Some Properties of Soluble Monoamine Oxidase Preparations from Guinea-Pig Liver

A thesis presented by

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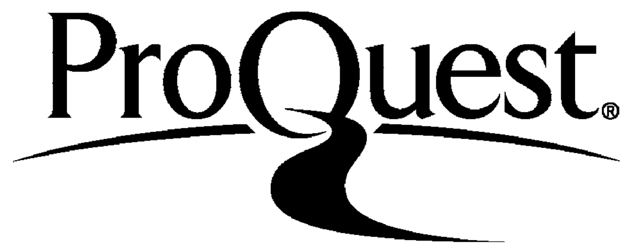

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This thesis is dedicated to

Dr. J. R. Lagnado

and

Miss J. B. Weiss

Abstract

Monoamine oxidase (MAO) (Monoamine: O_2-oxido-reductase (deaminating) E.C.1.4.3.4) was partially purified from soluble extracts of guinea-pig liver, which contain about 10 per cent of the activity present in whole homogenates. Higher yields of enzyme were recovered in soluble extracts derived from sonicated homogenates.

The substrate or inhibitor specificity of the soluble enzyme was very similar to that for crude particulate fractions or mitochondrial fractions when measured by manometric assay with molecular oxygen as final electron acceptor. The behaviour of the enzyme in other oxido-reductase systems, namely in the reduction of tetrazolium salts and NAD, were studied extensively. Marked differences in substrate specificity were found for the soluble enzyme depending on the nature of the final electron acceptor and on the type of preparation used as source of enzyme. The soluble enzyme showed a marked dependence on added NAD for tetrazolium reduction, the extent of this effect varying according to the substrate tested. The NAD dependence was correlated with the ability of various amine substrates to stimulate the enzymic reduction of NAD in the absence of tetrazolium salts. NAD had no effect on the enzyme when assayed manometrically. However, soluble enzyme derived from sonicated homogenate showed marked alterations in its substrate specificity in the NAD-reductase assay. In addition, the effect of added NAD in the tetrazolium-reductase assay was negligible compared to that seen in unsonicated preparations. Various data led to the conclusion that the NAD-dependent step(s) in the tetrazolium-reductase system of soluble enzyme preparations was probably inactivated by sonication, although the total amine-tetrazolium reductase activity was in fact increased by sonication.

These findings led to the formulation of possible pathways involved in the enzymic reduction of NAD and tetrazolium by monoamines. These are discussed in the light of current concepts about the chemical constitution of the enzyme.

Various attempts were made to detect isoenzymes of monoamine oxidase by gel electrophoresis. In the best preparations, only one mobile (anodic) form of the enzyme was demonstrated after polyarylamide gel electrophoresis of soluble extracts from guinea-pig liver.

SOME ABBREVIATIONS AND SYMBOLS USED IN THIS THESIS.

cyt.	cytochrome
DH_2	reduced substrate
Fp	flavo protein
g	force of gravity
NHI	nonhaem iron - containing flavo protein
hr	hour
M	molar
PMS	phenazine methosulphate

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CHAPTER ONE

INTRODUCTION

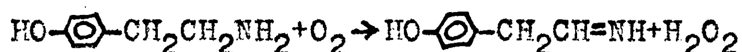
SECTION I: A REVIEW OF MONOAMINE OXIDASE.

A: General Survey of Earlier History of Amine Metabolism.

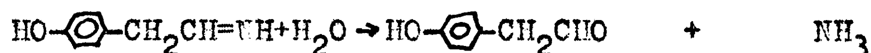
In 1928, Hare(1) described an enzyme present in liver extracts of rabbit, rat, pig, sheep, ox and dog. The enzyme was shown to catalyse the oxidative deamination of tyramine with the formation of stoichiometric amounts of ammonia and hydrogen peroxide. This enzyme was named by her 'tyramine oxidase'.

Later, Hare-Bernheim (2) conducting detailed studies with guinea pig liver extracts, demonstrated that one, two or even four atoms of oxygen could be absorbed per molecule of tyramine oxidised, depending on the pH, concentration and age of the extract. Bernheim(2) observed a decrease in enzyme activity with increasing time of incubation. This she attributed to product inhibition. As ammonium sulphate and p-hydroxypheny-lacetic acid were not inhibitory, Hare-Bernheim suggested p-hydroxyphenylacetaldehyde as a possible candidate. Indeed, she showed that cell-free extracts of an incubation mixture containing tyramine as the substrate inhibited oxidative deamination of tyramine by about 80 per cent. She explained that the inability to detect the aldehyde was due to a Cannizzaro type of reaction in which the aldehyde would condense with an available molecule of tyramine to form a Schiff's base.

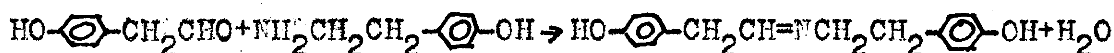
Accordingly, she proposed the following equations to show the stages of the oxidative deamination:-



Tyramine → imine + hydrogen peroxide



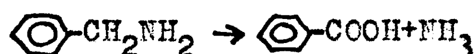
p-Hydroxyphenylacetaldehyde + ammonia



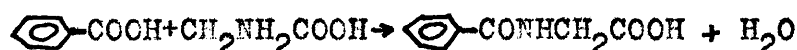
Cannizzaro type of reaction Schiff's base

Perhaps it may be of some interest to state that Alivisatos *et al.*(3,4) have recently applied the concept of the formation of a Schiff's base by an aldehyde and an amino group to explain the incorporation of indoleamines and their aldehydes during mitochondrial protein biosynthesis in vitro.

Before the discovery of 'tyramine oxidase' by Hare(1) much evidence had been accumulated concerning the metabolism of biogenic amines in organisms(5-11). Such evidence, however, came mainly from pharmacological and physiological experiments. Thus Schmiedeberg(5) by feeding benzylamine to dogs produced the first convincing evidence that it was metabolised. He recovered hippuric acid, the glycine conjugate of benzoic acid, from the urine and suggested the formation of ammonia in the course of the reaction:-



benzylamine → benzoic acid



benzoic acid + glycine → hippuric acid

Later, both Schmiedeberg(6) and Minkowsky(7) isolated free benzoic acid.

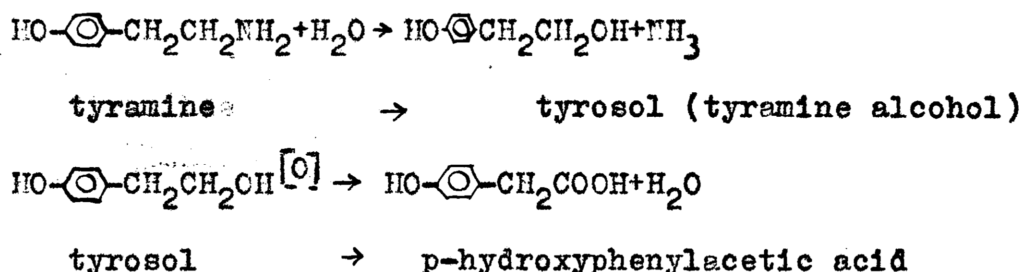
Minkowsky(7) incubated benzylamine with minced rabbit

tissues and isolated benzoic acid. This showed that deamination could also occur in vitro.

Mosso(8) injected benzylamine subcutaneously into dogs and recovered more than 90 per cent as hippuric acid in the urine.

Ewins and Laidlaw(9,10) investigated the metabolism of tyramine and tryptamine in rabbits, cats and dogs and found that when certain tissues were perfused, complete deamination occurred and the corresponding acids were recovered.

Ewins and Laidlaw(9), believing the deamination occurred first by hydrolysis and finally by the oxidation of the alcohol intermediate, proposed the following equation for the deamination of tyramine:-



Similarly Guggenheim and Löffler(11) studies the metabolism of phenylethylamine and isoamylamine in rabbits. Guggenheim and Löffler(11) fed them phenylethylamine and recovered the acid. Rabbit's liver was perfused with isoamylamine and isovaleric acid was similarly isolated(11). These workers, too, assumed the first reaction to be hydrolytic; the oxidation step occurred in the second stage resulting in an acid. It was Hare who first demonstrated by manometry that the first step of the deamination was oxidative and not hydrolytic. Later(2) she showed that no deamination occurred under anaerobic

conditions.

The discovery of tyramine oxidase(1,2) stimulated great interest in the metabolism of the metabolism of the biogenic amines in the early thirties. Various investigators (12-15, 17-19) extended the study to a number of amines in many other tissues.

Thus Pugh and Quastel(12,13) demonstrated that slices and extracts of nervous tissues (brain) and liver could catalyse the oxidative deamination of both aliphatic and aromatic monoamines and also showed that the enzyme was not identical with Krebs' amino acid oxidase. These workers(13) demonstrated isovaleraldehyde as an intermediate of isoamylamine oxidation by precipitation of its phenylhydrazone. They drew attention to the characteristic smell of isoamyl alcohol during oxidation. Philpot(14), and Pugh and Quastel(13), confirmed Hare's claim that oxygen was required for the deamination.

Philpot detected p-hydroxyphenylacetaldehyde as a product of tyramine oxidation by its phenylhydrazone.

Richter(15) extended this method to other monoamines by trapping the aldehydes with semicarbazide and crystallising them as their phenylhydrazones.

Richter found that semicarbazide did not inhibit oxygen uptake of the guinea pig liver extracts as expected when isoamylamine was used as substrate. Weiner(16) confirmed Richter's observation and thought this apparent discrepancy to mean that isovaleraldehyde is not further metabolised. Perhaps this insensitivity may be explained by the fact that isoamyl alcohol is easily formed. The alcohol is, of course,

insensitive to semicarbazide. We will refer to this topic in our results.

In 1937, Pugh and Quastel(13) Blaschko *et al.*(17,18) and Kohn(19) concluded that the 'aliphatic amine oxidase', (12,13) the adrenaline oxidase of Blaschko *et al.*(17,18) and the tyramine oxidase of Hare(1) were identical. The conclusion was based on distribution, competitive studies with mixed substrates and inhibitor studies. These workers(13,18,19) suggested the term 'amine oxidase' for the enzyme.

Later, Zeller(20) confirmed the claim of Blaschko *et al.* (18) that amine oxidase had no effect on the diamines, putrescine and cadaverine, and it did not attack histamine. He therefore proposed the term 'monoamine oxidase' to distinguish it from diamine oxidase which differs not only in substrate specificity but also in its distribution and inhibitor specificity, e.g. in being sensitive to carbonyl reagents such as semicarbazide and cyanide. (See tables I and II).

This term 'monoamine oxidase' (MAO) is the name by which it is still commonly known. Recently, according to the classification of the International Enzyme Commission(21) it is more specifically known as "monoamine oxidase" (monoamine: O_2 oxidoreductase (deaminating), EC 1.4.3.4).

Alles and Heegaard(22) conducted an extensive study on the distribution, substrate and inhibitor specificities of MAO from various sources and concluded that the enzyme appeared to be a group of multiple enzymes or a family of closely related enzymes. This was at a time when there was

TABLE I: SUBSTRATE SPECIFICITIES OF AMINE OXIDASES FROM ANIMAL TISSUES.

Enzymes	Source of Enzyme	Act upon a terminal Amino group: $-CH_2NH_2$	$-CH_2NHR$ eg. Epinephrine	$-CH_2NR^1R^2$ eg. Hordenine	Act upon: Histamine	1-methyl-histamine	Main or Preferential substrate	Ref.
Monoamine oxidase (MAO)	Liver and other mammalian tissues	++	++	++	-	+	Tyramine	23,27,65
	Human Plasma	++	++	++	-		Tyramine	28
Diamine oxidase (DAO)	Pig kidney	+	-	-	++	±	Histamine Cadaverine	23,27,65
	Human placenta	+	-	-	++		Cadaverine	23,65
Plasma Amine oxidases	Pig plasma	++			++	+	Benzylamine	23,65
	Human plasma	++			++	+	Benzylamine	23,65
	Ox plasma	+	-	-	±	+	Spermine	23,65
*Bacterial Amine oxidase	Aspergillus Niger	++	+		++			26

++ = very strongly oxidised

± = poorly oxidised

\- = not oxidised

x = reduces cytochrome C

TABLE II: INHIBITOR SPECIFICITIES OF AMINE OXIDASES

Inhibitor	Monoamine oxidase (MAO)	Diamine oxidase (DAO)	Spermine oxidase (SO)	Benzylamine oxidase	Amine oxidase from Aspergillus niger
Amphetamine,	+		-		
ephedrine	+				
Pyridoxamine,		+	+		
thiamine (B1)				+	
d,l Trans-phenylcyclopropylamine (Parnate)	+	-		+	
Isonicotinylisopropylhydrazide (Iproniazide IIH)	++	+		+	
Isoniazid,	-	+			
Aminoguanidine	-	+	+	+	
Amidines	+				
Hydrazinophthalazine (Hydralazine)	+	+	+	+	
Cyanide, hydro-		+	+	+	
xylamine,		+			
Semicarbazide	-	+	+	+	+
Quinacrine	+	+	+		
Mercuric chloride,	+				
silver nitrate,	+			+	
p-chloromercuribenzoate	+			+	
(PCMB)	+	-			

Modified from F. Buffoni, ref. 65; T.L. Sourkes, ref. 48; and Pletscher ref. 52.

+ = inhibition

- + no inhibition

no concept of the existence of isoenzymes. Subsequent research has either tended to support or contest this view.

B: Monoamine oxidase (MAO) E.C. 1.4.3.4) its distribution and properties.

1. Species distribution of MAO.

MAO is very widely distributed in the plant, animal and bacterial kingdoms(23-26).

In invertebrates it occurs in molluscs, echinoderms, annelids and in insects (the cockroach)(23). The richest source of MAO appeared to be the hepato-pancreas ('liver') of the cephalopods, sepia and octopus(23,27).

In plants, it is especially concentrated in pea seedlings(23,24).

In bacteria, MAO is found in *Bacilus pyocyanea, Escherichia coli*(25) and in *Aspergillus niger*(26).

The enzyme has been found in all the representatives of vertebrates tested(23,27). In mammals, the richest deposits are the liver, kidney, gut and brain, in that order. Rat kidney is an exception as it is reported to lack the enzyme(23). In man, however, the richest sources are the submaxillary and parotid glands(23). Recently, the 'classical' MAO was detected in human plasma(28) held for a long time to lack the enzyme(23). The plasma enzyme first described by Werle and Roewer(29), Hirsch(30) and Tabor *et al.*(31) was later identified as spermine oxidase. However, the recent isotopic studies of Otsuka *et al.*(28) on human plasma leave little doubt that their plasma enzyme is not spermine oxidase but the classical MAO thus indicating that human plasma

TABLE III

DISTRIBUTION OF MONOAMINE OXIDASE IN ORGANISMS

	Source	Reference
Vertebrates:	Mammals, birds, reptiles, amphibians, fishes (bony and cartilaginous) eg. Torpedo marmorata	23, 27
Invertebrates:	Molluscs, echinoderms, annelids, insects - cockroach	
Plants:	Higher plants such as pisum sativum L. (pea), Cannabis indica, Momordica balsamina	24, 27
Bacteria:	*B. aeruginosa*, *E. coli*, *B. pyocyanea*,	25
	Aspergillus niger	26

contains both oxidases.

It is necessary to stress in passing that great care must be taken before concluding that the enzyme is absent or plentiful in a particular species, organ or tissue. Studies made with a few substrates may not give a true measure of MAO in a particular material since the substrate specificities vary greatly with enzymes from different sources(16). Table III briefly summarises the distribution of MAO.

2. Intracellular distribution of monoamine oxidase.

There is considerable evidence to show that the enzyme is mainly particulate(32-42) in guinea pig liver, brain, kidney and many other organs of many animals. Some activity is also found in microsomes(32,33). De Duve *et al.*(37) confirmed the claim of Cotzias and Doles'(32) and Hawkins(33) that MAO is mainly particulate in distribution. De Duve *et al.* believed the bulk of MAO activity (ca.80 per cent) to be of mitochondrial origin. Oswald and Strittmatter(36) investigated the distribution in guinea pig and rat tissues and found that about 70-76 per cent of the enzyme activity was mitochondrial while some activity was also present in microsomes. More recently, Schnaitmann *et al.*(41) applied more carefully controlled techniques to the study of the subcellular distribution. These authors found that MAO was so concentrated in the mitochondrial outer membrane that it could be used as a marker. Tipton(42) applied similar methods of digitonin treatment, mitochondrial swelling, sonication, coupled with phospholipase digestion and centrifugation to show that in rat liver the enzyme is mainly found in the

outer mitochondrial membrane. In rat brain, however, the concentration of MAO in the inner membrane is considerable(42). The possible significance of this will be referred to later in connection with iso-enzymes.

Weissbach et al.(35) claimed that guinea pig liver, and to a much lesser extent, other tissues of several mammalian species, also contained a soluble form of MAO which could be extracted by conventional methods. Since they obtained their 'soluble' MAO from hypotonic aqueous tissue homogenates, it was possible that some of the enzyme activity might have arisen through mitochondrial lysis. This is conceivable in the light of the more recent findings of Schnaitmann et al.(41), and Tipton(42). Particles can easily detach from the mitochondrial membrane into the supernatant, which may not easily be sedimented by ultracentrifugation(38). Since Weissbach et al.(35) did not say whether or not their enzyme preparation contained succinic oxidase activity, it is difficult to draw any firm conclusion.

Later, Oswald and Strittmatter(36) applying more carefully controlled methods of mitochondrial extraction (i.e. by using 0.25M sucrase homogenates) to the study of the intercellular MAO distribution, confirmed the claim of Weissbach et al.(35). Oswald and Strittmatter did not detect succinic oxidase activity in the soluble MAO fraction of guinea pig liver.

The possibility, therefore, should not be overlooked that there is a distinct cell supernatant MAO with properties itentical with or overlapping those of the mitochondrial and

TABLE IV: SUBMITOCHONDRIAL LOCALIZATION OF MONOAMINE OXIDASE IN RAT LIVER AND BRAIN

According to K.F. TIPTON (Modified) See ref. 42.

Units of activity are expressed as atoms of oxygen consumed min^{-1} mg protein^{-1} in cases of cytochrome oxidase and MAO, and as moles NAD or dye reduced min^{-1} mg protein^{-1} in the cases of malate and succinate-dehydrogenases.

