group interaction was not responsible for the TNBS stimulation.

Belleau and Morgan (1963) suggested that the acetylenic groups of MAO inhibitor drugs may react with SH groups. The possibility of Clorgyline perturbing the membrane structure by interaction with protein SH groups was suggested in the present experiments by the result that arsenite reduced by 50% and Hg^{2+} which itself increased the rate of TNBS incorporation, abolished the Clorgyline effect. However, after membrane treatment with Clorgyline the number of protein SH groups measured with DTNB did not decrease indicating that Clorgyline did not interact with SH groups. Perhaps, aresenite and Hg^{2+} induce conformational changes in the membrane which reduces the effect of Clorgyline. Acetylene groups are known to react with metal ions, however pretreatment of the membranes with EDTA has no effect on the Clorgyline stimulation of TNBS indicating that interaction of Clorgyline with metal ions is not responsible for the effect.

ANS a compound which interacts with protein hydrophobic groups (Stryer, 1965) and 4-hydroxquinone which due to its ring structure is also hydrophobic in nature both reduce by 95% the Clorgyline effect (see Table 24). This suggests that Clorgyline perturbs

the mitochondrial membrane through either protein or lipid hydrophobic regions or both with the aromatic moiety of the drug. This is further supported by the observation that propargylamine which has no hydrophobic aromatic ring, does not affect the rate of TNBS incorporation in concentrations of up to 10 mM.

The question arises of whether or not Clorgyline stimulation of TNBS incorporation is related to the interaction of Clorgyline with MAO. Pretreatment of the mitochondrial membranes with Clorgyline to irreversibly inhibit the MAO followed by dialysis to remove any free Clorgyline, does not affect the stimulation of TNBS by Clorgyline. This would indicate two things: first that the perturbation of the membrane by Clorgyline is reversible whereas MAO inhibition is irreversible and second the interaction of MAO with Clorgyline is not part of the increase in TNBS incorporation. However, MAO is totally inhibited at Clorgyline concentrations of 10 μM whereas the stimulation of TNBS is measurable only at concentrations of 0.1 mM Clorgyline and above. Therefore, if the Clorgyline MAO interaction does result in a change which would result in a greater reactivity of its amino groups, the TNBS assay is not sensitive enough to measure it.

6.3 Properties of Membrane Bound MAO.

When lysed mitochondria were incubated with low concentrations of trypsin (50 μg/mg of protein), over the observed time period little MAO activity was lost (< 15%) with all substrates used (see Table 25). However, increasing the trypsin concentration 4 fold and over the same incubation period MAO A lost 70% of its activity whereas MAO B lost only 25% of its activity. A similar selective loss of MAO A over MAO B with trypsin was observed by Yang, Goridis and Neff (1972) with rat brain MAO. It has been recognized for some time that the rate of proteolysis depends upon the conformation of the protein

substrate (Rupley, 1967). Therefore, the limited digestion of MAO B as compared to MAO A may be due to restrictions in orientation or different conformations of similar proteins induced by the membrane environment which exists around the enzyme. However, it is also possible that part of the difference in the rate of hydrolysis of MAO A and MAO B may be due to differences in two different protein structures.

When intact or lysed mitochondria are subjected to 50°C MAO B lost activity at a faster rate than MAO A while the activity towards tyramine, a common substrate, was lost at an intermediate rate. Squires (1972) also has shown that MAO A was more heat stable than MAO B. There are several factors that can contribute to the heat stability of a protein, for instance Scheraga, Némethy and Steinberg (1962) showed that the strength of a hydrophobic bond increases with temperature up to about 60°C suggesting that as the temperature increases hydrophobic bonds would help maintain protein conformation. However, since most proteins denature as the temperature increases then other factors must be involved in protein stability such as hydrogen bonds. The environment of the membrane bound protein may also influence the heat stability. For instance enzymes bound to hydrophobic supports are less heat stable than enzymes bound to hydrophilic supports (Goldstein, 1973). Therefore, the differences in heat stability of MAO A and MAO B may be due to protein differences, environmental differences or both.

The Arrhenius plot of membrane bound MAO showed an interesting result, with tyramine, serotonin and benzylamine as substrates, a single discontinuity was observed at 26.8°C (see Fig. 27). The decrease in activation energy above the transition point reflects a change in the catalytic site (conformational change) at the transition temperature (Sineriz, Farias and Trucco, 1973). Breaks in Arrhenius

plots have been most commonly interpreted for membrane functions as the result of a phase change in the lipid environment (Kumamoto, Raison and Lyons, 1971; Sechi et al., 1973). When a phospholipid is heated to higher temperatures, it reaches a transition point (melting point) and exhibits a very high degree of molecular motion. That this phase change involves primarily the aliphatic chains of the phospholipids has been confirmed by X-ray analysis and by the observation that the temperatures at which the transition occurs falls in an order that parallels the melting point of the constituent fatty acids (see Chapman, 1969). Phospholipids that contain shorter carbon chains or unsaturated chains, exhibit phase changes at lower temperatures (Chapman, 1969). In some reports correlations have been found between transition temperatures of membrane lipids using electron paramagnetic resonance of spin labelled lipids and breaks in Arrhenius plots of enzymes which supports the phase change theory of Arrhenius plot breaks (Raison et al., 1971; Eletr,Zakim and Vessey, 1973; Morrisett et al., 1975).