Treatment	Description	Source	Enzyme content (total units)			
			Mono-amine oxidase (MAO)	Cyto-chrome oxidase	Malate dehydro-genase	Succinate dehydro-genase
1 Large Amplitude Swelling	Swollen mitochondria	Liver	1.8	80	17000	180
		Brain	0.8	116	10000	170
	Outer membranes	Liver	1.7	8.55	267	8.2
		Brain	0.25	43.1	347	0
	Inner membranes	Liver	0.347	66	16600	205
		Brain	0.21	116	7800	198
2 Phospho-lipase diges-tion	Soluble portion of outer membranes	Liver	1.27	0	11200	0
		Brain	0	0	356	0
	Particulate portion of outer membranes	Liver	0	6.2	11.6	1.6
		Brain	0.158	1.2	15.4	0
	Inner membranes	Liver	0.56	47.0	0	117
		Brain	0.206	12.1	0	27

microsomal enzymes. Table IV deals with intracellular distribution of MAO.

3. Physiological role of MAO.

The main role of MAO is said to be the oxidative deamination of some of the important amines of biological and pharmacological interest. Most naturally occurring amines are substrates (Table V). There has been considerable speculation concerning the significance of the widespread distribution of MAO. It is now well established that the enzyme catalyses the oxidative deamination of many pharmacologically active monoamines(23,27,43,44). Thus it has been suggested by Bhagvat et al.(43) that the presence of relatively large amounts of MAO in intestinal mucose implies, in this case, a protective function of MAO. The enzyme, by virtue of its activity would prevent any toxic monoamines produced in the intestine by the bacterial decarboxylases, from entering the circulatory system. These authors(43) also stressed the constancy of MAO's presence in placenta, where it has been shown that the monoamine content can be quite high in certain physiological states.

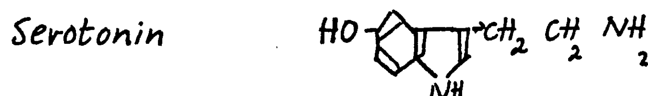
Adrenaline and related pressor amines have been suggested as some of the physiological substrates for MAO(27). In this connection, it may be stated that Schayer et al.(45,46) have shown that as much as 50 per cent of adrenaline and noradrenaline injected into rats is metabolised by MAO. Other metabolic pathways of catecholamine metabolism are known to exist(47).

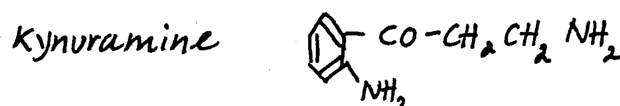
In plants, MAO may play a pivotal role by supplying the precursors of auxins. Indole acetic acid, a well-known

TABLE V: SUBSTRATES OF MONOAMINE OXIDASE

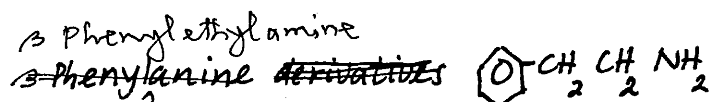
A: CYCLIC COMPOUNDS

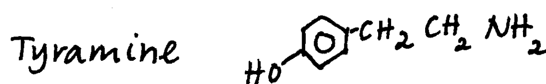
1 Tryptophan derivatives:

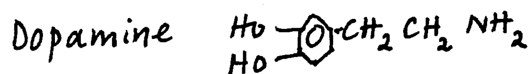


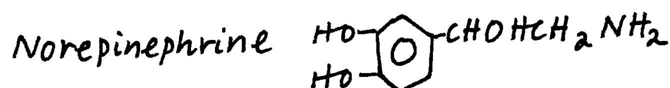


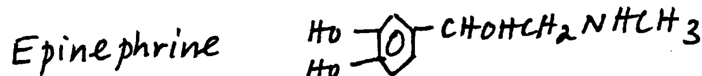
2 Phenylalanine derivatives:



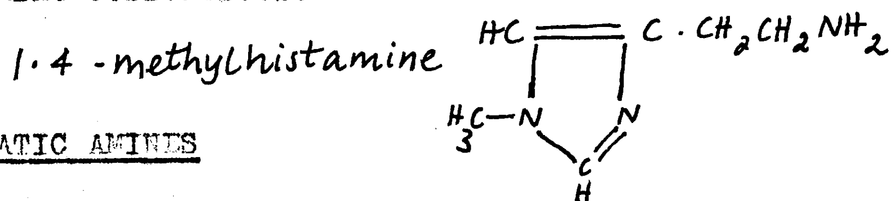




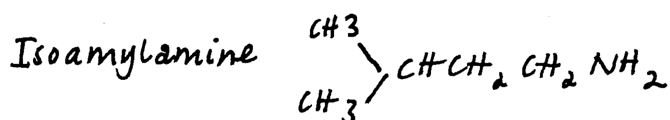


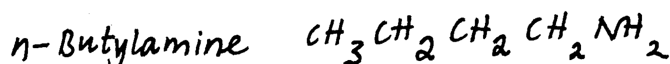


3 Histamine derivatives:



B: ALIPHATIC AMINES





plant hormone (auxin) is an oxidation product of indole acetaldehyde, the main metabolite of tryptamine oxidation. So far, MAO is the only enzyme known to catalyse tryptamine oxidation.

There are claims that MAO may be active in alkaloid synthesis in plants(27,48).

In mammalian brain the enzyme may be especially important in regulating the levels of serotonin(16,23).

The necessity of MAO for the metabolism of aliphatic monoamines in brain and other organs(12,13,35) as well as the metabolism of aromatic monoamines(1,2,12-20,23,27,29,35) is well known.

There are claims that MAO may supplement the action of DAO. Thus 1,4-methylhistamine (cf. table V) is said to be oxidised mainly by MAO instead of DAO(23).

Perhaps owing to the newly developed methods of preparing relatively pure and active MAO, better information may soon accumulate on the physiological functions of MAO.

4. <u>Mechanism of the reaction catalysed by monoamine oxidase</u>.

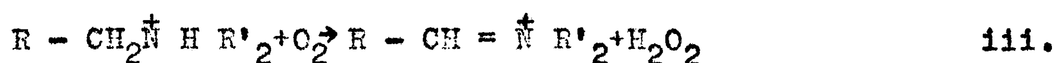
The 'tyramine oxidase' - catalysed reaction was originally formulated by Hare-Bernheim(2) thus:-

$$RCH_2NH_2+O_2 \rightarrow R-CH=NH+H_2O_2 \quad \text{(1st step)} \qquad \text{i.}$$

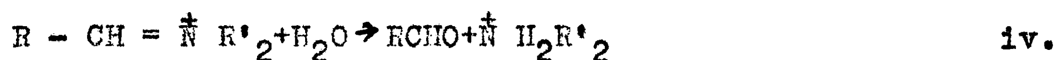
$$R-CH=NH+H_2O \rightarrow RCHO+NH_3 \quad \text{(2nd step)} \qquad \text{ii.}$$

In these series of reactions, the imino group formed in the first step spontaneously and nonenzymatically hydrolyses to form an aldehyde and ammonia. Richter(15) modified the Hare-Bernheim equation to include hordenine; a tertiary amine, which is ionic. The modified equation according to Richter,

therefore, is as follows:-



$$R - CH_2\overset{+}{N}\ H\ R'_2 + O_2 \rightarrow R - CH = \overset{+}{N}\ R'_2 + H_2O_2 \qquad \text{iii.}$$



$$R - CH = \overset{+}{N}\ R'_2 + H_2O \rightarrow RCHO + \overset{+}{N}\ H_2R'_2 \qquad \text{iv.}$$

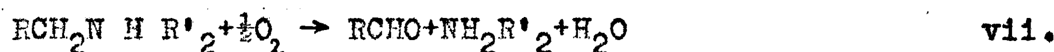
The overall equation is the sum of iii and iv:-

$$R - CH_2\overset{+}{N}\ H\ R'_2 + O_2 \rightarrow RCHO + \overset{+}{N}\ H_2\ R'_2 + H_2O \qquad \text{v.}$$

This modified formula, (v) although correct, presupposes the absence of catalase. In crude tissue suspensions where catalase is active, the hydrogen peroxide, H_2O_2 formed at iii is broken down by catalase thus:-

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2}O_2 \qquad \text{vi.}$$

The overall equation is the sum of v and vi:-



$$RCH_2N\ H\ R'_2 + \tfrac{1}{2}O_2 \rightarrow RCHO + NH_2R'_2 + H_2O \qquad \text{vii.}$$

Under ideal conditions, therefore, one molecule of amine is oxidised by an atom of oxygen. Sometimes, however, the oxygen absorbed exceeds that expected according to equation vii. This apparent discrepancy may be due to two main factors:-

(a) The hydrogen peroxide may escape the catalatic reaction (vi) and may take part in a peroxidatic reaction, eg. the peroxidation of an alcohol, eg. isoamyl alcohol, to its corresponding acid.

(b) The aldehyde (vii) may be further oxidised to the corresponding acid by aldehyde dehydrogenase. Both reactions can occur in liver and other tissues.

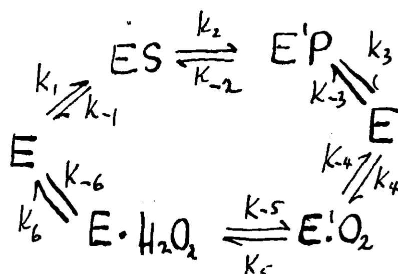
(c) A third factor may be the spontaneous oxidation of the aldehyde to the corresponding acid by air. Creasey(49) found

that the excess oxygen consumption when tyramine was used as substrate for rat liver MAO was caused by this factor. He corrected for it by including KCN in the reaction mixture. KCN stops this spontaneous oxidation without affecting the general oxygen uptake.

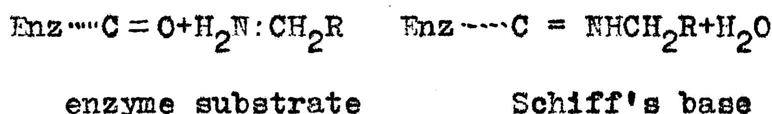
Belleau et al.(50) demonstrated that MAO could show stereospecificity. They also suggested on the experimental evidence, that the enzyme-substrate interaction involved a three-point attachment; the rate-controlling step in oxidation included the breaking of a C-N bond.

The need for adequate oxygen supply for the catalytic activity of MAO has been stressed(1,2,13,14,19). Thus, liver MAO activity, with tyramine as substrate, was thrice greater in 95 per cent oxygen than in air(19). This suggests, by analogy to other enzymes, that MAO reacted directly with molecular oxygen and behaved as a typical flavoprotein oxidase eg. D-amino acid and xanthine oxidases. Recently Tipton(51) reinvestigated the kinetics of MAO-catalysed reactions in relation to the effects of oxygen and substrate concentrations on catalysis. By keeping the oxygen concentration constant and varying that of the substrate or vice versa, Tipton obtained reciprocal plots of initial velocities in which the slopes remained constant irrespective of the concentration of the second substrate. This was interpreted as being consistent with a reaction mechanism in which a modified form of the enzyme is produced.He supported this claim by product inhibition and the demonstration of half-reaction at very low oxygen tension. The formation of a modified form of the

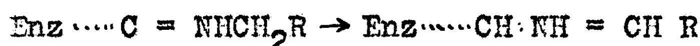
enzyme indicates that the flavin prosthetic group is either fully of partially reduced in the presence of substrate. According to Tipton then, the enzyme is partially bound to the product, hydrogen peroxide, thus:-



Tipton's finding is reminiscent of a mechanism given by Blaschko(23). In this reaction, the enzyme-substrate interaction is initiated by a reaction between the carbonyl group in the enzyme and the NH_2 group of the substrate:-



In the second stage the double bond shifts:-



The new double bond is then hydrolysed and the amino (NH_2) group is oxidatively removed from the enzyme. This may mean, however, that oxidation is not necessarily the first step in deamination by MAO.

This mechanism was postulated to apply to those amine oxidases such as pea seedling MAO amd MAO from some animal and bacterial sources which are sensitive to carbonyl reagents and which are now known not to attack N- substituted amines(23).

5. Inhibitors of monoamine oxidase.

(General references 23,27,52,66).

Perhaps the most extensively studied single property of monoamine oxidase is its inhibitor specificity.

So far, five main types of inhibitors are described. Representatives of each group would be found in Table VI. These five main groups may be simply classified as follows:-

A: Hydrazine type of inhibitors which include isonicotinyl-isopropylhydrazine (iproniazid, marsilid or IIH), - phenyl-isopropylhydrazine (pheniprazine or catron), and isopropyl-hydrazine;

B: Cyclopropylamines, represented by transphenylcyclopropy-lamine (tranylcypromine or parnate);

C: Amidines, represented by pentamidine;

D: Ephedrine and amphetamine type of inhibitors and the harmala alkaloids, eg. harmaline and related compounds;

E: The miscellaneous class of inhibitors including dyes such as methylene blue, certain aliphatic alcohols and quina-crine.

The inhibitors are subclassified into readily reversible, less readily reversible and miscellaneous inhibitors.

(i) Readily reversible:- Generally, when any of the 2αH atoms of a substrate of MAO is substituted by -OH or -CH_3 group, it affects the enzyme - substrate complex formation. Such substrates are in fact inhibitors. Both ephedrine and amphetamine have a substitution in the α-C atom, cf. table VI. They are competitive inhibitors which are readily reversible(23). Of the phenylisopropylamine series (amphetamine group), the

general rule is that compounds with the side chains:- $CH_2CH.MeNH_2$ or $-CH_2CH$ Et.NH_2 are inhibitors(23). This rule is equally true in the indole and 5-hydroxyindole series(23).

Harmaline and the related harmala alkaloids (cf. table VI) are also powerful, competitive and easily reversible MAO inhibitors(23,52,64).

When an animal, eg. the rat, is pretreated with harmaline before administering such potent MAO inhibitors as iproniazid or pheniprazine (catron), the action of the latter inhibitors is antagonised(52,64). The effect of catron is completely suppressed while that of iproniazid (IIH) is greatly reduced (52,64).

It is known that harmaline and related compounds compete with the hydrazine derivatives, IIH and Catron for the same site on the enzyme(61,64). This antagonistic effect is not displayed toward the cyclopropylamines which probably attack a different site on the enzyme(52).

Gorkin(38), working with rat liver MAO, found that harmine inhibited serotonin oxidation 1,000 times more strongly than that of tryptamine or tyramine.

(ii) <u>Less readily reversible or irreversible inhibitors</u>.

This group comprises the amidines with the common radical -C-:(NH)NH_2 eg. pentamidine; hydrazine derivatives, eg. IIH and catron; and the cyclopropylamines, eg. parnate(23,52-58).

The ability of amidines to inhibit MAO varies from species to species. Thus pentamidine is known to inhibit rabbit liver MAO about 50 times more strongly than the guinea pig enzyme, while the squid liver enzyme is hardly affected(23).

The cyclopropylamines, eg. parnate, and the hydrazine derivatives - such as IIH and catron - are competitive and irreversible inhibitors(52). Generally, both groups of inhibitors need aerobic preincubation for full inhibition(52,53), but parnate may not require any preincubation depending on the substrate(52). Parnate itself is said to be the active inhibitor species, attacking and inhibiting the enzyme chemically (52,58). Belleau and Moran(53) recovered 14C labelled parnate unchanged.

The mode of action of the hydrazine derivative of inhibitors such as iproniazid is still very speculative. Hare(1) and Davison(53) demonstrated that KCN has no effect on MAO. Davison(53,66) found, however, that KCN sensitises the enzyme towards IIH. He suggested that iproniazid forms a highly oxidative product which is the effective inhibitor. This author observed also that isopropylhydrazine was a better inhibitor than iproniazid(53).

Recently, Smith _et al_.(57) and Kory and Miglioli(56) demonstrated that the actual inhibitor formed by IIH, cyclopropylhydrazine or benzylhydrazine is volatile. The formation of the inhibitor is dependent on KCN or thiourea. It can be formed in the absence of tissue at high pH up to pH 12(56), and it requires oxygen. The actual process of inhibition, however, is independent of oxygen. The absorption spectrum of the inhibitor is different from that of any known chemicals of the hydrazine group(56).

It is reported(52,60) that the hydrazine types of inhibitors, eg. iproniazid can also inhibit other enzyme systems. Such enzymes are: choline dehydrogenase, aromatic amino acid

decarboxylase and diamine oxidase (DAO). The concentration of IIH to produce such inhibition is however greater than that for MAO(52,60).

It has been shown that while generally MAO inhibitors may increase the storage of endogenous and exogenous amines in many species, they may not have any significant effect in others, and in some cases they actually stimulate the depletion of amines(52,63). Thus Goldberg and Shideman(63) showed by fluorescent assays that administration of the potent MAO inhibitor, parnate, to cats and rats caused a marked depletion of the myocardial catecholamines of cat while that of rat was increased as expected. Pletscher(52) suggested this strange phenomenon might be due to the fact that in the cat the myocardial catecholamines (norepinephrine and epinephrine) are mainly metabolised by the catechol 3-O- methyltransferase (COMT) pathway c.f. figure 1.

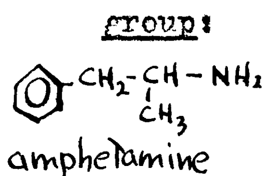
(iii) Miscellaneous Inhibitors.

Quinacrine, once a very common antimalarial drug, was found to inhibit flavin linked enzymes in oxidative systems (67-69). Allegretti and Vukadinovic(70) found that the oxidation of epinephrine by MAO was inhibited by quinacrine both in vivo and in vitro. Liver and kidney MAO were more strongly inhibited than brain and heart MAO. This difference in inhibition was thought to be either due to the greater difficulty of quinacrine to penetrate to the brain and heart enzyme than to the liver and kidney MAO or possibly due to the fact that the brain and heart MAO hold more tenaciously to the flavin prosthetic group than liver and kidney enzyme(70). As it is

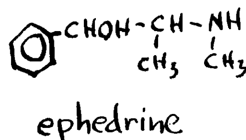
TABLE VI: INHIBITORS OF MONOAMINE OXIDASE

(i) Readily reversible inhibitors:-

Amphetamine and ephedrine group:

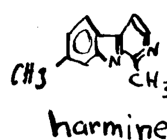


amphetamine

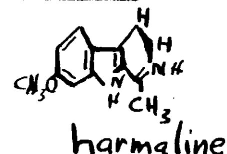


ephedrine

Harmalines:



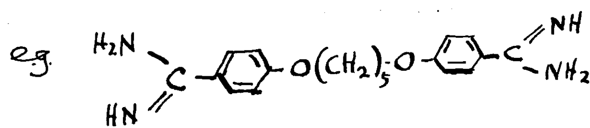
harmine



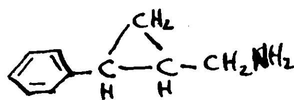
harmaline

(ii) Less readily reversible inhibitors:-

Amidines e.g.

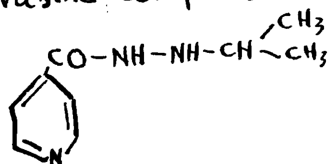


pentamidine

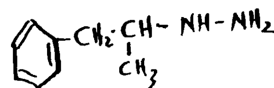


phenylcyclopropylamine (or tranylcypromine)

Hydrazine compounds



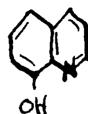
iproniazid (IIH) or Marsilid



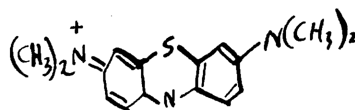
pheniprazine (catron)

(iii) Miscellaneous:

o-phenanthroline



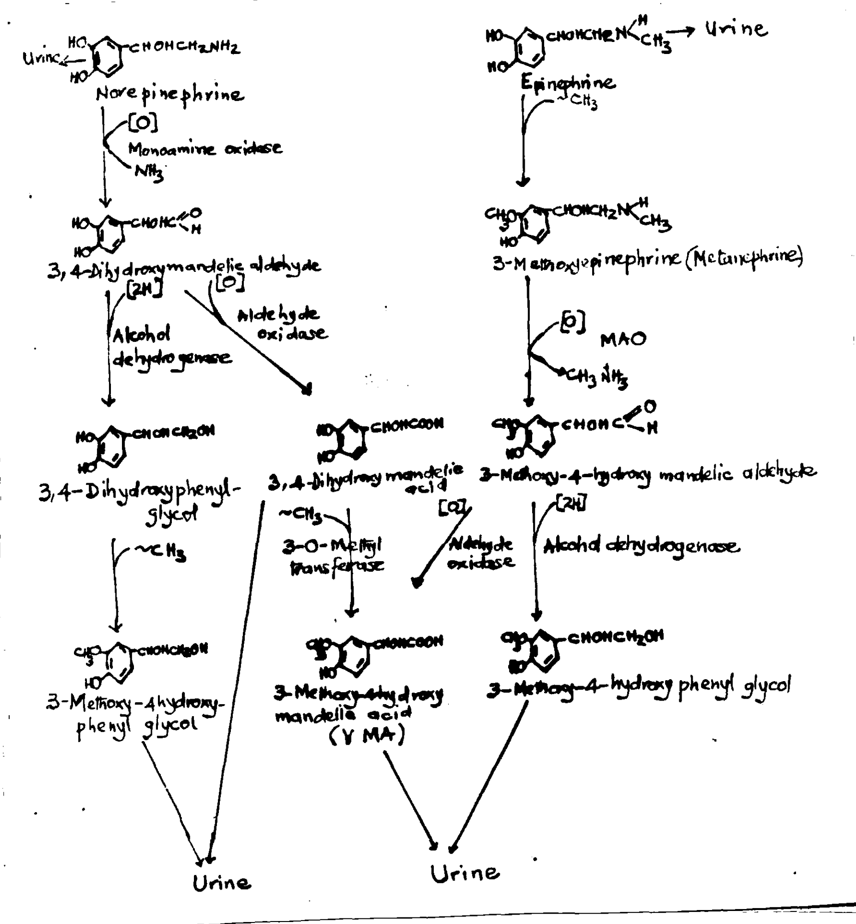
8-OH-quinoline



methylthionine chloride (methylene blue)

FIG. 1. SOME PATHWAYS OF CATECHOLAMINE METABOLISM SHOWING ORTHO-METHYLATION.

Phenylalanine → Tyrasine → Dopa → Dopamine → Norepinephrine → Epinephrine



now known that though MAO from liver and other organs are quicker to inhibit than brain enzyme, the effects last longer in the brain(52), this may mean that the differences in inhibition were due to the firmer binding of the liver and kidney MAO to the flavin than the brain and heart enzyme.

Lagnado and Sourkes(71) showed that quinacrine strongly inhibited rat brain MAO. This inhibition was partly reversed by FMN.

A number of organomercurials were also shown by these authors to inhibit MAO. This was interpreted as inhibiting mainly the SH groups of the enzyme(71).

Lagnado and Sourkes(72) also showed that a large number of metals may inhibit MAO as the result of the inhibition of sulphydryl. groups and probably other groups such as the amino group of the enzyme. Some of the inhibition by p-chloromercuribenzoate -PCMB- may be reversed though not too readily by glutathione(72). The inhibition of MAO by PCMB has been confirmed by other workers(73,74).

Lagnado and Sourkes(72) also demonstrated that British anti-Lewisite (BAL) may protect the enzyme against inhibition by Ag^{+} ions. A number of other heavy metals known to inhibit enzymes by complexing with SH groups were also found by these authors to inhibit MAO.

O-phenanthroline which forms chelates with Fe is shown by some workers to inhibit MAO(73,74) but it is claimed by others to activate it(75).

Other metal chelators such as hydroxyquinolines, eg. quinolinol; the naphthols, eg. α and β-naphthol may also

inhibit MAO not necessarily by complexing with metals such as Cu but instead by their phenolic function by forming charge transfer complexes with flavins(74). Such transfer complex formation has been demonstrated for amino acid oxidase and MAO(74).

Activators, cofactors and prosthetic groups of monoamine oxidase.

Lagnado and Sourkes(72) made an extensive study on the effects of metal ions on MAO and found stimulation by a number of salts, some of which were at very low concentrations of the order 10^{-6} to 10^{-5} moles/litre. These compounds include cuprous chloride, cupric sulphate, cadmium chloride, cobaltous nitrate, ferrous sulphate, mercuric acetate, silver nitrate, sodium selenite, uranyl acetate and stannous chloride. At higher concentrations these salts were definitely inhibitory.

For some time there was much speculation as to whether MAO contains flavin prosthetic groups or not. It is quite possible that while copper may not be necessary for enzyme activity in certain cases, it may be required by the same enzyme obtained from different species. In other words, these may well represent the heteroenzymes of MAO.

A similar case in point is the observation on the properties of *Aspergillus niger* enzyme(26) which combines the properties of the classical MAO and those of DAO(65,84). This peculiar crystalline enzyme from *Aspergillus niger* was shown by Muraoka and associates(26) to reduce cytochrome C both aerobically and anaerobically, but the anaerobic reduction was more pronounced. Polyamines were not substrates.

Perhaps especially striking was the observation that tyramine, tryptamine and serotonin, typical MAO substrates were the best substrates for the anaerobic reduction of cytochrome C. Histamine, a classical substrate for DAO, was an equally good substrate for cytochrome C reduction. The reduction of cytochrome C by histamine was stimulated by such electron carriers as menadione and α- and β-naphthoquinone.

Ethylene diamine, formerly thought to be inert as a substrate to MAO, was found to reduce cytochrome C without any noticeable oxygen uptake.

These peculiar observations pose the question as to whether the enzyme is a diamine or a monoamine oxidase. They also raise another question as to the relation of MAO to components of the electron transport chain.

The question concerning the possible participation of MAO in the electron transport chain is of some interest.

The insensitivity of MAO to cyanide and azide, known inhibitors of the cytochrome system of enzymes, may not be cited as immutable evidence against the involvement of MAO in the electron transport chain. After all, it is now known that the inhibition of the cytochrome chain by azide and cyanide is not necessarily due to the specific poisoning of metal catalysis(85).

Carbon monoxide, a more specific inhibitor of copper- and iron - containing enzymes, is also known not to inhibit all enzymes containing these metals(85).

Axelrod *et al.*(76) and Rossiter(77) found marked decrease in D-amino acid oxidase activity of riboflavin

deficient rat liver homogenates. As MAO and D-amino acid oxidase have similar properties in that they are both insensitive to cyanide, and also catalyse similar reactions with the production of H_2O_2, Hawkins(78) compared the activities of D-amino acid oxidase, known to be FAD-linked, and that of MAO in rat liver in riboflavin deficiency. She found D-amino acid oxidase activity was only about 20 per cent of the control while that of MAO was about 50 per cent. Restoring riboflavin in the diet readily reactivated D-amino oxidase activity but that of MAO was restored with great difficulty. By supplementing riboflavin with inositol, she readily reversed the deficiency effect on MAO. Hawkins was unable to conclude exactly whether riboflavin was required by MAO as a prosthetic group or whether it was needed for the enzyme synthesis.

Lagnado and Sourkes(79) Distler and Sourkes(80) and Youdim and Sourkes(81) confirmed Hawkins' results.

By selectively omitting ingredients known to affect rat growth, Lagnado and Sourkes(79) found that by omitting riboflavin from the diet, MAO activity was greatly reduced. By omitting pyridoxine no deficiency effects were observed; however, the omission of both riboflavin and pyridoxine appeared to slightly alleviate the riboflavin deficiency effect. The results of such experiments are summed up in Table VII.

No ready explanation of these results is apparent at the moment.

Nara _et al._(82) claimed that purified beef liver mitochondrial MAO contained both copper and FAD. Erwin and

Hellerman(74) showed that a highly purified bovine kidney MAO Contained FAD which they however failed to isolate as it was too tightly linked with the enzyme. These authors(74) showed that the enzyme exhibited seven to eight sulphydryl equivalents per 100,000 g. of protein. Tipton(73), later, succeeded in isolating FAD from purified pig brain MAO. He later showed that when the FAD was removed the enzyme was inactivated; restoring the FAD reactivated the enzyme(83).

The requirement of copper is not so clear. Some authorities claim that copper is necessary for full enzyme activity(82) but others contest this(74).

So far, copper has been found in a large number of MAO preparations from different sources including the pea seedling enzyme(24,65) and the plasma MAO(28) which also contains pyridoxalphosphate.

It may well be that oxygen requirement is in fact minimal, being required mainly for reoxidising a reduced prosthetic group, such as FAD, which is now proved to be the prosthetic group of some MAO preparations(73,83). In connection with the minimal requirement of oxygen, it is worth while to state that Weissbach _et al._(68) have demonstrated that MAO oxidises N,N-dimethyltryptamine -N- oxide faster anaerobically than aerobically. These authors showed that high tensions of oxygen inhibit the oxidation(86).

That MAO may enter the electron transport chain through cytochrome b5 is also worth considering since Tipton(42) appears to have detected this cytochrome in MAO-enriched submitochondrial preparations from rat tissues. Indeed,

TABLE VII: EFFECT OF RIBOFLAVIN (Vitamin B_2) AND PYRIDOXINE (B6) DEFICIENCIES ON MAO ACTIVITY OF RAT LIVER

Activity is expressed as $\mu l. O_2$ absorbed/hr/100 mg wet wt. tissue. All values are corrected for substrate blank.

Diet	Week 1	3	4	5	6	7
A. Tyramine						
complete	230	225	352	-	390	270
Deficient in B6	340	234	352	340	354	375
Deficient in B2	98	165	132	132	60	75
Deficient in both	254	210	228	128	155	105
B. Isoamylamine						
complete	94		132	-	90	90
Deficient in B6	102		136	106	120	105
Deficient in B2	70		76	63	30	30
Deficient in both	72		84	72	60	45

Data: From Lagnado and Sourkes see ref. 48,79.

cytochrome b_5, formerly thought to be confined to microsomes (87), is now found to be also present in mitochondria(88).

It is also worth noting that the two enzymes found to be more or less exclusively associated with the outer mitochondrial membrane are:- MAO and a rotenone insensitive NADH - cytochrome C reductase(89).

The view that oxygen requirement may be only minimal is however very difficult to reconcile with other experimental data showing the strong dependence of MAO on high oxygen tension(1,2,13,14,19,51). Furthermore, Tipton's(51) recent postulated reaction mechanism for pig brain MAO suggests the involvement of the superoxide anion ($O_2^{\bullet}$ or $HO_2^{\bullet}$) in amine oxidation. This could indicate a similar pathway to milk xanthine oxidase(154,156).

Homogeneity of monoamine oxidase.

The view that MAO was a single enzyme species distinguished from DAO by distribution, substrate and inhibitor specificities (see table XVIII) was first challenged when Alles and Heegaard(22) published their extensive studies on MAO. Since then there has been much controversy as to whether MAO is a single or a multiple enzyme with overlapping substrate and inhibitor specificities(16,29,36,91,92,120).

Werle and Roener(29) claimed to have separated from rabbit liver, by acetone treatment, an aliphatic amine oxidase catalysing the destruction of butylamine from an aromatic amine oxidase. This claim is subject to confirmation.

Hagen and Weiner(91) ahd Hope and Smith(92) demonstrated the existence of different substrate specificities by MAO

from different organs of the same animal (mouse). Weiner (16) demonstrated similar results with MAO from different nervous tissues. Blaschko(23) commenting on such findings stated: "The differences shown are such that it must be assumed either that enzymes from different tissues are entirely different proteins or that each organ contains a mixture of oxidases in differing proportions".

Oswald and Strittmatter(36) confirmed the findings of Hagen and Weiner(91) and Hope and Smith(92) with their studies on different tissues of rat and guinea pig (see table VIII). These authors(36) found remarkable differences in Km values not only between the two animals but also among the different organs of the same species.

By means of heating experiments, they showed convincingly that the metabolism of certain substrates were more profoundly affected than others. Oswald and Strittmatter remarked: "These differences might indicate that:

(a) various tissues contain different monoamine oxidases
(b) contain a different group of monoamine oxidases
(c) contain different proportions of several monoamine oxidases common to several tissues".

Youdim and Sourkes(81) and Gorkin <u>et al</u>.(59) also arrived at similar conclusions by means of inhibitors.

These results suggest indirectly the possible existence of different molecular species of MAO. Progress for clarifying this state of affairs has been hindered by the great difficulty in obtaining relatively pure, soluble and active MAO preparations. While this research was in progress, several papers have since appeared describing such preparatior

methods(73-75,82,90,93).

Preparation of monoamine oxidase.

Various methods have been recently developed by modifying the former conventional methods of enzyme extraction.

It has been known for years that acetone treatment of MAO to yield powder gives a relatively active enzyme which is stable(27). It is also known that MAO is stable to freezing and thawing(94), or to freeze drying(93).

Treatments with bile salts or with lysolecithin yield relatively clear solutions(27). "Soluble" forms of MAO have been recently obtained by mitochondrial lysis followed by sonication and sedimentation at high speeds(95). Guha et al.(93) showed that sonication of sucrose mitochondria from rat liver yielded a soluble active enzyme which could be purified 350-fold by repeated chromatography on DEAE cellulose. Similarly, Gorkin(38) showed that sonication of mitochondria in an atmosphere of hydrogen converted the membranes into fragments which were sedimented by spinning at high speed for two hours and purified by chromatography, density gradient electrophoresis or by Sephadex G 200 filtration.

The recent methods for preparing MAO include the use of sonication, detergent treatment, high speed centrifugation, ammonium sulphate fractionation, chromatography and repeated freezing and thawing techniques(73-75,82,90).

SECTION II: THE ENZYMIC REDUCTION OF TETRAZOLIUM SALTS AND OTHER DYES BY MONOAMINES.

A: Historical.

Hare(1) found that neither methylene blue nor 1-naphthol-2-sulphonate-2:6-dichloroindophenol were reduced in her anaerobic experiments in presence of liver extracts and tyramine. Dyes with more positive potentials were so rapidly reduced by the extracts alone that she could not draw any conclusions.

Philpot(14) later found that methylene blue and toluylene blue strongly inhibited the enzyme activity. Among the other dyes she tested, o-bromophenolindophenol and o-creosolindophenol were not inhibitory in presence of substrate (tyramine).

Danzami(96) first applied the tetrazolium method (blue tetrazolium) to the study of MAO activity in isolated liver mitochondria.

Francis(97,98) extended this technique histochemically to the distribution study of the enzyme in tissues.

Blaschko(27) found that the tetrazolium method did not parallel the Warburg manometric technique. He observed that acetone powder could still absorb oxygen in the presence of amines while 2,3,5-triphenyltetrazolium (TTC) could not be reduced. This led Blaschko to conclude that a factor(s), or possibly a second enzyme was necessary for catalysing tetrazolium reduction in such preparations.

Lagnado and Sourkes(94) demonstrated that the tetrazolium technique could serve as an important method for studying MAO activity in isolated tissues. These authors also found

that the ferricyanide, methylene blue and 2,6-dichloropheno-lindophenol methods could be employed in the measurements of MAO activity. They observed that the tetrazolium salts, (TTC) and neotetrazolium chloride (NTC) could not be reduced either by washed mitochondria or well-dialysed crude tissue extracts. In the presence of either purine, NAD, hypo-xanthine or inosine as a cofactor, together with a supernatant fraction, the ability to reduce TTC and NTC was reconstituted.

These authors also found that a heat stable, non-dialysable factor could replace the chemicals already mentioned. Neither the nondialysable factor nor the chemicals alone could restore the ability of the washed mitochondria for reducing tetrazolium; the supernatant fraction was also essential. Among the substances studied, nicotinamide and ademine strongly inhibited the cofactor function of NAD. Cyanide, azide, atabrine, o-phenanthroline and PCMB inhibited tetrazolium reduction.

Blaschko's suggestion for the requirement of additional factors for reducing tetrazolium was confirmed and extended.

Weissbach et al.(99) repeated Lagnado and Sourkes' experiment on mitochondria with a different tetrazolium salt. These authors postulated that aldehyde was the possible reductant of tetrazolium. The cofactors of Lagnado and Sourkes(94) were not required in the systems with INT as the dye.

The results of other workers in recent years in other enzyme systems have shown that different tetrazolium salts accept electrons at different places in the respiratory

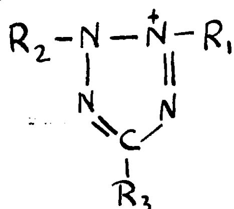
chain (100-103).

Since the experimental work of this thesis largely involved the use of tetrazolium salts as final electron acceptor of the amine oxidase system of guinea pig liver, it may be of some interest to present the reader with a brief review of tetrazolium salts, with particular reference to their application in enzymology to oxidoreductase systems.