If the results of the MAO Arrhenius plots can be interpreted by the above explanation, then MAO A and MAO B must be in similar lipid environments as they have the same transition temperatures. Using differential scanning calorimetry Blazyk and Steim (1972) found that a thermotropic phase transition in mitochondria and in a water dispersion of mitochondrial lipids centered around $0^{o}C$. Although, the transition for MAO does not reflect this 0^{o} transition, it probably represents the transition of the micro environment or boundary lipids (see Jost et al., 1973) associated with the enzyme. In addition, the effect of the lipid phase change induced conformational change has been investigated for some enzymes. For example, Zakim and Vessey (1975) found that UDP-glucose, UDP-mannose and UDP-xylose inhibit microsomal

UDP-glucuronyltransferase at 6°C but none of these compounds affected the activity at 37°C. The authors found that these significant alterations in the regulatory properties of the enzyme were associated with temperature-induced phase changes within the lipid portion of the microsomal membrane. In some preliminary experiments to investigate MAO activity above and below the transition temperature, it was found that Deprenil inhibition was not changed for any substrate at 10°, 16° and 37°C. Therefore, the change in the enzyme observed by the break in Arrhenius plot, does not have an effect on the binding or selectivity of Deprenil.

There are other possible explanations for breaks in Arrhenius plots which would explain the observed transitions with MAO. These include thermally induced reversible changes in enzyme protein structure (Massey, Curti and Granther, 1966) and diminuation of partial inhibition above the transition temperature (Morrisett et al., 1975).

6.4 Lipid Peroxidation.

When low concentrations of ferrous ammonium sulfate (20 μM) are added to KCl suspensions of intact mitochondria, over a 60 min incubation period there was an extensive fall in turbidity as compared to suspensions without added ferrous ion. That the turbidity changes were due to ferrous ion is indicated by the fact that in suspensions with EDTA present, little change in turbidity was observed over the 60 min. period (Fig. 28). In concurrence with the loss in turbidity, over the same time period, there was an increase in the amount of lipid peroxides produced as measured by the amount of malonylaldehyde formed.

This situation has been extensively investigated by Hunter et al., (1963) and the authors suggested that the swelling and disruption of the mitochondria as measured by the turbidity changes was the result of

initial damage and permeability change when the lipid peroxide was formed. The end result of mitochondrial lipid peroxidation in morphological terms has been shown by McKnight et al. (1965) to be the production of mitochondrial membrane ghosts. The authors found that after lipid peroxidation the general size and shape was the same as the parent mitochondria, but there was a considerable decrease in their density and dry mass.

The oxidation of polyunsaturated fatty acids is the process of lipid peroxidation and in the rat liver mitochondria the most likely fatty acids to undergo oxidation with the formation of malonaldehyde are arachidonic and decahexaenoic acid, 22:6 (Dahle, Hill and Holman, 1962). The exact relationship of membrane damage to lipid peroxidation is still obscure but two major possibilities exist. Direct damage as a consequence of peroxidation of unsaturated fatty acids with the resultant schism of the fatty chains. The residual esterified short acyl fragment would then tend to escape from its previously protected hydrophobic environment (see Lubin, Shottet and Nathan, 1972). Alternatively membrane damage could reflect a secondary effect of lipid peroxide formation since these products are well known to react with proteins (Desai and Tappel, 1963) and to inhibit enzymes (McKnight and Hunter, 1966). In all probability the sum total of membrane alterations due to lipid peroxidation is most likely a combination of the above possibilities.

At the end of the peroxidation reaction and after centrifugation it was found that the pellet had lost 80% of the protein and 45% of the phospholipid (see Table 26) which is similar to that as reported by McKnight and Hunter (1966). Similar results were obtained when mercaptoethanol or BSA were present during peroxidation. It was found also that during the course of peroxidation, the total number of

protein SH groups decrease about 41% (see Fig. 29). This however was not surprising as it has been known for some time that peroxides react with SH groups (Lewis and Wills, 1962). It was interesting to note though, that a similar loss of SH groups was observed at both 4° and 25°C which contrasted with the finding of Lewis and Wills (1962) that the reaction of peroxides was greatly decreased by reducing the temperature. The results showed that mercaptoethanol had no effect on the SH content during peroxidation but BSA had a slight protective effect which may indicate the higher reactivity of the BSA SH groups compared to some of the mitochondrial SH groups (see Table 26).