B: Some properties of tetrazolium salts.

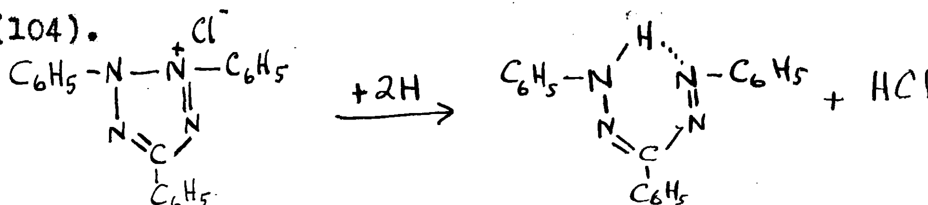
Tetrazolium salts (TTZs) are quarternary ammonium salts with a carbon skeleton and four nitrogen atoms, one of which is quarternary, thus conferring saltlike properties on the product.

All tetrazoles are synthesised from the parent compound, formazan, with the general formula:-



The nature of the substituents R_1, R_2, R_3 varies from salt to salt, but all the common salts are substituted in all the three positions by phenyl groups.

Tetrazoles are colourless or weakly coloured, water-soluble salts which are readily reduced to intensely coloured water - insoluble and non-autoxidisable formazans, as shown for TTC(104).



2,3,5-triphenyl tetrazolium chloride (TTC) pale yellow — TTC formazan red

Formazans are soluble in organic solvents and each has

its characteristic absorption maxima at which it can be conveniently measured spectrophotometrically.

C: The enzymic reduction of tetrazolium salts.

Kuhn and Jerchel(105) were the first to demonstrate tetrazolium reduction in organisms (bacteria and germinating seeds). Since then TTZ's have been widely used in solving biological problems especially in enzymology.

Perhaps of the enzyme systems most extensively studied, those involving the pathways of tetrazolium reduction by succinate, and by the reduced nicotinamide nucleotides have received the greatest attention(100-103,105-112). Thus Brodie and Gots(105) showed that the reduction of TTC by yeast alcohol dehydrogenase, a flavoprotein diaphorase, caused a simultaneous oxidation of NADH.

Kun(107) demonstrated that the amino acid oxidases could catalyse the same reaction. The glycolytic enzymes, however, required mitochondria for catalysing the reaction.

Shelton and Schneider(108) showed that purified xanthine oxidase, and NADH/cytochrome c reductase of bacterial origin were able to reduce TTC and NTC with a concomitant oxidation of NADH.

Brodie and Gots(109) found that purified crystalline rabbit muscle alcohol dehydrogenase, yeast alcohol dehydrogenase and glyceraldehyde dehydrogenase from Escherichia coli were capable of reducing NAD at 340 mμ, but incapable of reducing TTC and NTC. These enzymes lacked the flavoprotein diaphosase system. These authors, therefore, extracted a flavoprotein enzyme from E. coli and destroyed

its dehydrogenase activity by sonication. In the presence of this bacterial enzyme, rapid reduction of TTZs by NADH could be followed. By prolonged dialysis of the extract against dilute HCL and acid potassium phosphate, the flavin component was separated from the apoenzyme. This apoenzyme lost its power to reduce NTC in the presence of NADH. Reconstitution of FAD reactivated the system 60 per cent but neither FAD nor NADH alone, nor both together, could result in TTZ reduction. They also showed that the rate of NTC reduction was proportional to FAD concentration. These observations showed convincingly that the enzymatic reduction of the tetrazoles by reduced nicotinamide nucleotides required a flavin-linked diaphorase system.

These diaphorase systems have been shown to include the dicoumarol-sensitive, menadione-NAD(P)H oxidoreductases (EC.1.6.5.2)(110) and lipoamide dehydrogenase (E.C.1.6.4.3) (111).

D: Sites of interaction of tetrazolium salts with the respiratory chain.

That the mechanism of enzymic reduction of tetrazoles is a complex reaction has been stressed in recent years by several authors(100-103,110,111). Thus Nachlas et al.(100), Lester and Smith(101), Slater(102), Slater et al.(103), Vesco Guiditta(110) and Guiditta and Strecker(111) have shown that a number of factors may determine the point of reduction of a single tetrazole by an enzyme preparation.

Slater(102) and Slater et al.(103) have shown that the point of reduction of NTC by rat liver homogenates may vary

depending on whether oxygen was present or absent (aerobic and anaerobic conditions); on the tissue concentration - dilution effect of cofactors, the presence or absence of added quinones, eg. vitamin K_3 (menadione), the presence or the absence of added cytochromes when succinate was the substrate. Slater(102) found that under aerobic conditions, about 90 per cent of NTC reduction by liver homogenates in the presence of succinate, occurred at the level of cytochrome C through an unknown factor, and 10 per cent was reduced at or near the level of ubiquinone. Under anaerobic conditions (by carrying out the experiment in Thunberg tubes), he found that the proportion of the two sites was changed. NTC reduction was about 50 per cent at each side. Slater also observed that the rate of NTC reduction by rat tissue homogenates with succinate as the substrate was not proportional to the amount of tissue added. This was contrary to what was found with beef heart muscle preparations. When external electron carriers such as phenazine methosulphate and menadione were added in the presence of ascorbic acid (vitamin C), the rate of NTC reduction was proportional to the tissue concentration. However, the point of NTC reduction shifted from the level near or at cytochrome C, where it was antimycin A sensitive, to a level insensitive to antimycin A. Cyanide and azide had no affect on the system.

In the presence of ubiquione, the succinate-NTC reductase reaction was proportional to the tissue concentration. This would mean that another pathway was in operation.

While NTC couples at two points on the respiratory chain,

as stated, TTC was shown to couple at only one point, i.e. at the cytochrome oxidase level.

Similarly, 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3' (3,3'-dimethoxy-4,4'-biphenylene) ditetrazolium chloride (nitro blue tetrazolium or NBT) was shown by Slater et al.(103) with the succinate electron transport chain at one point only, that is at the ubiquinone level. Two other tetrazoles, INT and N^1-4,5-dimethylthiazol-2-yl-CN-diphenyltetrazolium bromide (MTT) interacted with the chain at two points, i.e. at the level of cytochrome C and at or near ubiquinone level.

So far, none of the tetrazolium salts tested has been shown to react directly with soluble succinate dehydrogenase. Nachlas et al.(100) found that in the presence of cytochrome b, NBT and INT were reduced by the soluble succinate dehydrogenase. If phenazine methosulphate was added to the soluble succinate dehydrogenase, all the tetrazolium salts tested were readily reduced. This would mean that electrons were transferred by the primary dehydrogenase - a flavo-protein, too tetrazolium salts through phenazine methosulphate as an intermediate electron carrier.

While NTC couples with the succinate respiratory chain at two sites as shown by Slater et al.(103), it has been shown by Lester and Smith(101) to couple with the electron transport chain at a region of the NADH-flavoprotein level in the presence of NADH-linked substrates. This would mean that NTC interacts with the electron transport chain of rat liver preparations at three specific sites which are suggestively similar to those proposed by Slater(113) and others for oxi-

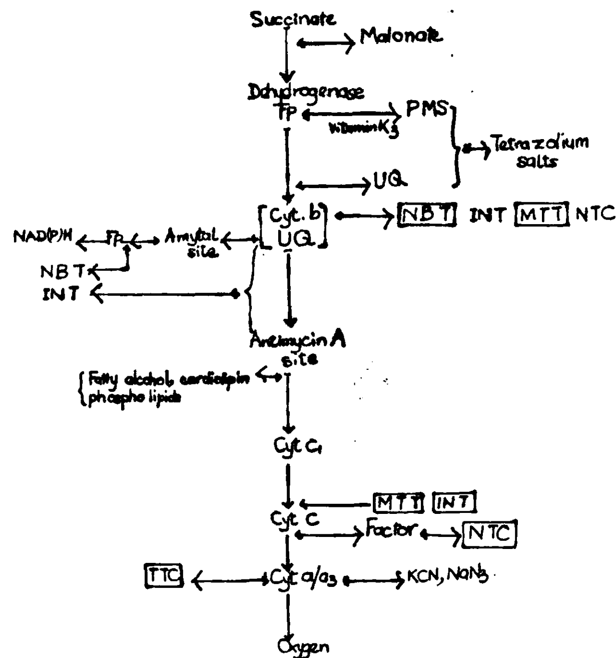
dative phosphorylation(114). The fact that tetrazolium salts have been found by Clark and Greenbaum(115) to inhibit oxidative phosphorylation in rat liver mitochondria is worth mentioning.

So far, it appears no definite prediction could be made about the point of interaction of a tetrazolium salt with the electron transport chain on the basis of its site of interaction with the chain in another enzyme system. Vosco and Giuditta(110) have shown that the pathways of NADH-tetrazolium reductase in mitochondria and microsomes from the same source may be different depending on the assay conditions. The tetrazolium reductase system of the microsomal preparation was shown to be sensitive to SH-inhibitors but the mitochondrial enzyme was unaffected. The microsomal system could also catalyse NADH cytochrome C reductase reaction and was slightly antimycin A- and amytal-sensitive. In the presence of NADH, INT, NBT and MTT were rapidly reduced by the microsomal system, while NTC and TTC were not. These authors have shown that lipoamide dehydrogenase, NADH-ferricyanide reductase and NAD(P)H menadione reductase may all contribute to tetrazolium reduction by flavoproteins.

Similarly, Lester and Smith(101) have shown that a number of quinones, including ubiquinone and menadione, cytochromes, long chain fatty alcohols and detergents eg. triton x - 100, may all be required for tetrazolium reduction by isolated beef heart muscle mitochondria which had been subjected to acetone fractionation in order to selectively extract lipids from the perparation.

The scheme given in Fig. 2 briefly summarises some of the points of interaction of tetrazolium salts with the electron transport chain of mammalian tissues.

FIG. 2. SOME SITES OF INTERACTION OF TETRAZOLIUM SALTS WITH THE ELECTRON TRANSPORT CHAIN IN ANIMAL TISSUES.



▭ = main sites for the aerobic reduction of tetrazolium salts.

CHAPTER TWO

MATERIALS AND METHODS

A: MATERIALS.

Tyramine and tryptamine were obtained from Koch Light Laboratories Ltd., Colnbrook, Bucks.

5-Hydroxytryptamine (serotonin), isoamylamine and other amines were bought from British Drug House (B.D.H.).

Tetrazolium salts were purchased from Koch Light Ltd., and Sigma Chemicals Ltd., London.

1-Isonicotinyl-2- isopropylhydrazide (marsilid phosphate or IIH) was a generous gift from Roche Products Ltd., Manchester Square, London. All other chemicals were purchased from Sigma Product Ltd., Armour Chemicals Inc. and B.D.H. Ltd., and were of the highest quality available.

Adult guinea pigs of both sexes were used and they were obtained from Alderwood, General Livestock Supplies, Camden Town, London.

B: EXPERIMENTAL METHODS.

The reduction of INT, NAD and oxygen by soluble and particulate MAO preparations from guinea pig liver.

1. Preparation of soluble and particulate monoamine oxidase.

Unless otherwise specified, the following method was employed routinely for preparing soluble MAO.

Adult guinea pigs (irrespective of sex) were killed by cervical dislocation. The liver was immediately removed and washed in cold tap water and chilled on ice, it was then cut into small pieces with a pair of scissors and weighed

after removing fat deposits and connective tissues.

Aqueous homogenates were prepared by homogenizing 1 part of tissue with 5 parts cold distilled water in an M.S.E. homogenizer at 0-4°C. for 3 minutes with cooling at one minute intervals. Further steps for enzyme extraction were as follows:-

Centrifugation: The homogenate was first spun at 20,000g for 20 minutes in a refrigerated M.S.E. "High speed 18" centrifuge, the residue was discarded, and the supernatant fluid was further centrifuged at 100,000g for 2 hours in a refrigerated M.S.E. "Superspeed 40" centrifuge. The residue obtained was resuspended in 0.01M sodium phosphate buffer, pH 7.6 and was designated PARTICULATE ("P") MAO.

Ammonium sulphate ($(NH_4)_2SO_4$) precipitation.

The high-speed supernatant was made 25 per cent with respect to $(NH_4)_2SO_4$ by the dropwise addition of a saturated solution of the salt (pH8) at 0-4°C. and with continual stirring to prevent any local precipitation. After standing in the cold for 30 minutes the solution was centrifuged at 10,000g for 10 minutes at 0-4°C; the precipitate was discarded. The supernatant was brought to 40 per cent saturation with ammonium sulphate solution. The final residue obtained was dissolved in 0.01M phosphate buffer, pH 7.6 and was dialysed overnight in the cold against several changes of the same buffer. The soluble preparation obtained will be referred to as soluble or 'S' MAO.

Preparation of the acid fraction from 'S' MAO.

Part of the 'S' MAO was further fractionated by acidi-

fication (at 0-4°C) to pH 5.0 with 0.5N acetic acid. The residue obtained after spinning the extract for 5 minutes at 10,000g (at 0-4°C) was dissolved in 0.05N phosphate buffer, pH 7.6 and was dialysed overnight against changes of the buffer. The resulting preparation was designated, soluble acid or S pH5 MAO.

It is to be noted that the SpH5 MAO could be prepared from an undialysed S-MAO. In fact that was the method frequently adopted as it saved time.

Any modifications in the preparation of enzyme will be described alongside the experimental results.

2. The enzymic reduction of tetrazolium salt (INT) by amines.

It is not known with certainty whether tetrazolium salts accept electrons from the dehydrogenase or from some other component of the monoamine oxidase system (see introduction: anaerobic reduction of dyes). The enzymic reduction of INT by MAO substrates will, therefore, be referred to as amine-tetrazolium (INT) reductase activity.

Principle:

Substrate (2H) + Tetrazolium → substrate (oxid.) + Formazan.

The method is based on the change of colour of tetrazolium salts from pale coloured or colourless water soluble salts when in the oxidised state to intensely coloured water insoluble and non auto-oxidisable formazans when reduced in enzymic dehydrogenase reactions where tetrazolium salts act as final electron acceptors(100-112). The formazan produced from 2-(p-iodophenyl)-3-(p-nitrophenyl -5-phenyl tetrazolium chloride (INT) is readily soluble in ethyl acetate and absorbs maximally

at 520 $m\mu$ in a Hilger bench colorimeter at which wavelength it can be conveniently measured.

Reagents:

(a) Substrate: 0.1M amine solution in 0.1M sodium phosphate buffer, pH 7.6

(b) Tetrazolium salt: 0.00396M INT solution in 0.1M phosphate buffer, pH 7.6

All solutions (a,b) were kept in the cold in dark bottles.

(c) Buffer: 0.1M-Na_2HPO_4/NaH_2PO_4 buffer pH 7.6 was used in all assays except when stated otherwise.

Amine/INT reductase assay: The standard reaction mixture contained in 2.0 ml, 20 μ moles amine; 50 μmoles phosphate buffer pH 7.6; 1.2 μmoles tetrazolium salt (INT); enzyme up to 0.5 ml. Water was added to make a final volume of 2.0 ml; water was substituted for amine in substrate controls.

The ingredients, excluding substrate and dye (INT), were pipetted into 13 mm.x 100 mm. test tubes and preincubated for 2 minutes in a water bath maintained at 37°C. The reaction was started by adding the dye and then the substrate (at 37°C) in rapid succession.

This method served to reduce or abolish endogenous tetrazolium reduction which was observed when INT was preincubated with the enzyme; probably this was due to the utilization of oxygen and/or endogenous substrates.

The reaction was stopped after incubation for 10 or 15 minutes (depending on the enzyme activity) by adding 3 ml cold neutralised 50 per cent trichloroacetic acid per

tube. 10 ml ethyl acetate was added to each tube to extract the formazan. The tubes were vigorously shaken and the ethyl acetate layer was pipetted off into suitable cuvettes and the OD was read at 520 mμ in a Hilger bench colorimeter.

In earlier experiments 50 per cent trichloroacetic acid (unneutralised) was used instead of the neutralised acid. It was found that the unneutralised acid caused excessive adsorption of formazan to the enzyme protein thus making extraction difficult with subsequent inaccurate reading.

<u>Activity measurements:</u>

Activity was expressed in terms of u moles formazan (iodoformazan) on the basis of a calibration curve constructed for iodoformazan. The molar extinction coefficient of the iodoformazan obtained by chemically reducing INT with spectrophotometrically measured amounts of NADH in presence of phenazonium methosulphate was identical to that of commercially purchased iodoformazan, both of which gave a molar extinction coefficient of 13300 in ethyl acetate. On the basis of the calibration curve (fig. 3) it was found that the OD/520 mμ for 0.5 μmole formazan in 10 ml ethyl acetate was 0.650.

The molar extinction coefficient for some stocks of INT purchased from Koch-Light Ltd., was 6820 when chemically reduced. However, when identical quantities of the INT were reduced enzymically in an amine INT reductase reaction, the OD at 520 mμ was identical to that obtained for the same quantity of INT from Sigma Chemical which gave a molar extinction coefficient of 13300.

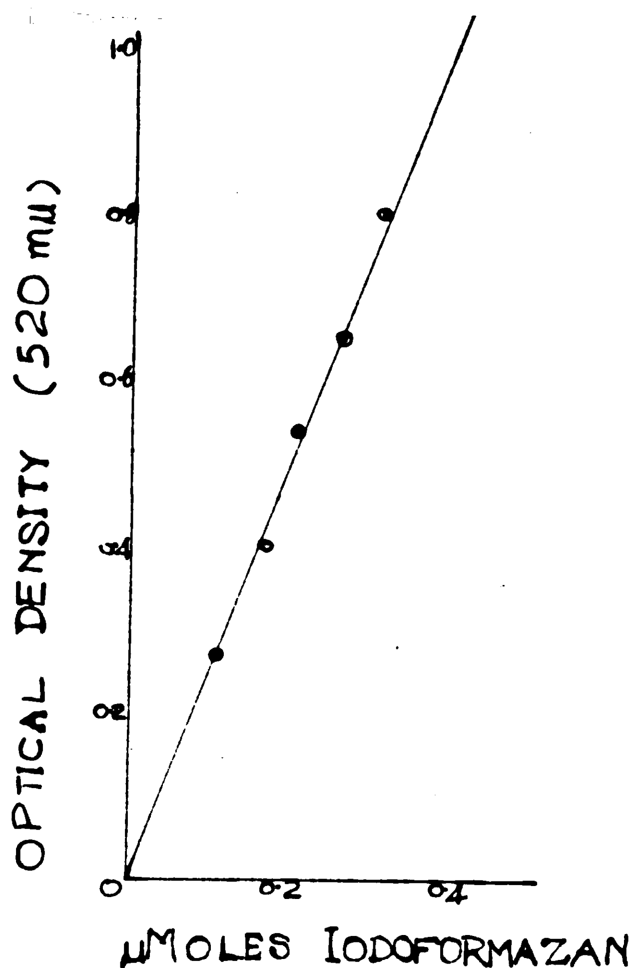
The lower extinction coefficient of the former INT was

probably due to impurities.

All the results reported in this thesis in terms of u moles formazan were, therefore, based on the molar extinction coefficient of the Sigma type iodoformazan since it was found to be in agreement with what was obtained by other authors(110).

FIG. 3. THE CALIBRATION CURVE FOR IODOFORMAZAN.

The calibration curve was constructed by dissolving accurately weighed amount of commercially purchased iodoformazan in 100 ml ethyl acetate. The stock solution was then diluted with the solvent and the various concentrations were measured at 520 mμ as indicated in the figure.



3. Activity measurement by substrate disappearance method.

Principle: The oxidation of kynuramine by MAO can be conveniently assayed measuring the decrease in absorption at 360 mμ due to kynuramine disappearance(116). The fall in optical density is directly proportional to the enzyme activity and is independent of aldehyde oxidase and other enzymes which may be present(116). Traces of diamine oxidase may interfere, as DAO also oxidises kynuramine though of about a tenth of rate of MAO(116).

Reagents: 0.00307M kynuramine dihydrogen bromide in 0.1M sodium phosphate buffer, pH 7.6 kept in the cold in a dark bottle

0.25M sodium pyrophosphate buffer, pH 8.8

Reaction mixtures and procedure: The following ingredients were pipetted into quartz glass cells of 1 cm light path containing a final volume of 3.0 ml:- 0.6 μmoles kynuramine; 125 μmoles pyrophosphate buffer, pH 8.8; enzyme up to 0.2 ml and water to make up the final volume. Water was substituted for the substrate in blanks. After 5 minutes of preincubation in a Beckman model DB spectrophotometer (c 30°C), the reaction was started by adding the substrate and readings were taken every 2 minutes for 20 minutes.

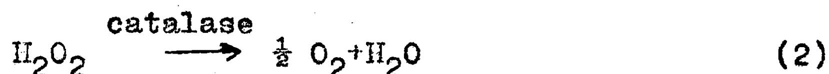
The enzyme activity was expressed in terms of the OD change at 360 mμ per 100 mg protein/hr. in excess of the OD change due to endogenous enzyme activity.

4. Measurement of MAO by oxygen uptake.

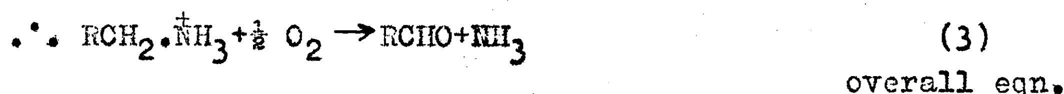
The Warburg manometric technique.

Principle:

$$RCH_2.NH_3+O_2+H_2O \xrightarrow{MAO} RCHO+NH_3+H_2O_2 \quad (1)$$



$$H_2O_2 \xrightarrow{catalase} \tfrac{1}{2}\, O_2+H_2O \quad (2)$$



$$\therefore\ RCH_2.\overset{+}{N}H_3+\tfrac{1}{2}\, O_2 \rightarrow RCHO+NH_3 \quad (3)$$

overall eqn.

The manometric method is based on the stimulation of oxygen consumption by tissues in the presence of amine substrates(1,15,16). The reaction is catalysed by MAO as shown in equation (1). One molecule of oxygen is absorbed per molecule of substrate metabolised with the concurrent formation of ammonia, aldehyde and hydrogen peroxide in stoichiometric amounts (eqn 1). The hydrogen peroxide is destroyed by catalase present in the reaction medium (eqn 2), thus contributing half a molecule or an atom of oxygen per molecule of substrate oxidised. In the presence of catalase, therefore, the overall reaction is as shown in equation (3), which is the sum of the first two equations.

The aldehyde intermediate can be trapped by adding semicarbazide to the reaction mixture to prevent further oxygen uptake due to the oxidation of the aldehyde to the corresponding acid(15,16).

Activity measurements.

MAO activity was expressed as μ moles of oxygen consumed per hour/100 mg protein in excess of the endogenous respiration. Unless otherwise stated, the values were based on the results

of the first 30 minutes of incubation, when oxygen uptake was linear as a function of time.

Reaction mixtures.

The enzyme preparation (1.0 - 1.5 ml) was pipetted into the main well of the Warburg flasks containing the following ingredients:-

In main well: 50 μmoles $Na_2HPO_4/NaH2PO_4$ buffer pH 7.6

In side arm: 20 μmoles substrate (neutralised), or water In control mixtures.

centre well: 0.1 ml 40 per cent KOH and filter paper.

Water was added to the main well as required to make a final volume of 2.5 ml.

Semicarbazide (neutralised) at 0.016M (final conc^n) was added in most cases to trap the aldehyde intermediate.

The gas phase was air.

Procedure:

Stoppered flasks were attached to their manometers and incubated at 37°C in Warburg water bath. After 5 minutes preincubation the taps were closed and two or three readings were taken in rapid succession to check whether the temperature equilibration was adequate and whether the apparatus was airtight.

The reaction was started by tipping the substrates from the side arm into the main well ("zerotime"). Readings were taken every 5 or 10 minutes depending on the enzyme activity, for at least an hour.

5. Protein estimation.

This was done by the method of Lowry *et al.*(118).

Reagents.

A. 2 per cent sodium carbonate in 0.1N NaOH

B. 0.5 per cent copper sulphate in 1 per cent sodium citrate.

C. 1 ml reagent B mixed with 50 ml of reagent A.

D. Folin-Ciocalteu reagent diluted HO 2.3 with water, giving a solution 1N in acid. Reagents C and D were prepared daily and discarded after use.

Method:

0.8 ml of the sample containing 10-100 mg. protein was mixed with 4 ml. of reagent C at room temperature and was allowed to stand for 10 minutes. 0.4 ml. of reagent D was rapidly added with shaking and thorough mixing. The colour developed was read after 30 minutes in the Hilger bench colorimeter at 700 mμ.

A standard crystalline bovine serum albumin was simultaneously run with each protein determination.

CHAPTER THREE

RESULTS

Unless otherwise specified, guinea pig liver tissue was used throughout this thesis. Any change in tissue will be indicated alongside the experimental results.

THE REDUCTION OF TETRAZOLIUM SALT (INT), NAD AND MOLECULAR OXYGEN BY SOLUBLE (S AND S-pH5) AND PARTICULATE (P) MONO-AMINE OXIDASE PREPARATIONS FROM GUINEA PIG LIVER.

SECTION I:

A. The enzymic reduction of tetrazolium salts by amines.

1. Soluble preparations: (S. MAO) etc.

The result for amine/INT reductase activity of S-MAO is presented in table IX.

It can be seen that all the substrates reduced INT enzymically, though to varying degrees. Tryptamine was the best substrate for the system and enzyme activity towards it was more than fourfold that seen for isoamylamine, benzylamine, -phenylethylamine and tyramine.

Since Lagnado and Sourkes(79,72) had already shown that amine/tetrazolium reductase activity of rat tissues, using neotetrazolium chloride (NTC) as acceptor could be stimulated by NAD and other cofactors including hypoxanthine, the effects of these cofactors on the amine/INT reductase system of the S-MAO was investigated. It was found that apart from NAD, which was effective, none of the other cofactors was stimulatory. (NAD (0.15 mM final conc^{n}) consistently caused a 5-10 fold stimulation of the enzymic INT reduction by

TABLE IX. NAD STIMULATION OF INT REDUCTION BY SOLUBLE MAO (S-MAO) IN THE PRESENCE OF AMINES OR ACETALDEHYDE.

	Substrate	Additive	Final molar conc. (M)	μ moles formazan/ 100 mg protein/hr.	μ moles "extra" formazan/ 100 mg protein/hr.	% Stimulation
1.	Water (blank)	-	-	1.87	-	-
		NAD	1.5×10^{-4}	3.46	1.59	85
2.	Isoamylamine	-		6.38	4.51	-
		NAD	1.5×10^{-4}	50.5	47.04	940
3.	Benzylamine	-		4.37	2.50	-
		NAD	1.5×10^{-4}	7.2	3.74	50
4.	Phenylethylamine	-		5.27	3.4	-
		NAD	1.5×10^{-4}	13.00	9.54	181
5.	Serotonin	-		10.60	8.73	-
		NAD	1.5×10^{-4}	24.00	20.54	135
6.	Tyramine	-		7.23	5.36	-
		NAD	1.5×10^{-4}	27.9	24.44	421
7.	Tryptamine	-		25.87	24.00	-
		NAD	1.5×10^{-4}	27.86	24.4	2
* 8.	Acetaldehyde	-	-	10.3	8.43	-
		NADP	1.5×10^{-4}	10.3	8.43	-
		NAD	1.5×10^{-4}	28.0	24.54	191

Legend: The standard reaction mixture contained in a final volume of 2.0 ml: 20 μmoles amine substrate (pH 7.6); 50 μmoles sodium phosphate buffer (pH 7.6); 1.2 μmoles INT; enzyme up to 0.5 ml. (about 4 mg protein). Water was added to make up the volume. Water was substituted for substrate in blanks. Incubation was for 10 minutes.

Further details are given under "Methods".

* 0.05 per cent (final conc^{n}) of acetaldehyde was used in this experiment.

"Extra" formazan was that in excess of the endogenous enzyme activity. (i.e. no substrate), except in the case of (1) where it refers to the effect of NAD on endogenous tetrazolium reduction.

isoamylamine and tyramine and about 2-3 fold of that by phenylethylamine and serotonin but it had no significant effect with tryptamine as substrate. NADP was not a cofactor in this system. This was in agreement with the findings of Lagnado and Sourkes who observed that NADP had no effect on their system.

NAD also caused a slight stimulation of the endogenous enzymic activity (85 per cent) but this effect was increased with longer incubation. This endogenous enzyme activity was either abolished or very greatly reduced in other experiments by longer preincubation with the ingredients in the absence of INT in which case probably the endogenous substrates were inactivated towards INT reduction by being oxidised by such aerobic preincubation in the absence of INT. If the preincubation step was omitted by adding INT and enzyme simultaneously, the endogenous enzyme activity could be so great as to mask the effect of amines, especially if NAD was present.

Nicotinamide and adenine were found by Lagnado and Sourkes to strongly inhibit the cofactor functions of NAD in the amine/NTC reductase system of rat tissues, were not inhibitory in our system. In fact, nicotinamide was routinely used in the amine/INT reductase system of S-MAO of guinea pig liver to protect NAD from breaking down . This may reflect differend properties between the rat and guinea pig enzymes. However, since I have confirmed with the guinea pig enzyme that dialysis abolishes NTC reductase activity(94), which could be restored by added purines, it is very probable that the differences may be due to the fact that the tetrazolium salts, INT and NTC,

accept electrons at different places in the amine/tetrazolium reductase electron transport system. A parallel observation has been demonstrated for the succinate/tetrazolium reductase systems by Slater and others(100,103).

Succinic dehydrogenase activity was not detected in the S-MAO preparation. Such S-MAO preparations, however, contained active aldehyde dehydrogenase activity which was about twofold stimulated by NAD in the INT reductase assay when acetaldehyde was the substrate.

2. <u>Particulate preparations</u>.

Table X shows a typical result when the amine/INT reductase activity was assayed for the particulate system.

In the particulate system also, tryptamine was the best substrate for demonstrating enzymic tetrazolium reduction and was about 77 fold as efficient as isoamylamine.

When the NAD effects on the two amines to reduce INT enzymically in the system are compared, it will be seen that while NAD strongly stimulated enzymic INT reduction by isoamylamine that by tryptamine was slightly inhibited. This apparent inhibition of INT reduction when tryptamine was substrate was in fact due to the stimulation of endogenous INT reduction by NAD. Thus, though tryptamine reduced INT enzymically to the same extent in the presence and absence of NAD (see table X No. 5), when the endogenous value was deducted a smaller value was obtained for tryptamine. Again if the data of tables IX and X are compared it will be seen that though there was NAD stimulation of the endogenous enzyme activity in both the S and P systems, NAD stimulated enzymic

INT reduction by tryptamine to such an extent, that the deduction of the endogenous value did not show inhibition of the enzymic activity for this substrate. In fact, though NAD did not appear to stimulate INT reduction by tryptamine in any of the enzyme systems being described, NAD was found to stimulate the enzyme activity in the initial stages by abolishing a lag phase of a minute or two which was consistently seen in almost all experiments involving tetrazolium reduction.

Table X indicates that generally the pathway for INT reduction by tryptamine was different from that of all the other amines which were found to be aided to a certain extent in all cases.

TABLE X. THE EFFECTS OF NAD ON INT REDUCTION BY PARTICULATE(P) MAO PREPARATION IN THE PRESENCE OF MONOAMINES.

Added substrate	Additive	Final molar (M) conc^n.	µ moles formazan/ 100 mg protein/ hr.	µ moles 'extra' formazan/ 100 mg protein/ hr.	% stimulation
1. None	-		11.1		-
	NAD	1.5×10^{-4}	16.2	5.1	46
2. Isoamyl-amine	-		13.3	2.2	-
	NAD	1.5×10^{-4}	32.2	16.0	627
3. Tyramine	-		21.5	10.4	-
	NAD	1.5×10^{-4}	42.7	26.5	155
4. Serotonin	-		46.5	35.4	-
	NAD	1.5×10^{-4}	52.8	36.6	3
5. Trypt-amine	-		155.0	143.9	-
	NAD	1.5×10^{-4}	155.0	135.8	-3 *

* negative sign before a number shows inhibition.

Legend: The particulate preparation used was the residue obtained by centrifuging the first supernatant at 100,000g for 2 hours. It was resuspended in phosphate buffer and dialysed overnight against the buffer.

All other conditions are as in table IX.

3. <u>The effects of prolonged centrifugation of the S MAO on NAD stimulation of amine/INT reductase activity.</u>

The first two results just described suggested that tryptamine oxidation by the two enzyme systems might operate through a different pathway from that of the other amines tested. Attempts were therefore made to dissect this out physically. As it had been reported by Seiden and Westley (95) and Gorkin(38) that part of the MAO present in sucrose mitochondria from rat tissues (brain and liver) could be released in a soluble form which does not sediment after centrifugation at 100,000g for an hour, it was decided to increase the time of centrifugation at 100,000g for more than three hours. This prolonged centrifugation at 100,000g was intended for removing any lysed mitochondrial or microsomal particles which might be present in the S-MAO. After such treatment, the different fractions (supernatant and redissolved residue in 0.01 phosphate buffer pH 7.6) were assayed for amine/INT reductase activity. The result obtained by centrifugation at 100,000g for 3 hours is shown in table XI.

A much higher stimulation of INT reductase activity by NAD was observed. Isoamylamine oxidation in the supernatant was found to be absolutely dependent on added NAD while that of tyramine was 8390 per cent stimulated. Unexpectedly, a twofold stimulation of INT reduction in the presence of tryptamine as substrate was observed when NAD was incorporated with the system (supernatant fraction). The effect of NAD on serotonin oxidation was the same in the supernatant as seen for the whole enzyme (unspun) system, about 80 per cent

stimulation in both cases.

INT reduction by the re-dissolved residue was not greatly affected by NAD when it was assayed with the mono-amines though a 9 per cent inhibition of the reaction by NAD was observed when tryptamine was the substrate. Such redissolved residues, however, showed active tetrazolium reduction with the substrates (on bases of specific activity). The enzyme activity (specific activity) was about fourfold for the first three substrates (see table XI) and about twice for tryptamine over that for the whole enzyme (unspun).

The endogenous enzyme activity was negligible for both fractions derived from the unspun enzyme showing that the variations in NAD effect on the two systems had nothing to do with the endogenous enzyme activities.

With longer centrifugation time at 100,000g (i.e. between 6 and 9 hours), all the substrates tested became definitely dependent on NAD to reduce INT enzymically. In the case of tryptamine as the substrate, in the absence of NAD there was a lag phase for at least 8 minutes when the ingredients including INT were incubated at 37°C. Isoamylamine and tyramine did not give any visually detectable INT reduction by the supernatant when all the ingredients were incubated at 37°C for an hour in the absence of NAD. However, even at room temperature the presence of 1.5×10^{-6} M (final conc^{n}) of NAD in the reaction mixture resulted in visible tetrazolium reduction by all the substrates tested. NAD stimulated reduction of INT by tryptamine was not further in the supernatant fraction by further high speed centrifugation

1000,000g for 6 or 9 hours though the lag phase was either abolished or greatly reduced by NAD.

TABLE XI. THE EFFECTS OF NAD ON AMINE/INT REDUCTASE ACTIVITY OF S-MAO AFTER SPINNING THE ENZYME AT 100,000g FOR 3 HOURS.

Fraction	Substrate	Additive	Final molar concⁿ (M)	μ moles formazan per 100mg protein/hr.	% stimulation
Whole enzyme (unspun)	1. Isoamylamine	-		4.50	-
		NAD	1.5×10^{-4}	27.02	500
	2. Tyramine	-		6.8	-
		NAD	1.5×10^{-4}	27.4	300
	3. Serotonin	-		10.0	-
		NAD	1.5×10^{-4}	18.0	80
	4. Tryptamine	-		18.5	-
		NAD	1.5×10^{-4}	18.4	-
Supernatant	1. Isoamylamine	-		0.00	-
		NAD	1.5×10^{-4}	3.00	- *
	2. Tyramine	-		0.225	-
		NAD	1.5×10^{-4}	19.10	8390
	3. Serotonin	-		1.70	-
		NAD	1.5×10^{-4}	3.09	82
	4. Tryptamine	-		2.2	-
		NAD	1.5×10^{-4}	7.3	232
Redissolved residue	1. Isoamylamine	-		4.01	-
		NAD	1.5×10^{-4}	8.1	102
	2. Tyramine	-		5.0	-
		NAD	1.5×10^{-4}	10.2	104
	3. Serotonin	-		8.1	-
		NAD	1.5×10^{-4}	8.51	5
	4. Tryptamine	-		15.0	-
		NAD	1.5×10^{-4}	13.7	-9 **

* Not possible to determine the NAD stimulation because of zero OD for the control.