Lipid peroxidation does not release any active MAO into the 10,000 x g supernatant although some residual activity was found in the pellet. Control incubations showed a preferential decrease of MAO A activity probably due to a combination of very low protein concentrations (dilution denaturation) coupled with the buffered KCl media. Lipid peroxidation itself reduces the enzyme activity to 30% of the control value for each substrate. This finding is in agreement with Ottolenghi, Bernheim and Wilber (1955) who found that MAO was inhibited during lipid peroxidation induced by ultraviolet light or ascorbic acid. Mercaptoethanol did not protect the MAO activity but BSA did to a limited extent which may indicate that MAO was inhibited by the reaction of peroxides and the enzymes SH groups. This is supported by the finding of Rapara, Klyashtorin and Gorkin (1966) that oxidized fatty acids inhibited MAO by interaction with SH groups. However, MAO inhibition by the interaction of peroxides with other components of the enzyme cannot be ruled out as it is known that peroxides also react with primary amino groups of amino acids (Chio, Reiss, Fletcher and Tappel, 1969). Ferrous ion induced lipid peroxidation has been shown to inactivate a number of other mitochondrial enzymes such as all

the isocitrate dehydrogenase, 80% of the 3-hydroxybutyrate dehydrogenase and 36% of the malate dehydrogenase (McKnight and Hunter, 1966).

After lipid peroxidation, the overall heat stability of MAO is decreased which indicates that because of the large loss of lipid and protein from the membranes, the environment contributes to the heat stability of the enzyme (Fig. 30). However, the different rates of inactivation of MAO A and MAO B due to heat were still observed and the substrate selectivity of the enzyme with Deprenil was similar to that of the untreated preparation. Therefore, if as suggested by Houslay and Tipton (1973) the environmental lipids of MAO are responsible for the differential properties of MAO, then lipid peroxidation does not alter the enzymes environment to the extent where the differential properties are abolished.

6.5 Effect of Phospholipases on MAO.

Phospholipase C enzymes catalyze the removal of the phosphorylamine moieties from some phosphoglycerides and the resultant diglyceride has no detergent properties (see Coleman, 1973). It has been found that treatment of certain enzymes with phospholipase C reduced their activity. For example Duttera, Byrne and Ganoza (1968) found microsomal glucose 6-phosphatase lost 80 to 90% of its activity after 70% of the lipids had been hydrolyzed. The enzyme could be reactivated by addition of lipid of which phosphatidyl ethanolamine was the most effective. This showed that the enzyme required phospholipid for activity, however, it is as yet unclear as to whether the lipid is required in the enzymatic reaction or for conformational stability.

Treatment of rat liver mitochondria with phospholipase C released 60% of the phospholipid phosphoryl moieties from the membrane.

However, there was no decrease in the MAO activity with tyramine, serotonin or benzylamine as substrates. In addition, no MAO activity was lost when a solubilized MAO preparation was treated with phospholipase C which removed 95% of the phospholipid phosphoryl groups. This indicates that in contrast to glucose-6 phosphatase (Duttera, Byrne and Granoza, 1968) MAO does not require lipids for activity which can be hydrolyzed by phospholipase C. It was also observed that after phospholipase C treatment of membrane bound and soluble MAO that the tryptic digestibility, heat stability, Arrhenius plots and the inhibitor selectivity of Deprenil were similar to that of untreated membrane bound MAO. Therefore, electrostatic interactions between the polar heads of the phospholipids and the charged groups of the enzyme cannot be the major source of stability of MAO or a factor involved in the formation of the multiple forms. It should be pointed out however, that the residual (5%) phosphoryl groups not cleaved may be required for MAO activity or confer properties to the enzyme.

In contrast to phospholipase C, phospholipase A hydrolyzes specific ester bonds in phosphoglycerides to form monoacylphosphoglycerides (see Coleman, 1973). These products are effective detergents which can disrupt membranes (see Singer, 1971) and in some cases have been shown to inhibit enzymes unless serum albumin is present to remove them (Fleischer, Casu and Fleischer, 1966). Phospholipase A has been used by a number of workers to release enzymes from membranes. Fleischer et al., (1966) solubilized β-hydroxybutyric dehydrogenase from beef heart mitochondria by the action of phospholipase A and Awasthi, Ruzicka and Crane, (1970) correlated the release of NADH-dehydrogenase from mitochondria with the hydroylsis of cardiolipin. In addition, a number of enzymes have been reported which when depleted of phospholipid by the action of phospholipase A loose activity and are reactivated by the addition of phospholipids (see Coleman, 1973). Therefore, phospholipase A is a useful tool in solubilizing

membrane bound proteins and in studying lipid-protein interactions during the investigation of lipid dependent enzymes.