** Inhibition by NAD.

4. <u>The effects of NAD concentration on amine/INT reductase activity of S-MAO.</u>

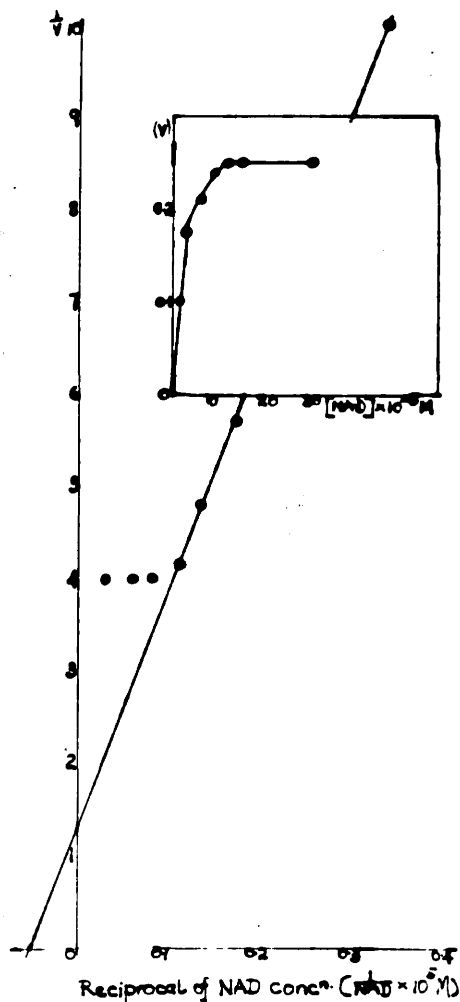
NAD effects on enzymic INT reduction by amines was investigated further by determining the concentration of it necessary for cofactor activity in the system. Since isoamylamine showed the greatest requirement of NAD in the amine/INT reductase systems already described, it was chosen as the ideal substrate for the investigation.

It was found that the stimulation of enzymic INT reduction by isoamylamine in presence of added NAD was proportional to the amount of NAD added, as shown in Fig. 4. It will be observed that the velocity/NAD concentration curve appeared to follow the general types of curves obtained for systems governed by Michaelis-Menten equation. When the initial velocity of the reaction was plotted as a function of NAD concentration, the K_m was found to be $2x10^{-5}$M. Reciprocal plots of $\frac{1}{NAD}$ / $\frac{1}{v}$ gave an intercept on the $\frac{1}{NAD}$ axis from which the apparent K_m was found to be $2x10^{-5}$M.

The NAD concentration plots showed that added NAD very quickly saturated the amine/INT system of S-MAO and caused no further stimulation of the reaction.

FIG. 4. THE EFFECTS OF NAD CONCENTRATION ON INT REDUCTION BY ISOAMYLAMINE IN THE PRESENCE OF S.MAO.

The reaction mixture comprised 20 μmoles isoamylamine (neutralised); 50 μmoles sodium phosphate buffer, pH 7.6; 1.2 μmoles tetrazolium salt (INT); 0.5 ml S.MAO (about 4 mg protein); NAD concentrations were as indicated in the graph. Water was added to make a final volume of 2.0 ml. Water replaced the substrate or NAD in controls.



B. Enzymic NAD reduction by amines.

Since it is not known for certain whether the NAD reduction by the system is due to the amines themselves or by an unknown product, though others have claimed that it is the aldehyde intermediate which reduced NAD(35), MAO activity as measured by this method will be simply referred to as "Amine/NAD reductase" activity for convenience.

In view of the NAD stimulation of enzymic INT reduction by amines already described in this thesis, attempts were made to assay the enzyme activity spectrophotometrically at 340 m using NAD as electron acceptor. The following reaction mixture was employed:-

A total volume of 3.0 ml in 1 cm quartz cells contained 6.7 μmoles amine substrate (neutralised); 4 μmoles/ml. nicotinamide; 1.06 μmoles NAD; 83.67 μmoles Na_2HPO4/NaH_2PO4 buffer, (pH 8.2); enzyme, 0.25-0.5 ml (about 4 mg protein in most experiments); water was added as required to make up the final volume. Water replaced the substrate in controls. The reaction mixture was preincubated for 10 minutes at about 30_0C in the Beckman model DB spectrophotometer. The reaction was started by adding the substrate.

1. Soluble MAO preparation.

It was found that NAD reduction could not be followed at 340 mμ because of the oxidation of the reduced NAD by the tissue preparation. Indeed, it was observed that after an initial increase in absorbance of about 0.150-0.250 OD per minute with isoamylamine, for example, as substrate, there was an equally rapid fall of the OD to zero. This confirmed the

findings of Weissbach et al.(35) who could not assay the enzyme activity at 340 mμ with a similar preparation. Attempts were therefore made to stop the NADH oxidation by the tissue by incorporating (in final concentrations) either 2μg/ml of antimycin A or 3.3×10^{-3}M sodium amytal, known potent inhibitors of the respiratory chain(122) in the reaction mixture.

These inhibitors, however, were ineffective in stopping the oxidation of NADH. This would, therefore, suggest that the mitochondrial NADH oxidases were absent from the system of the S-MAO.

Another factor which could also account for oxidation of NADH was the pH of assay (8.2). NADH is known to be more stable at high (alkaline) than low (acid) pH. Sodium pyrophosphate buffer (pH 8.8) was chosen instead of the sodium phosphate buffer stated above. Though a higher pH would be certainly more favourable than the chosen pH 8.8, at this pH there was no oxidation of NADH. The result of a typical assay is presented in table XII. This would suggest, therefore, that the oxidation of reduced NAD by the former system was due to the equilibrium of the reaction : NAD + ⇌ NADH + H^+ which was favoured by relatively acid pH 8.2 as compared to pH 8.8.

At acid pH the excess H^+ pushes the reaction towards NAD^+ while at alkaline pH the equilibrium shifts towards NADH + H^+.

TABLE XII. NAD REDUCTION BY S-MAO
IN PRESENCE OF MONOAMINES:
AT pH 8.8 IN SODIUM PYROPHOSPHATE BUFFER.

Exp.	Substrate	μ moles NADH/100 mg protein/hr.
1	Benzylamine	8.16
2	Isoamylamine	25.40
3	Tyramine	23.40
4	Serotonin	23.10
5	Tryptamine	21.8

Legend: In a final volume of 3.0 ml contained in 1 cm light path quartz glass cells were the following (in final concentrations):- 6.7 μ moles amine (neutralised); 4 μ moles nicotinamide/ml; 1.06 μ moles NAD; 150 μ moles sodium pyrophosphate buffer, (pH 8.8); enzyme: 0.25 ml (4 mg protein); water was added as required to make up the final volume. Water replaced the substrate in controls. After 10 minutes preincubation in the spectrophotometer, the reaction was started by adding the substrate. Readings were taken every minute for 30 minutes.

2. Acid fraction of S-MAO (i.e. S-pH5 MAO)

The results reported for the S-MAO were different from those obtained for the acid fraction derived from it (for preparation see the Methods section.

This S-pH5 MAO caused little oxidation of NADH at pH 8.2 (Na_2HPO_4/NaH_2PO_4 buffer) at which pH the enzyme activity was therefore assayed in order to compare the results with those obtained for a similar preparation by Weissbach *et al.* (35). Table XIII (below) compares the results obtained in this thesis with those for a similar preparation by Weissbach *et al.* The results of these authors were expressed as percentages, based on serotonin destruction as measured at 340 $m\mu$ in the presence of added NAD.

It will be seen that tyramine was the best substrate for NAD reduction enzymically according to the authors. According to them tyramine oxidation by soluble (acid fraction) from guinea pig liver extracts was 30 fold higher than that of β-phenylethylamine. Isoamylamine was not tested (figures in brackets).

Data by the present author show that β-phenylethylamine, and not tyramine, was the best substrate for the S-pH5 MAO in the amine/NAD reductase system; followed by isoamylamine; tyramine was the third best substrate. In fact, with some preparations, the rate of oxidation of β-phenylethylamine was twice that of tyramine (see table XIII). The authors also claimed that excess aldehyde dehydrogenase was required for full enzyme activity (for serotonin). This claim, however, could not be confirmed by the present author in his experiments. These discrepancies might be probably either

TABLE XIII. NAD REDUCTION BY S-pH5 MAO IN THE PRESENCE OF MONOAMINES

Exp.	Substrate	μ moles NADH/100 mg protein/hr.
1	Isoamylamine	32.5
2	β-Phenylethylamine	33.1 (6) a
3	Tyramine	16.2 (180)
4	Serotonin	10.0 (100)
5	Tryptamine	9.27 (93)

a = Data in brackets represent values obtained by Weissbach et al.(ref. 35) for a similar enzyme preparation from the same source. Results obtained by these authors were expressed as percentage activities relative to serotonin oxidation, taken as 100 per cent.

due to strain differences or to the differences in centrifugation speeds during enzyme extraction.

A comparison of the results of the amine/NAD reductase system (tables XII and XIII) with those of the amine/INT reductase system (tables IX-XI) will show that apart from β-phenylethylamine, those substrates which were most sensitive to NAD stimulation in the amine/INT reductase system were also the best substrates for the amine/NAD reductase system of guinea pig liver soluble MAO extracts.

3. Mitochondrial suspensions (substrate).

An attempt was made to study the enzyme activity in mitochondrial suspensions in relation with their ability to reduce NAD enzymically as compared with the enzyme activity in the soluble systems.

Mitochondria were prepared according to the method described by Hogeboom(119) but with slight modifications in centrifugation speeds as follows:-

A 10 per cent adult guinea pig liver homogenate in 0.25 M sucrose was prepared by homogenizing 1 part tissue with 9 parts 0.25 M sucrose in a Potter-Elvehjem homogenizer for 2 minutes at 0-4°C, with cooling at 30 second intervals. All subsequent steps were also carried out at 0-4°C. The nuclear fraction was isolated by centrifugation at 2,000 *g* for 10 minutes and was discarded. The supernatant was then centrifuged at 10,000 *g* for 10 minutes. The mitochondrial pellet obtained was resuspended in 10 ml 0.25M sucrose and was respun at 10,000 *g* for 10 minutes. This preparation should be assumed to be contaminated with microsomes.

The reaction mixture was as already described.

There was a comparatively rapid enzymic reduction of NAD with a similar rapid oxidation of NADH by the system in the presence of tyramine. This gave rise to fluctuations in apparent NAD reduction with time.

By incorporating either 2µg/ml antimycin A or $3.3x10^{-3}$ M amytal (final concentrations) in the reaction mixture, it was nevertheless possible to follow enzymic NAD reduction by tyramine for 15 minutes only. After that time the OD gradually declined (see fig. 5b). This was especially noticeable when amytal was the inhibitor. Antimycin A is known to be a more potent inhibitor of NADH oxidation by tissue than amytal(122). Whether the decrease in absorbance was caused by mitochondrial breakdown or not was not investigated. It could be possible however, that NADH was oxidised by NADH-cytochrome C reductase type of reaction. Fig. 5b shows NADH oxidation by mitochondrial suspensions in the presence and absence of respiratory chain inhibitors.

Generally it was observed that the rate of amine/NAD reductase reaction in the soluble MAO systems was faster than in the mitochondrial system. This was contrary to what was expected since MAO is known to be mainly mitochondrial in distribution(32-42).

FIG. 5b. TYRAMINE/NAD REDUCTASE ACTIVITY OF SUCROSE MITOCHONDRIAL SUSPENSIONS IN SODIUM PHOSPHATE BUFFER, pH 7.6.

The complete reaction mixture of 3.0 ml contained 6.7 μmoles tyramine hydrochloride (neutralised); 83.67 μmoles sodium pyrophosphate buffer/ml, pH 8.8; 1.06 μmoles NAD; 4 μmoles/ml nicotinamide; 0.5 ml enzyme; antimycin A or amytal was present in the same strength as indicated in 5a ii. Where semicarbazide was present (~~see~~ 5b iv), it was in a final concentration of 13.3×10^{-3}M.

Water was added as required to make up the final volume. It replaced the substrate in blanks.

iii no semicarbazide
iv + semicarbazide

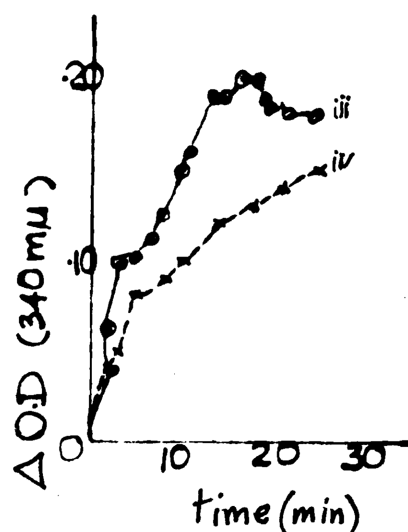
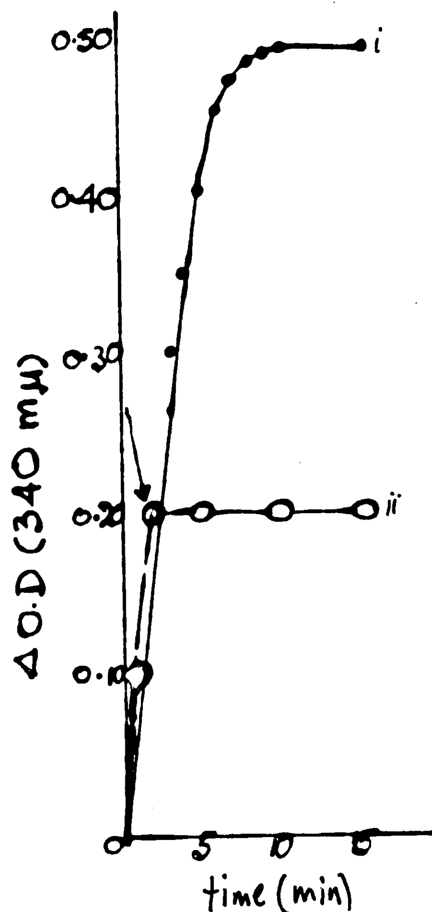


FIG. 5a.

NADH oxidation by guinea-pig liver sucrose mitochondrial suspensions in phosphate buffer, pH 7.6. The reaction mixture contained in 3.0 ml: 0.5 µmole NADH; 0.5 ml enzyme (about 4 mg protein); 83.67 µ moles sodium phosphate buffer pH 8.2. Water was added as required to make a final volume of 3.0 ml. Water replaced NADH in controls. At the arrow, either antimycin A (2 µg/ml) or sodium amytal 3.3×10^{-3}M (final concn) was added (curve ii).



C. Comparison of substrate specificities in the amine/INT reductase and amine/NAD reductase systems of S-pH5 MAO.

The acid fraction derived from the S-Mao was found to be more active in reducing INT in presence of added monoamine substrates than the S-MAO itself. NAD, which was obligatory in the amine/INT reductase system of S-MAO for oxidising some substrates in particular, isoamylamine, was found to be dispensable when the acid fraction was assayed for its ability to reduce INT. Indeed, the presence of NAD in the reaction mixture caused the inhibition of tryptamine oxidation, though that of isoamylamine and tyramine was still twice stimulated in some experiments. When the amine/INT reductase activity is compared with that of NAD reduction, it would appear that the two systems are not equivalent. The reduction of NAD was linear for at least 30 minutes; and isoamylamine and β-phenylethylamine were the best substrates for the system. For the INT reductase system, however, tryptamine which, incidentally, was the poorest substrate (apart from benzylamine) for NAD reduction, was consistently the best substrate for tetrazolium reduction. Whatever the reasons for such discrepancies, it is apparent that NAD can act as a hydrogen carrier under the experimental conditions and could conceivably act as an intermediate carrier in tetrazolium reduction.

Table XIV compares the results of the two oxide reductase systems. A comparison of tables XIV(B) and IX will reveal that there are apparent differences between S-MAO and its acid fraction. Table XIV(B) shows that in the presence of added NAD the enzyme activity with tyramine as substrate was twice that of tryptamine while in table IX the same value was obtained for both substrates.

TABLE XIV. SUBSTRATE SPECIFICITIES OF ACID FRACTION OF S-MAO PREPARATION IN TWO OXIDOREDUCTASE SYSTEMS (A & B)

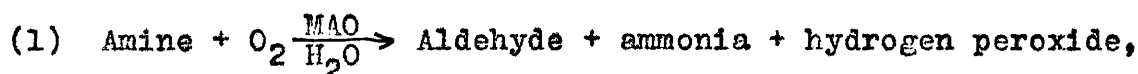
Oxidoreductase system	Substrate	Addition	Activity in μmoles per 100 mg protein per hour
A[x] Amine/NAD reductase	1. Isoamylamine	-	32.5
	2. β-Phenylethylamine	-	33.1
	3. Tyramine	-	16.2
	4. Serotonin	-	10.0
	5. Tryptamine	-	9.2
B[xx] Amine/INT reductase	1. Isoamylamine	-	9.0
		0.15mM NAD	24.2
	2. β-Phenylethylamine	-	12.4
		0.15mM NAD	16.2
	3. Tyramine	-	23.9
		0.15mM NAD	51.9
	4. Serotonin	-	18.2
		0.15mM NAD	18.6
	5. Tryptamine	-	27.1
		0.15mM NAD	24.8

[x] activity was expressed μ moles NADH

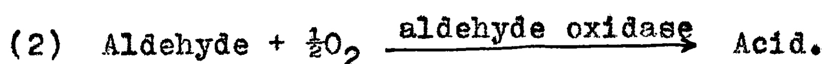
[xx] activity was expressed μ moles formazan.

D. The reduction of molecular oxygen by S and P-MAO preparations. The Warburg manometric technique.

Table XV shows the results obtained when MAO activity was assayed by the conventional Warburg manometric technique, measuring oxygen uptake by the soluble and particulate MAO preparations. The oxidation of serotonin was about 150 per cent stimulated by 0.15mM NAD (final concn.). NAD, however, did not stimulate the oxidation of any of the other substrates. It was, therefore, presumed from the results (of table XIV) that the aldehyde intermediates were further oxidised since 0.016M semicarbazide (final concn.) inhibited the oxygen uptake; and in the case of the last three substrates (tyramine, tryptamine and serotonin), it also abolished pigment formation. The degree of inhibition, as measured by following the reaction to completion, clearly indicated that an atom of oxygen was used up per molecule of substrate oxidised according to the following equation:-



(1) Amine + $O_2 \xrightarrow[H_2O]{MAO}$ Aldehyde + ammonia + hydrogen peroxide,



(2) Aldehyde + $\frac{1}{2}O_2 \xrightarrow{\text{aldehyde oxidase}}$ Acid.

(3) $H_2O_2 \xrightarrow{\text{catalase}} \frac{1}{2}O_2 \quad H_2O$

∴ Net O_2 uptake = sum of eqn 1+2 - eqn 3
= $(1.5 - 0.5\ O_2) = O_2$, i.e. two atoms of oxygen.

The inhibition of oxygen uptake during the oxidation of isoamylamine contradicted the result obtained for the particulate MAO in that semicarbazide did not inhibit oxygen uptake in that system (cf table XV). Richter(15) and Weiner(16) had

shown earlier that semicarbazide had no observable inhibitory effect on oxygen consumption of guinea pig liver MAO homogenates when isoamylamine oxidation was measured. Weiner believed this might probably mean that isovaleraldehyde was not further metabolised. But the fact that isoamyl alcohol is readily detected by the smell in manometric techniques suggests that thereason for the discrepancy may be more complex than hitherto anticipated.

It may be possible that these seemingly contradictory results for oxygen uptake by the soluble and particulate MAO extracts are in fact indications that different pathways operate in the two systems for isoamylamine metabolism. Similarly in the particulate system the metabolic pathway of isoamylamine may differ from that of the other monoamines. It is also likely that the semicarbazide was directly inhibiting the S-MAO.

TABLE XV. THE EFFECTS OF SEMICARBAZIDE ON THE OXYGEN UPTAKE BY S-MAO.

Substrate	Additive	Final molar conc. (M)	µ/moles oxygen/ 100 mg protein per hr.	Per cent inhibition
1. Isoamylamine	-		9.4(49.40)	-
	Semi-carbazide	1.6×10^{-2}	3.75(49.36)	60
2. Tyramine	-		11.0	-
	Semi-carbazide	1.6×10^{-2}	4.59	58
3. Tryptamine	-		8.76	-
	Semi-carbazide	1.6×10^{-2}	4.16	52
4. Serotonin*	-		11.8	
	NAD	5×10^{-4}	31.3	165 *

Data in brackets represent oxygen uptake values by the particulate fraction of the same enzyme preparation.

* Semicarbazide was absent from the reaction mitures.

Legend. Soluble(s) MAO was prepared from liver homogenate as described under Methods.

For details of reaction mixture see Methods.

E. <u>The effects of iproniazid (IIH) and tranylcypromine (TCP) on MAO.</u>

1. <u>Amine/INT reductase systems of S and P MAO preparations.</u>

It was reported by Davison(66) and Pletcher(52) that IIH may require aerobic preincubation for at least 10 minutes before it can effectively inhibit MAO. Pletcher stated that while this is true for IIH, TCP may not require such treatment when certain MAO substrates are used. It was, therefore, of some interest to study the effects of the inhibitors on the enzyme preparations in the tetrazolium assay system.

Table XVI (A & B) summarises the results obtained under the specified conditions of assay.

It is clear from table XVI that the inhibitors strongly inhibited the enzyme activities of both preparations on aerobic preincubation. The soluble enzyme was especially sensitive to the inhibitors since the enzymic INT reduction by the amines was abolished by the inhibitors after the preincubation. It is also clear from the table that without aerobic preincubation, the enzyme activity was hardly affected by IIH, while TCP showed a definite inhibition (isoamylamine, 80 per cent; tyramine, 76 per cent; tryptamine, 60 per cent). This then confirms the results of Davison and Pletcher who measured MAO activity by other methods of assay. It also means that the soluble enzyme was MAO and similar if not identical to the particulate enzyme. The data also showed clearly that tryptamine oxidation by the system (particulate) was the most resistant to the effects of the inhibitors. It should be noted that owing to the weak enzyme activity (S-MAO) with tyramine and isoamylamine as

substrates, NAD was also included in the system. This, however, did not alter the results.

TABLE XVI(A). THE EFFECTS OF IPRONIAZID (IIH) AND TRANYLCYPROMINE (TCP) ON INT REDUCTION BY PARTICULATE MAO IN THE PRESENCE OF AMINES.

Substrate	Inhibitor	Final molar conc. (M)	Aerobic pre-incubation (10 min)	μ moles formazan /100 mg protein/ hr.	% inhibition
1. Isoamylamine	-		-	33.7	-
	IIH	2.5x10^{-3}	-	32.4	4
	"	"	+	1.5	96
2. Tyramine	-		-	80.7	-
	IIH	2.5x10^{-3}	-	80.0	1
	"	"	+	10.7	87
3. Tryptamine	-		-	121	-
	IIH	2.5x10^{-3}	-	121	0
	"	"	+	25.6	79
1. Isoamylamine	-		-	33.7	
	TCP	2.5x10^{-3}	-	6.6	80
	"	"	+	0.0	100
2. Tyramine	-		-	80.7	-
	TCP	2.5x10^{-3}	-	19.1	76
	"	"	+	2.93	96
3. Tryptamine	-		-	121	-
	TCP	2.5x10^{-3}	-	48.4	60
	"	"	+	5.13	96

NAD was present in experiments with isoamylamine and tyramine.

TABLE XVI(B).* THE EFFECTS OF IPRONIAZID (IIH) AND TRANYLCYPROMINE (TCP) ON INT REDUCTION BY SOLUBLE(S) MAO IN THE PRESENCE OF AMINES.

Substrate	0.15mM NAD	Inhibitor	Final molar conc. (M)	μ moles formazan 100 mg protein/hr.	% inhibition
1. Isoamyl-amine	-	-		4.2	-
	-	IIH	$2.5x10^{-3}$	0.0	100
	+	-		25.5	-
	+	IIH	$2.5x10^{-3}$	0.0	100
2. Tyramine	-	-		5.7	-
	-	IIH	$2.5x10^{-3}$	0.0	100
	+	-		23.0	-
	+	IIH	$2.5x10^{-3}$	0.0	100
3. Trypt-amine	-	-		23.3	-
	-	IIH	$2.5x10^{-3}$	0.0	100
		-			
1. Isoamyl-amine	-	-		3.86	-
	-	TCP	$2.5x10^{-3}$	0.00	100
	+	-		28.5	-
	+	TCP	$2.5x10^{-3}$	0.00	100
2. Tyramine	-	-		6.0	-
	-	TCP	$2.5x10^{-3}$	0.0	100
	+	-		24.4	-
	+	TCP	$2.5x10^{-3}$	0.0	100
3. Trypt-amine	-	-		24.5	-
	-	TCP	$2.5x10^{-3}$	0.0	100

* There was 10 minutes aerobic preincubation in all experiments in this section.

E. 2. Amine/NAD reductase system of S-pH5 MAO.

The enzymic NAD reduction by amines was strongly inhibited by 1.67 10^{-3} M (final concentrations) of IIH or TCP when it was preincubated 10 minutes at room temperature with the reaction mixture in the absence of substrate. Under this condition, serotonin and isoamylamine oxidation was completely abolished by either of the inhibitors, while that of tyramine or tryptamine was strongly inhibited. ~~when NAD reductase activity was measured with the substrates~~. Unlike amine/INT reductase system which required aerobic preincubation of IIH and the enzyme at 37°C before inhibition of the enzyme activity could be shown, the amine/NAD reductase system could be inhibited at least 25 per cent by IIH without aerobic preincubation. This would mean that the two systems were not equivalent. This also suggests that amine/NAD reductase system is more sensitive to IIH effect than amine/INT reductase system.

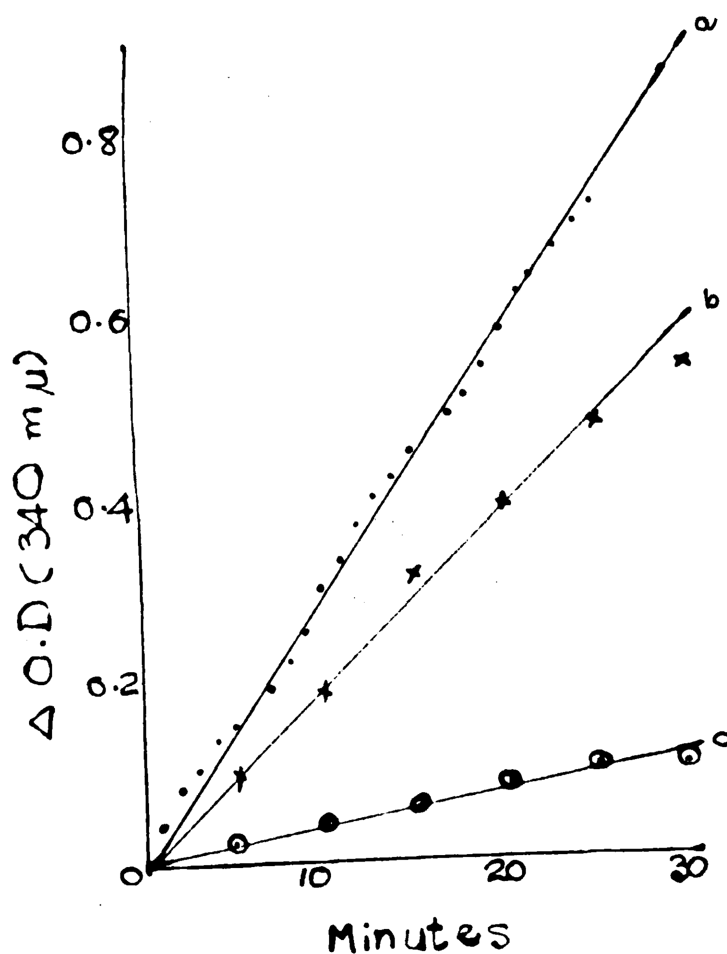
The enzymic/NAD reduction by acetaldehyde was not affected by 1.67×10^{-3} (final conc.) of IIH. The effects of IIH on the enzymic-INT reduction by acetaldehyde was not so consistent. There were contradictory results on several occasions, in other words, INT reduction by the same enzyme preparation with acetaldehyde as substrate was inhibited in certain experiments while under the same experimental conditions, stimulation was observed in certain cases with IIH as the inhibitor.

Fig. 6 shows the effects of these inhibitors on tyramine oxidation in the amine/NAD reductase system.

FIG. 6. THE EFFECTS OF MAO INHIBITORS ON THE NAD REDUCTION BY S-pH5 MAO IN THE PRESENCE OF TYRAMINE.

The complete reaction mixture contained in 3.0 ml of solution, 6.7 μmoles tyramine (neutralised); 8.367 μmoles sodium phosphate buffer, pH 8.2; 1.06 μmoles NAD; 12 μmoles nicotinamide; 0.25 ml enzyme (S-pH5 MAO, about 4 mg protein); and water to make up the final volume. Water replaced the substrate in blanks.

For the inhibition experiments (curves b and c), either iproniazid (IIH)(b) or tranylcypromine (TCP)(c) was present in a final concentration of 1.67×10^{-3}M.



F. Some factors affecting endogenous reduction of INT by MAO preparations.

During the inhibitor studies just described, there was a consistent reduction of INT by endogenous substrates in the tissue preparations used. The endogenous INT reduction by the soluble (S) and particulate (P) enzyme preparations were stimulated by iproniazid, a known potent MAO inhibitor. This was especially pronounced when the control mixture (substrate blank) contained INT and iproniazid during the preincubation period. In the case of isoamylamine, for example, the OD observed for a completely inhibited reaction was less than the OD obtained for the endogenous INT reduction in the presence of iproniazid. This iproniazid stimulated endogenous INT reduction by the tissue preparations was enhanced by added NAD at the final concentration tested (0.15mM). The stimulatory effects were in fact purely additive, in other words, the combined stimulation by IIH and NAD was the sum of the individual effects. Parnate did not raise the endogenous INT reduction, nor did NAD affect the reaction of parnate with the enzyme extracts in its ability to inhibit the endogenous tetrazolium reduction. Parnate is claimed to act chemically on MAO, that is to say parnate is said to be the effective species which inhibits the enzyme(52,58). It was found that by preincubating the extracts with iproniazid in the absence of the dye, INT, the endogenous tetrazolium reduction was greatly lessened both in the presence and the absence of the inhibitor. The extra OD due to NAD alone was similarly suppressed by the inhibitors. From these observations it would appear that there

are some endogenous substrates which can reduce INT. This reaction was stimulated by iproniazid which, according to Davison's postulate, hydrolyses to form a highly oxidised compound which is the effective inhibitor(66). It will be of some interest to state that a number of workers have recently detected a volatile MAO inhibitor formed by iproniad (56,57). Since undialysed enzyme preparations were found in a preliminary study to greatly stimulate endogenous INT reduction, all the extracts assayed in the experiments described were carried out on overnight dialysed preparations in several changes of 0.01 M sodium phosphate buffer (pH 7.6).

Data for the experiments are presented in table XVII.

TABLE XVII. THE EFFECTS OF VARIOUS TREATMENTS AND MAO INHIBITORS ON THE ENDOGENOUS INT REDUCTION BY SOLUBLE(S) AND PARTICULATE (P) MAO PREPARATIONS.

Tissue preparation	Treatment	Activity in µ moles formazan/100mg protein/hr.			
		Control	NAD 0.15mM	iproniazid (IIH) (0.025M)	NAD/IIH (0.15mM) (0.15mM)
SOLUBLE(S) ENZYME	Preincubation with inhibitor and dye, INT	1.80	4.32	6.3	9.2
	% stimulation	-	140	250	411
S-MAO	Preincubation in the absence of dye	1.8	2.48	3.38	4.51
	% stimulation	-	38	88	151
		Control	NAD 0.15mM	Parnate (0.025M)	NAD/parnate (0.15mM) (0.025M)
S-MAO	Preincubation with inhibitor and dye	1.75	2.08	1.73	1.74
	% stimulation		19	-	-
		Control	NAD	IIH (0.025M)	NAD/IIH
P-MAO	Preincubation with inhibitor and the dye	8.2	12.5	16.8	18.3
	% stimulation	-	52	107	123
		Control	NAD	IIH	NAD/IIH
P-MAO	Preincubation with inhibitor in the absence of dye	8.1	11.8	8.3	8.2
	% stimulation	-	46	3	1

From these data, it is clear that the percentage stimulation of the endogenous INT reduction was greater in the soluble fraction than in the particulate. However, from preliminary experiments, it was found that by freezing and thawing the endogenous enzyme activity was greatly decreased. This process also decreased the affinity of the enzyme for the substrates in the amine/INT reductase system. Thus while it became increasingly difficult to demonstrate the oxidation of some amines, e.g. tyramine, by this method, that of tryptamine oxidation was comparatively easy to show. Oxygen uptake measurement was not markedly affected. This then would suggest the presence in tissues of a tetrazolium reductase pathway for the amines which may be affected by ageing. The pathway appeared to differ for the amines. Iproniazid, a known MAO inhibitor, could act as a fairly good substrate for the system under certain conditions.

SECTION II: THE EFFECTS OF SONICATION ON THE ACTIVITY OF SOLUBLE MAO PREPARATIONS FROM GUINEA PIG LIVER.

In the series of experiments described in the preceding section soluble MAO preparations were derived from high speed centrifugation of hypotonic, aqueous tissue extracts (cf. Methods). The "soluble" enzyme present in such high-speed supernatants could have arisen partly at least from lysed and fragmented mitochondria and microsomes, which are known to contain about 70 and 20 per cent, respectively, of the total MAO activity of sucrose homogenates of guinea pig liver(36). Indeed it was shown by Seiden and Westley(95) that sonication of rat brain mitochondrial suspensions in phosphate buffer released a considerable proportion of the MAO into solution which did not sediment after centrifugation at 100,000g for an hour. More recently Gorkin(38) reported similar MAO preparations. In the following experiments to be described, therefore, the preparation of soluble MAO from guinea pig liver was modified to include, after homogenisation, a sonication step. This was done as an attempt to obtain more enzyme.

Procedure: The intital homogenate was sonicated (at 0-4°C) by high frequency oscillations (20 kilocycles per second) (1.0 - 1.2 mAmps) for 30 minutes in an M.S.E. Mullard 60 watt ultrasonic disintegrator with a 1/8" diameter probe. The treatment was interrupted at 5 minute intervals to avoid overheating. All subsequent steps in the enzyme preparation (e.g. centrifugation speeds and ammonium sulphate fractionations) were as already described under "Methods".

The soluble MAO preparation obtained after sonication will be referred to as "S.S" or "S.Sonicated MAO", and its corresponding acid-treated derivative will be labelled as "S.S-pH5". Similarly the particulate preparations derived

from the sonicated homogenates will be designated as "P.S" or "P sonic MAO".

The ability of the soluble extracts derived from sonicated homogenates to catalyse the enzymic reduction of INT, NAD and molecular oxygen by monoamines will now be described and compared to the data for the unsonicated homogenate extracts given in the preceding section.

The reduction of INT, NAD and oxygen by soluble (S.S and SS-pH5) MAO preparations (derived from sonicated homogenates) by monoamines.

A. The effects of sonication on Amine/INT reductase activity of:-

1. S.S.MAO: It was found that sonication of the homogenate resulted in a marked increase in the amine/INT reductase activity of the soluble fractions derived from it with all the substrates tested. From the data shown in table XVIII, it is clear that sonication had little or no effect on the amine/INT reductase activity of either the homogenate or the high-speed residue (P Sonic) derived from it when tryptamine was tested as substrate. The negative value obtained for tyramine in the (unsonicated) homogenate-tyramine/INT reductase system was caused by a high endogenous value, coupled to the observation that there was a relatively long lagphase (not less than 5 minutes) for INT reduction in the presence of tyramine, whereas dye reduction in the absence of added substrate showed no lagphase. Sonication reduced the lagphase during tyramine oxidation to about 2 minutes. Similarly, the data in table XVIII show that the endogenous rates for the

TABLE XVIII. AMINE/INT REDUCTASE ACTIVITY OF MONOAMINE OXIDASE PREPARATIONS DERIVED FROM A TYPICALLY SONICATED LIVER HOMOGENATE OF GUINEA PIG.