MAO is not released from the outer mitochondrial membrane by the action of phospholipase A and less than 20% of the enzyme activity was lost. In addition the Arrhenius plots, tryptic digestability (Fig. 33) and inhibitor selectivity of Deprenil (Fig. 34) was similar to those of the intact mitochondrial enzyme. No indication could be inferred as to the relation between bound lipids and the above functions because the protein lipid content of the pellet had only been reduced by 25% as compared to control suspensions. Therefore, phospholipase A would appear to have only limited accessibility to the phospholipids associated with the 40,000 x g pellet over the incubation period used (20 min). This pellet may in fact be isolated outer-mitochondrial membrane as Backman, Allmann and Green (1966) and Tipton (1967) have used phospholipase A for its isolation but they used a 60 min contact period. However, the overall heat stability of MAO A and MAO B was reduced indicating that some heat stabilizing factors (lipid or protein) were released from the membrane by phospholipase A treatment of mitochondria (see Fig. 32).

As with phospholipase C, phospholipase A has a greater accessibility to the environmental lipids around MAO after the enzyme had been solubilized (see Section 5.6). This is indicated by the fact that gel filtration of the solubilized, phospholipase A treated preparation shifts the elution pattern of MAO from the slightly retarded peak of the untreated preparation to a fully retarded peak with a phospholipid content 93% less than the original mitochondrial enzyme. In addition, the gel filtration results indicate that the solubilized enzyme preparation consists of large protein-lipid units solubilized by detergent which are broken down into smaller units by the action of

phospholipase A. Similar to the membrane bound enzyme, phospholipase A treatment of solubilized MAO had little effect on the activity of MAO which indicates that the enzyme does not require phospholipids for activity. In comparison, Tzagoloff and Maclennan (1965) removed 80-85% of the phospholipid associated with cytochrome oxidase by phospholipase A treatment and found a parallel loss in enzymatic activity. Their lipid depleted preparation contained 63.3 µg phospholipid/mg of protein whereas in the present work MAO retained almost full activity with a phospholipid content of only 14 µg/mg protein (a 92% reduction in the MAO phospholipid content).

The present results agree with the findings of Harada et al. (1971) that phospholipase A treatment has little effect on MAO activity. However, even though similar results were obtained by Erwin and Hellerman (1967) with the bovine kidney enzyme, the authors suggested that a non-specific lipid requirement might be fulfilled by the detergent used to solubilize the enzyme. In the case of cytochrome oxidase Tzagoloff and Maclennan (1965) found that detergent could reactivate the lipid depleted enzyme by 20%. However, this suggestion can be ruled out in the present work as the sonicated soluble MAO preparation (see Section 2.5.1) treated with phospholipase A lost little activity. In contrast, Tipton (1972) found that his preparation of rat liver MAO lost activity when treated with phospholipase A. However, a direct comparison with the present results was not possible as no quantitative figures were reported by Tipton (1972) who incubated his preparation for 6 h which in itself may have reduced the activity of the enzyme. In a recent report, Bernsohn and Spitz (1974) found that in fat deficient rats, the brain MAO activity decreased to 57% of control values and that by feeding the deficient animals either linoleic or linolenic acids, the MAO activity increased to control values and in some cases beyond. This may indicate a fatty acid requirement for enzyme activity although in the light of the

above discussion, this could also indicate that fatty acids may be required for synthesis of the enzyme or for the binding of the enzyme to the membrane, however, the results of Bersohn and Spitz (1974) could also be brain specific and not apply to the rat liver enzyme.

Oreland (1972) found that when pig liver MAO is solubilized by the removal of phospholipids with organic solvent, the Km value of the enzyme with benzylamine as substrate was lowered. The author suggested that removal of phospholipids also removes a diffusion barrier for the substrate or possibly a conformational change in the enzyme structure that resulted in an increased affinity of the substrate for the enzyme. However, the Km values of lipid depleted rat liver MAO and and the membrane bound enzyme were similar (see Table 28) suggesting that for both MAO A and MAO B there are no lipid diffusion barriers and that the lipid depleted enzyme has a similar conformation to that of the membrane bound MAO. However, Houslay and Tipton (1975b) found a slight change in the reaction mechanism of rat liver mitochondria on solubilization which they suggested was due to conformational change.

The results of the tryptic digestibility and heat stability of the lipid depleted MAO are in agreement with those of Oreland and Ekstedt (1972) for the pig liver enzyme; that the membrane environment around the enzyme confers resistance to these modes of inactivation. As the lipids (and proteins) are removed a larger area of the protein is exposed and thus the rate of inactivation by trypsin and heat is increased (Fig. 38,40). However, as shown in the same figures, the different rates of inactivation of MAO A and MAO B are still observed. In addition, the selective inhibition of the lipid depleted preparation is similar to that of the membrane bound enzyme. These results plus

the observation that the lipid content of the phospholipase A preparation (14 ug/mg protein) is similar to that of the perchlorate treated preparation of Houslay and Tipton (1973) whose preparation had lost the differential properties of MAO A and MAO B, would indicate that phospholipids are not responsible for the formation of the multiple MAO forms as suggested by Houslay and Tipton (1973) and Neff and Yang (1974).

The Arrhenius plot of the lipid depleted MAO preparation showed similar temperature transitions as observed with the membrane bound enzyme (Fig. 39). This is in contrast to a number of mitochondrial enzymes which when solubilized no longer show transition temperatures but only a single slope in the Arrhenius plot which indicated that the lipids induced the temperature dependent conformational change (Raison, Lyons and Tomson, 1971; Abdul Matlib and O'Brian, 1975). Similar results to that observed for MAO were obtained by Sineriz, Farias and Trucco (1973) for (Ca^{2+})-ATPase of Escherichia Coli. These workers found that the enzyme had the same transition temperature after solubilization indicating that lipids were not responsible for the conformational change. In an earlier discussion (see Section 6.3), it was suggested from the membrane bound MAO Arrhenius plot that MAO A and MAO B were in similar lipid environments. However, because lipid depleted MAO retains the same transition temperatures as the membrane bound enzyme, these results would suggest that the conformational change is induced not by lipids but rather from within the protein itself.

In light of the previous discussion that the differential properties of MAO A and MAO B could not be abolished by the removal of enzyme bound phospholipids by the action of phospholipases or by altering the membrane environment by lipid peroxidation (see Section 5.4), some observations should be made on the results of Houslay and Tipton (1973). These workers found that treatment of a partially purified preparation of

rat liver MAO with the chaotropic agent sodium perchlorate under certain conditions (0.5 M perchlorate at 28°C for 8.5 min.) resulted in no loss of MAO activity. In addition, the differential heat stability and inhibitor specificity of Clorgyline were abolished which in the light of the fact that perchlorate had reduced the lipid content of the enzyme suggested that the membrane environment was responsible for the formation of MAO A and MAO B. However, in attempts to repeat these experiments, it was found that sonicated soluble, triton X-100 soluble and an MAO preparation solubilized as described by Youdim and Sandler (1968) and used by Houslay and Tipton (1973), all lost MAO activity when treated with perchlorate under similar conditions as above (see Table 30). In particular MAO A activity was especially sensitive to the action of perchlorate and the preparation in which detergent was used lost more MAO A activity than the sonicated soluble enzyme. This may indicate that when the enzyme is still bound to membrane fragments, which may result from sonication, the enzyme is more resistant to perchlorate inactivation. The nature of the perchlorate inhibition is not certain but chaotropic agents are potent protein denaturants and they also induce lipid peroxidation (Hatefi and Hanstein, 1974) which inhibits MAO even in the presence of mercaptoethanol to protect the SH groups (see Section 5.4). The loss of MAO A activity by perchlorate would then result in an enzyme preparation which would lose selective properties towards different substrates. This is indicated as shown in Fig. 43 by the loss of the biphasic inhibition curve with Deprenil. A similar objection can be made for the perchlorate soluble MAO, which solubilized only 14% of the MAO A activity but 30% of the MAO B was recovered (see Table 31) and although the selective inhibition pattern of Deprenil was still observed, the reduced MAO A activity is reflected in an increase

in the tyramine inhibition plateau to the 70% level (Fig. 44). Since Houslay and Tipton (1973) did not use a specific MAO A substrate, perhaps their preparation lost this activity also. However, their results could also be interpreted as a perchlorate induced conformational change independent of lipids, in which MAO still retains tyramine and dopamine oxidizing ability (They did use common substrates) and also resulting in the abolition of the differential properties. In general though, these results show that great care must be used when treating MAO with chaotropic agents and specific substrates must be used for MAO A and MAO B to determine any change in activity.

Since the present results indicate that lipids are not responsible for the formation of the multiple forms, their exact nature is still open to question. The results with the inhibitor propargylamine indicates that the principal factor governing the selectivity is the hydrophobic regions on the enzyme. Since the present results indicate that phospholipids do not control or constitute these regions as suggested by Neff and Yang (1974), the differences between MAO A and MAO B may be due to the sequence of non-polar amino acids that constitute these regions. In addition, because propargylamine does show some selectivity other factors may be involved in the selectivity such as polar regions (see Section 6.1). Another possibility would be similar or identical proteins folded in different ways to form MAO A or MAO B. That these two enzymes are similar proteins is indicated by the result that the transition temperature in their Arrhenius plots are the same. The different sensitivities of MAO A and MAO B after lipid removal to inactivation by trypsin or heat would be consistent with the two forms being conformational isoenzymes. However, it should be pointed out that the residual amount of lipid material still bound to MAO may confer properties to the enzyme and be responsible for the formation of the multiple forms.

6.6 Relationship of MAO to the Outer Mitochondrial Membrane.

There have been numerous models of membrane structure proposed (for a review see Singer, 1971) but for the purpose of the present discussion the fluid mosaic model, which has been presented in great detail, will be used (Singer and Nicholson, 1972). The model is consistent with the restrictions imposed by thermodynamics. In this model, proteins that are integral to the membrane are a heterogenious group of globular molecules, with the ionic and polar groups protruding away from the membrane into the water phase and the hydrophobic groups are buried in the non-polar region of the membrane. The majority of the phospholipid is organized into a discontinuous fluid bilayer, although a small fraction of the lipid may interact with the imbedded protein. The authors suggested that the fluid mosaic structure is analogous to a 2-dimensional solution of integral lipoproteins in the viscous phospholipid bilayer solvent. Recent experiments with a variety of techniques and with several different membrane systems are consistent with this model (Singer and Nicholson, 1972).

In the first step for the analysis of membrane proteins, Singer, (1971) proposed two categories, peripheral and integral. Peripheral proteins require only mild treatment to dissociate them from membranes such as high ionic strength or metal chelating agents. When released from the membrane, they are usually free of lipids and they are freely soluble and molecularly dispersed in neutral aqueous buffers. These criteria for perpherial proteins suggests that they are only weakly bound to membranes possibly through electrostatic interactions and they are not strongly associated with lipid material. In general, this class of proteins represents about 30% of the total membrane protein. The other 70% which are classified as integral proteins require hydrophobic bond-breaking agents for solubilization such as detergents, organic solvents and chaotropic agents. When

solubilized, they are usually associated with lipids and are usually insoluble or aggregated in aqueous buffers.

A search of the literature and the results from the present work suggests that MAO is an integral protein. The requirements for the dissociation of the enzyme from the outer mitochondrial membrane involves the use of hydrophobic bond-breaking reagents: detergents (see Table I), organic solvents (Hollunger and Oreland, 1970) and chaotropic agents (see Table 8). When MAO is solubilized the enzyme is associated with lipid material (Erwin and Hellerman, 1967; see Section 5.6) and the enzyme readily aggregates in aqueous buffers in the absence of detergent (Houslay and Tipton, 1973; Oreland, 1971). However, Capaldi and Vanderkooi (1972) classified MAO as a prepherial protein because it was solubilized by aqueous media. The enzyme preparation used in their study was pig liver MAO which in fact had been solubilized by organic solvent extraction and not aqueous media (Oreland, 1971) suggesting the protein is integral. In addition, Capaldi and Vanderkooi (1972) calculated the polarity of a number of proteins by summing the mole fractions of polar amino acids and found that the polarity index of MAO was lower than that of the highest one calculated in their group of integral proteins supporting the contention that MAO could be an integral protein.

As an integral protein, it can be assumed that a major portion of the surface of MAO will consist of water insoluble nonpolar amino acids (Capaldi and Vanderkooi, 1972). These groups may then interact with the nonpolar side chains of the lipids (fatty acids) suggesting that the enzyme may penetrate the lipid bilayer. In addition part of the enzyme may protrude above the membrane which would be polar in nature. These two protein regions interior (nonpolar) and exterior (polar) have been determined for some proteins. For example,

Ito and Sato (1968) found that when intact microsomal membranes were treated with trypsin, the cytochrome b_5 spectral activity was solubilized in a non-aggregating protein with a molecular weight of 12,000. However, if the membranes were treated with detergent, the b_5 spectrum was found associated with a protein of molecular weight of 25,000 which aggregated in the absence of detergent. If the detergent b_5 was treated with trypsin, the b_5 spectrum was again found in a soluble 12,000 molecular weight fragment. The authors concluded that cytochrome b_5 is a single polypeptide which is cleaved by trypsin hydrolysis into roughly two equal sized moieties: one contains the b_5 activity and is soluble but the other confers hydrophobicity upon the whole molecule and may be responsible for its attachment to the membrane. Since trypsin cleavage releases the b_5 carrying portion, this moiety is probably exterior to the membrane and the other interior. Similar results were obtained by Maroux and Louvard (1976) for aminopeptidase and maltases of the intestinal brush border membrane.

When mitochondrial membranes were treated with a concentration of trypsin which within 10 min had almost completely abolished NADH-cytochrome C reductase (Kuylenstierna et al. (1970), little of the MAO activity was lost and incubation for periods up to 40 min resulted in less than 15% of the activity being lost. (see Table 25). In addition no MAO activity was released from the membrane. Increasing the trypsin concentration 4 fold (200 µg/mg protein) and over the same incubation period MAO A lost 65% of its activity whereas MAO B lost only 23% of its activity. This suggests that MAO is predominantly an interior protein and that MAO B may be more interior than MAO A.

Further evidence that MAO is an interior protein is indicated by the result that free DTNB readily inhibits MAO (see Fig. 42) but DTNB-Sepharose inhibits membrane bound MAO by less than 5%, presumably due to

the membrane restricting the penetration of the bound DTNB to the enzymes SH groups. This is supported by the fact that soluble phospholipase A treated MAO was inhibited by DTNB-Sepharose by 93.4% (Table 29).

Trypsin hydrolysis can also be used to determine which side of the membrane an enzyme is located. Kuylenstierna et al. (1970) found that the outer mitochondrial membrane is impermeable to trypsin and that by studying the action of trypsin on lysed and intact mitochondria determined that NADH-cytochrome C reductase is most likely located near the outer surface of the outer mitochondrial membrane. However, the trypsin digestability of MAO in intact or lysed mitochondria are similar (see Table 25) indicating that the enzyme does not favour either side of the membrane and that access to the enzyme may be similar from both sides. Similar results with trypsin and MAO were obtained by Kuyhenstierna et al. (1970). Further evidence for similar accessability is suggested by the result that MAO is inhibited to similar extents in intact and lysed mitochondria by DTNB-Sepharose (Table 29) which due to the large bead size will probably not permeate the membrane. Tipton (1967) found that MAO substrates freely crossed the outer mitochondrial membrane which would support the contention that the active site of MAO may be accessable from either side of the membrane. However, Bloom et al. (1972) using a histochemical staining technique for the localization of MAO activity suggested from their results that MAO of the guinea pig kidney is localized only on the inner surface of the outer mitochondrial membrane.

An important feature of the enzymology of membranes is that some of its functions are vectoral, that is separation of the substrate

and products occurs on the two sides of the membrane as a result of the enzyme action. This has been shown to exist for the enzyme papain bound to a collodion film separating two compartments. The addition of substrate to one side results in the appearance of product only in the other side (Goldman, Kedem, Silman, Caplan and Katchalski, 1968). However, because the outer mitochondrial membrane is permeable to substances of molecular weight up to 10,000 (see Ernster and Kuylenstierna, 1969) this would suggest that MAO is not a vectoral enzyme.

It is assumed in the fluid mosaic model that only the integral proteins are critical to the structural integrity of the membrane (Singer and Nicholson, 1972). There is, however conflicting evidence of this in relation to MAO when sonication is used to solubilize the enzyme. Again this is related to the question of MAO being integral to the membrane. Thompson, Coleman and Finean (1967) found that mild sonication removed a large proportion of the MAO from rat liver mitochondrial membranes and X-ray diffraction indicated that the thickness of the membrane was reduced after sonication. The authors suggested that the enzyme was loosely bound. Similar results were obtained by Racker and Proctor (1970). They found that sonication of kidney cortex mitochondrial membranes at pH 9.6 followed by centrifugation at pH 6.0 resulted in no detectable MAO activity in the membrane. This also suggested that sonication removed the loosely bound enzyme. However, sonication of rat liver mitochondria in which the MAO had been labelled with [^{14}C] pargyline and under the conditions of Racker and Proctor (1970) showed that in both control and sonicated suspensions 50% of the label was in the supernatant (see Table 34). This suggests that Racker and Proctor (1970) inactivated the MAO by sonication and low pH and the enzyme protein was still attached to the membrane.

Alternatively, the present results could also indicate that MAO from rat liver mitochondria is more tightly associated to the membrane than MAO from kidney cortex mitochondria.

In contrast to the loss of MAO activity when rat liver mitochondrial membranes were resolved by the method of Racker and Proctor (1970), it was found by Thompson et al. (1967) that after mild sonication (1.3A for 7s) of the rat liver outer mitochondrial membrane, the supernatant, after sedimentation of the membranes, contained MAO activity. The amount of enzyme released was dependent on the concentration of the membrane suspension treated. However, these workers sonicated their membranes in sucrose media (0.88 M) which may have been able to retain membrane fragments with MAO attached in suspension after centrifugation whereas in the present work the less viscous medium phosphate buffer was used which may not be able to retain similar fragments.

Mild sonication has been used to solubilize a number of proteins from a variety of membranes but an interesting comparison to the mild sonication used to release MAO from the membrane by Thompson et al., (1967) and Racker and Proctor (1970) is to that when sonication is performed on the inner mitochondrial membrane. This membrane has regularly spaced projecting subunits (see Ernster and Kuylenstierna, 1969) which are released from the membrane by mild sonication (Tzagoloff, McConnell and MacLennan, 1968) or by treating the membrane with glass beads (Penefsky, Pullman, Datta and Racker, 1966). However, the outer mitochondrial membrane of rat liver does not contain any projecting subunits (Parsons, Williams and Chance, 1966; Smoly, Wakabayski, Addink and Green, 1971). This comparison taken in conjunction with the fact that in the majority of MAO solubilization procedures (See Table I), long sonication periods have been used ($>$ 20 min) suggests that this

procedure probably disrupts the outer mitochondrial membrane into small fragments existing as either closed vesicles or as small sheets which may still retain the original membrane structure (see Razin, 1972) and the "soluble" MAO activity. This is directly supported by the work of Verevkina et al. (1964) who found that the amount of MAO in the 12,000 x g supernatant increased with sonication up to 30 min but sedimented at 105,000 x g. An electron microscopic examination showed the size of the sonicated particles to be from 50-200 Å. Therefore, the present results on sonication together with the preceding observations indicates that even though MAO can be solubilized by sonication, it can still be regarded as an integral membrane protein.

The assignment of an organizational role to the mitochondrial membrane protein fraction (SP) first isolated by Criddle et al. (1961) and Green et al. (1961) was based mainly on its lack of enzymatic activity and on its presence in large amounts (30-45%). Originally, structural protein (SP) was thought to be homogeneous (Criddle et al. 1962) but Haldar, Freeman and Work (1966) with SP from rat liver mitochondria and Lenaz, Lauwers, Allmann and Green (1968) with SP from Ox heart mitochondria showed that with electrophoresis the SP was heterogenous. The isolation and purification of SP involves extraction of mitochondria with bile salt and detergents followed by ammonium sulfate precipitation and washing with organic solvent. It is not surprising therefore, that no enzymatic activity was observed since the conditions of isolation are harsh and may denature any enzymes present. However, isolation of SP from mitochondria in which the MAO had been labelled with (^{14}C)pargyline indicated that about 30% of the MAO protein was included in the SP fraction (Table 32). That SP may consist of

other enzymes is indicated by the results of Schatz and Saltzgaber (1969) who found about 70% of the ATPase protein was in the SP. Electrophoresis at basic pH of the SP showed that the label was associated both with the migrating SP and with the protein that remained at the origin (Fig. 46). Similar results were obtained when electrophoresis was performed with the detergent soluble mitochondria that also contained the SP fraction (Fig. 45). Since the label of both fractions migrated to the same position, indicating the similarity of both proteins, perhaps the MAO in the SP fraction is an aggregated form of the enzyme. However, the isolation of SP was performed a number of times and throughout a consistent 30% of the label precipitated with the SP fraction. It would be interesting to speculate that only MAO A or MAO B was included in the SP.

When SP was isolated by the method of Zahler et al. (1968) using acid extraction, 40% of the label was in the SP fraction, however, this label crossed dialysis tubing indicating that the MAO flavin-label complex had been extracted from the apoenzyme. This supports the finding of Sourkes (1968) that about 50% of the MAO flavin could be released by acid extraction. In addition, washing of the SP isolated by ammonium sulfate precipitation with either butanol or methanol at 50°C as described by Criddle et al. (1961) or with acetone as described by Richardson et. al. (1964) both removed the label from the enzyme. It is not surprising therefore, that Green et al. (1961) could not find any flavin in their SP but it may have contained the MAO apoenzyme.

In terms of the fluid mosaic model SP would be considered a heterogeneous group of integral proteins which includes some denatured enzymes (Singer, 1971). This is indicated by the fact that the

isolation procedure involves the use of detergents, after isolation the SP is insoluble in the absence of detergents and under certain conditions SP will bind lipid material (Richardson, Hultin and Fleischer, 1964). The present results would therefore support the contention that the SP is in part made up of enzymes (including MAO) and indicates that MAO is an integral protein which may be involved in the structural integrity of the membrane.

As an integral membrane protein, the lipid environment of MAO may have several functions in relation to the enzyme. A number of membrane bound enzymes require phospholipids for activity (for a review see Coleman, 1973) and recent evidence indicates that the lipid environment may be responsible for the multiple forms (Houslay and Tipton, 1973). However, as shown in Section 5.6 removal of lipid from the enzyme has little effect on the enzyme activity and although the present results showed that lipids protect the enzyme from inactivation by heat and trypsin, the differential properties of MAO A and MAO B were still observed. This indicated that the enzyme did not require phospholipids for activity or the formation of the multiple forms (see Section 6.5).

Lipids may provide the anchor that retains MAO to the membrane. Experimental evidence for this is provided by the work of Hollunger and Oreland (1970) who showed that pig liver MAO could be released from the membrane after extraction of the phospholipids by organic solvent. In addition, Olivecrona and Oreland (1971) showed that soluble MAO could be reattached to the membrane in the presence of phospholipids. Further support for this contention is indicated by the result that lipid deficient rats have a reduced brain MAO activity (Bernsohn and Spitz, 1974). The lipids may affect the membrane enzymes

by upholding an orderly arrangement in the membrane that allows the coupling of various enzyme reactions. An aldehyde dehydrogenase has been found tightly bound to mitochondrial membranes (Smith and Packer, 1972) and recently it has been reported that this enzyme, which may be responsible for catalysis of MAO products, is located in the outer mitochondrial membrane (Horton and Barrett, 1975). In addition, the lipids may constitute a solvent for the MAO products either protecting them from some unwanted side reactions or providing an alternative solvent for their own enzymatic catalysis.

The role of the outer mitochondrial membrane is still rather obscure. There is as yet no evidence that the outer membrane or its components maintain or promote oxidative phosphorylation or other energy linked functions. However, this membrane does contain a number of enzymes including MAO which as the present results suggest is an integral protein possibly involved in maintaining the membrane structure. Therefore, as the functions of the outer mitochondrial membrane become clear, functions of the enzyme in relation to the membrane will become clear.

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