Tissue preparation	Sonication	Added substrate	μ moles formazan/100mg protein/hr.	μ mole extra formazan/100mg protein/hr.
Homogenate	-	None	45.0	-
"	-	Tyramine	40.0	-5 *
"	-	Tryptamine	87.8	42.8
"	+	None	40.0	-
"	+	Tyramine	42.71	2.71
"	+	Tryptamine	98.1	58.1
Soluble MAO	-	None	3.68	-
	-	Tyramine	14.98	11.3 (55.6)
"	-	Tryptamine	43.28	39.6 (39.6)
"	+	None	1.64	-
"	+	Tyramine	68.34	66.7 (70.0)
"	+	Tryptamine	140.64	139.0 (139)
High speed residue	-	None	8.1	-
	-	Tyramine	41.9	33.8
	-	Tryptamine	105.1	97
"	+	None	8.1	-
"	+	Tyramine	41.9	33.8
"	+	Tryptamine	112.1	104.0

\+ refers to preparations derived from sonicated homogenate or to the homogenate itself.

\- similarly refers to unsonicated homogenate derivatives or to the actual homogenate itself.

* A negative value was obtained for this substrate.

Data in brackets represent values obtained in the presence of 0.15mM NAD (final conc^{n}.)

The original homogenate was divided into two parts, one of which was sonicated. Thus all enzyme preparations were derived from a single homogenate.

[illegible]: All the preparations were dialysed overnight in the cold against several changes of 0.01M sodium phosphate buffer, pH 7.6. For reaction mixture, etc. see Methods.

S-MAO derived from untreated homogenate was twice that obtained for a similar fraction derived from a typically sonicated homogenate. In each experiment, sonicated and control enzyme preparations were derived from the same initial tissue extract.

The observations on the endogenous rates of INT reduction would suggest that sonication may disrupt a system present in the liver tissue of guinea pig, capable of stimulating endogenous INT reduction, or may cause the inactivation of a carrier system responsible for endogenous INT reduction.

From data given (see table XVIII), it is obvious that the ability of the soluble (S.S.) MAO derived from the sonicated homogenate to catalyse NAD-stimulated reduction of INT by tyramine, had been either considerably reduced or practically abolished. It was also found in other experiments that not only did sonication decrease the NAD requirement by the amine/INT reductase system for oxidising some amines including tyramine, but it also decreased the NAD stimulation of endogenous INT reduction. Thus while the data in table XVII show that the endogenous reduction of INT could be about 140 per cent stimulated by NAD, the results reported in table XVIII show that NAD stimulation of endogenous INT reduction by the S.S-MAO preparation was not more than 2 per cent while that of the S-MAO was 143 per cent.

Further reference to the data (table XVIII) will reveal that the enrichment, in terms of specific activity, of the S.S.MAO by sonication of the homogenate was such that in the absence of added NAD, the enzymic reduction of INT by tyramine was increased almost six-fold as compared to that of the S-MAO,

while that of tryptamine oxidation was correspondingly increased about four-fold. It may be of interest to state that the increase in specific activity caused by sonication was not parallel to the amount of protein released from the homogenate into the soluble fraction. Thus in the typical experiment from which the data for table XVIII were compiled, while the specific activity (S.A.) of the S.S-MAO was 139 μmoles formazan/100mg protein/hr and that of the S.MAO was 39.6 μmoles formazan/100mg protein/hr with tryptamine as substrate, the protein concentrations of these two fractions were respectively 15.5mg and 12.8mg per ml enzyme. This result and similar observations would suggest that the sonication of the homogenate caused redistribution of enzyme activity resulting in the enrichment of the soluble fractions derived from them.

Before describing observations on the amine-INT reductase activity of the acid fraction of the S.S.-MAO, some variations in the ability of the S.S.MAO itself to reduce INT by amines in the absence of exogenous NAD will be described.

Though the S.S.-MAO did not normally show any dependence on added NAD when tyramine, isoamylamine or benzylamine were used as substrates this was not invariably the case. Thus, occasional preparations exhibited a moderate requirement for NAD for INT reduction in the presence or absence of added amine. This was never as pronounced as in the case of the S.MAO prepared from control (non-sonicated) homogenates. These preparations also displayed substrate specificities which were intermediate between those shown on the one hand, by the enzyme derived from control homogenates and that

from experimental (sonicated) homogenates. Such anomalies were found to be caused by incomplete sonication (see later, table XXIII).

The data in table XIX show that the indoleamines (serotonin and tryptamine) were the least dependent on NAD in the amine/INT reductase system of S.S MAO. It is also apparent that though benzylamine oxidation was very weakly stimulated by NAD in the former system (S.MAO), it was about thrice stimulated in the sonicated system. The unusual behaviour of benzylamine cannot be explained. However, the presence of a specific benzylamine oxidase(23,65) might account for this observation.

TABLE XIX. THE EFFECTS OF NAD ON AMINE/INT REDUCTASE ACTIVITY OF SOLUBLE (S.S.) MAO PREPARATION DERIVED FROM SONICATED HOMOGENATE

Added substrate	Additive	Final molar concn. (M)	µ moles formazan/ 100mg protein/hr.	µ moles extra formazan/ 100mg protein/ hr.	% stimulation
1 None	-		1.80	-	-
"	NAD	1.5×10^{-4}	2.5	0.7	39
2 Isoamyl-amine	-		29.8	28.0	-
	NAD	1.5×10^{-4}	46.7	44.2	58 (940)
3 Benzyl-amine	-		9.47	7.67	-
	NAD	1.5×10^{-4}	21.40	18.9	146 (50)
4 Tyramine	-		34.3	32.5	-
	NAD	1.5×10^{-4}	54.8	52.3	61 (421)
5 Serotonin	-		43.30	41.5	-
	NAD	1.5×10^{-4}	44.0	41.5	0 (135)
6 Tryptamine	-		81.9	80.1	-
	NAD	1.5×10^{-4}	82.4	79.9	0

"Extra" formazan values represent values due to stimulation of enzymic INT reduction by added substrate less those obtained in the absence of exogenous amines (in the case of the endogenous activity extra formazan value represent NAD stimulation).

Data in brackets were the percentage stimulation caused by added NAD as was reported in table IX.

2. S.S-pH5 Fraction.

The S.S-pH5 fraction was very active in reducing tetrazolium by amines. The inability of NAD to stimulate enzymic INT reduction by amines was absolute for this fraction and its ability to stimulate endogenous tetrazolium reduction was equally abolished by the sonic treatment of the homogenate. These observations were clearly different from those made on fractions derived from non-sonicated homogenates, on the one hand, and also from the occasional findings on the S.S. MAO of which the S.S-pH5 enzyme was a direct derivative, on the other hand. Table XX (A and B) compares the substrate specificities of the S.S.MAO and its S.S-pH5 fraction.

From the data presented, it is clear that there was a slight specific activity increase in enzyme activity in the acid fraction over that of the S.S.MAO. From the foregoing report, it may be inferred that not only did sonication of the homogenate result in the specific activity increase in the derived soluble fractions through enrichment and redistribution of enzyme but it also altered the requirement for NAD both in the amine/INT reductase system and in the endogenous INT reductase system, as was found in table XIX above.

TABLE XX (A & B). THE EFFECTS OF NAD ON AMINE/INT REDUCTASE ACTIVITIES OF SOLUBLE MAO PREPARATIONS OBTAINED FROM A SONICATED HOMOGENATE

μ moles formazan/100 mg protein/hr.

Substrate	(A) Fraction S.S.MAO		(B) Fraction S.S.-pH5 MAO	
	NAD (-)	NAD (+)	NAD (-)	NAD (+)
1 Isoamylamine	30.0	45.0 (940)	32.6	33.0 (147)
2 Benzylamine	10.2	23.0 (5)		
3 Tyramine	66.7	70.0 (421)	69.9	69.0 (117)
4 b-Phenylethylamine	41.5	40.2 (181)	43.4	43.2 (31)
5 Serotonin	84.0	83.9 (135)	90.0	90.0 (0)
6 Tryptamine	139.0	139.0 (2)	142.0	142.0 (-)

Data in brackets represent percentage NAD stimulation of similar fractions derived from unsonicated homogenate data from the preceding section (section 1).

3. The effects of substrate concentration on amine/INT reductase activity of soluble and particulate MAO preparations from guinea pig liver.

The effects of tryptamine and tyramine concentrations on the various enzyme preparations were investigated. In view of the weak enzyme (S-MAO) activity with tyramine, NAD 0.15mM (final conc^{n}.) was added to the system. Since NAD did not stimulate INT reductase activity of the particulate or the S.S.MAO preparations with tryptamine and tyramine as substrates NAD was excluded in experiments involving these preparations. Generally, tryptamine appeared to inhibit the S.S.MAO at high concentrations. Tryptamine added at high concentrations (between 10^{-3} and xx10^{-2} M final) also caused an immediate turbidity when added to the incubation mixture. This was not observed for any other preparations. Tyramine oxidation by the S.S.MAO was sluggish at 20×10^{-3}M (final conc.) and below. At 0.055M (final conc.) well above the range when tryptamine became inhibitory (see fig. 7), tyramine oxidation by the S.S.MAO became more vigorous. This was, however, similar to what was observed for the S.MAO preparation with the same substrate in the presence of added NAD. The apparent Km determined for tryptamine and tyramine oxidation by the various enzyme preparations are summarised in table XXI. The Km for tyramine oxidation by the S-MAO (in the presence of added NAD) as determined from S X V plots was 8×10^{-4}M. This was in good agreement with the Km for that substrate when Oswald and Strittmatter(36) determined it manometrically measuring oxygen consumption by rat liver

particulate enzyme. (These authors obtained a Km of $8.5x10^{-4}$ M). The Km of tyramine from reciprocal ($\frac{1}{v} x \frac{1}{s}$) plots, however, was $38.5x10^{-4}$M with the S-MAO of guinea pig liver. That for the S.S.MAO in the INT reductase assay system from S X V plots was $27x10^{-3}$M while the $\frac{1}{v} x \frac{1}{s}$ plots gave $40x10^{-3}$M. In the absence of added NAD, this may mean the affinity of the soluble enzyme (i.e. sonicated homogenate derivative) for tyramine was considerably reduced. It may also mean that the two soluble enzyme (S-MAO and S.S.MAO) preparations were not equivalent.

Tryptamine showed several peaks or plateaus with the S.S.MAO. There was a plateau well below $5x10^{-4}$M tryptamine concentration (final). The Km for that range from S X V plots was $2x10^{-4}$M while reciprocal ($\frac{1}{v} x \frac{1}{s}$) plots gave $5x10^{-5}$M. The Km for the range shown in fig. 7 will be found in table XXI. This value did not differ much from that obtained for the particulate enzyme or from those obtained for tyramine. In spite of the complexities of the tetrazolium assay it is apparent that the Km values for the substrates were comparable with those obtained from oxygen uptake measurements by other workers(19,36).

It is, however, necessary to state that these apparent values may not necessarily reflect the enzyme activity in the amine dehydrogenating system; in other words, these values may not be assumed to be specifically for amine dehydrogenation. For example, the reaction measured may be a second step which may be rate limiting. This rate limiting step may either be the oxidation of aldehyde by INT or it may involve a carrier

in the INT reductase system. None of these questions could be answered or correctly assessed since the mechanism of INT reduction by the system is not fully characterised.

It will be seen from table XXI the high Km values obtained for tyramine oxidation by the S.S.MAO as compared to what were obtained for it with the S.MAO preparation in the presence of added NAD. The value for the S.MAO was ten-fold lower than what was obtained for the S.S.MAO (reciprocal plots). However, the Km for tryptamine oxidation by the S.MAO was 47 fold higher than what was found for its oxidation by the S.S.MAO preparation. These results are suggestive that the properties of the two preparations towards the substrates had been reversed probably as the result of the sonication of the homogenates from which the S.S.MAO preparations were derived.

It is interesting to note that in spite of these high Km values NAD was generally not required to stimulate enzymic INT reduction by the substrates with such preparations.

FIG. 7. THE EFFECTS OF SUBSTRATE CONCENTRATIONS ON AMINE/INT REDUCTASE ACTIVITY OF SOLUBLE AND PARTICULATE MAO PREPARATIONS.

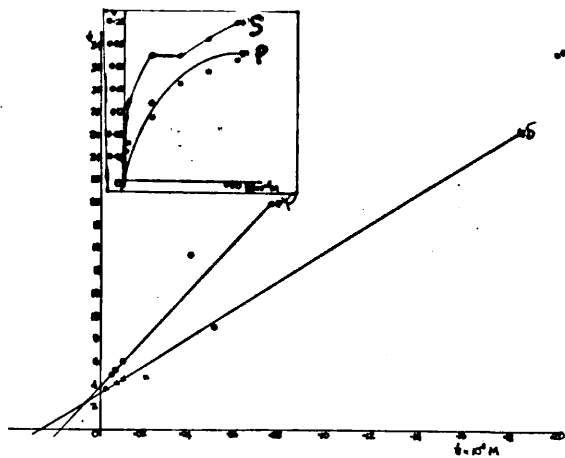
The complete model reaction mixture contained in 2.0ml of solution, 50 μmoles sodium phosphate buffer, pH 7.6; 1.2 μmoles INT; enzyme up to 0.5ml (about 4mg protein); and a series of (neutralised) substrate concentrations as indicated in the graphs (7a-e ii). Water was added as required to make up the final volume. Water also replaced the substrate in blanks.

The effects of tyramine concentrations on S MAO and P-MAO-INT systems are shown in fig. 7a in which S and P represent soluble and particulate respectively both for the inserts and the reciprocal plots. NAD 1.5×10^{-4}M (final conc^{n}) was incorporated in the S-MAO system of figure 7a.

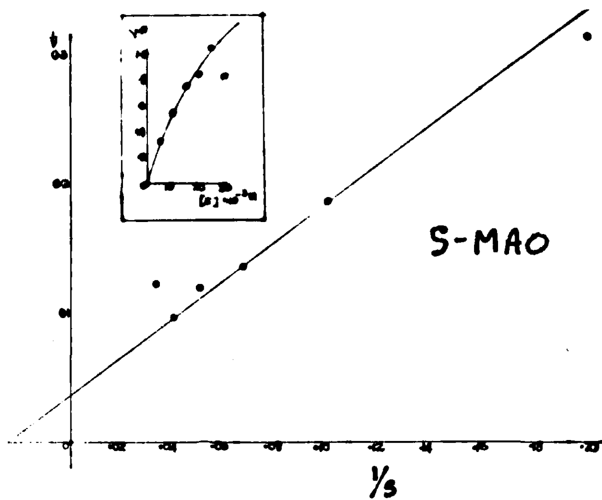
The effects of tryptamine concentrations on INT reductions by S-MAO and P-MAO are shown in figures 7 b and c respectively both for the inserts representing ordinary plots, and the reciprocal plots of both systems.

Fig. 7d shows the effects of tyramine concentrations on the S.S-MAO and INT reductase system (insert for ordinary plots).

Fig. 7e (i and ii) represent respectively the ordinary and reciprocal plots for tryptamine concentrations on S.S MAO-INT reductase system.



(a)



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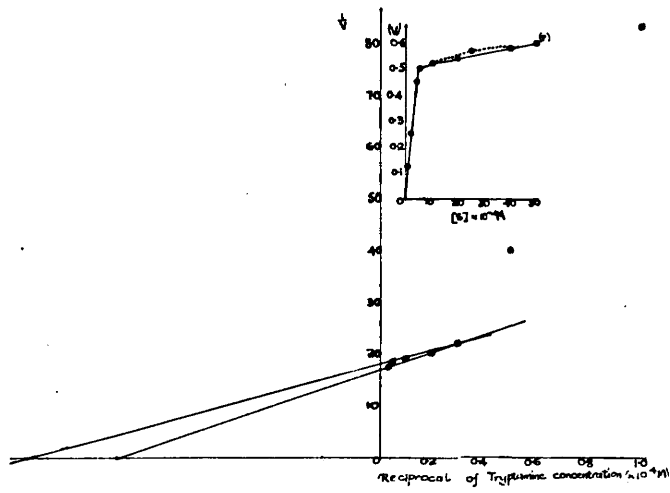
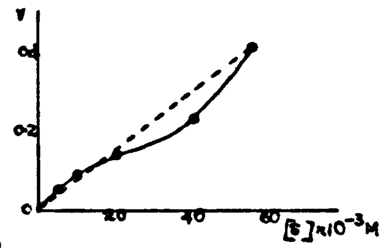
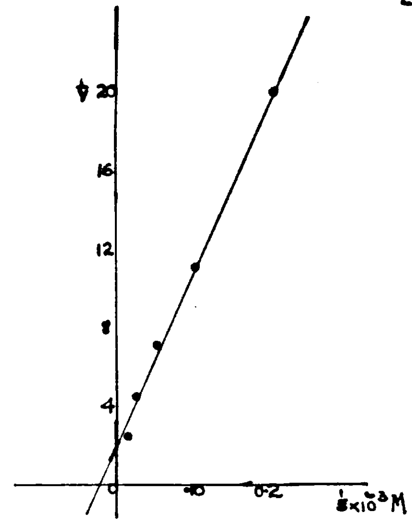
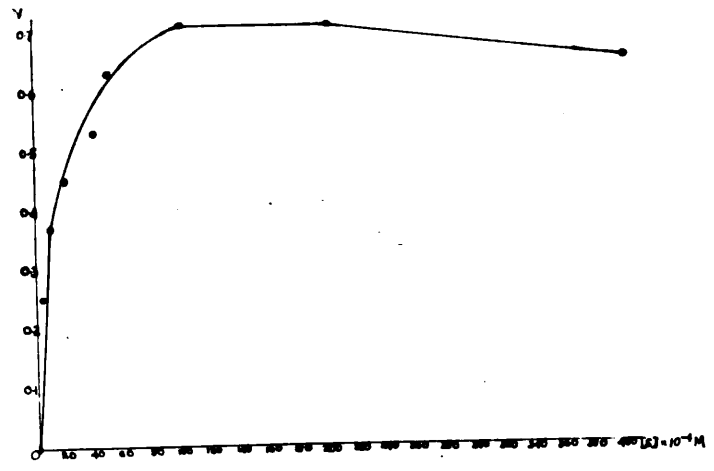


(c)

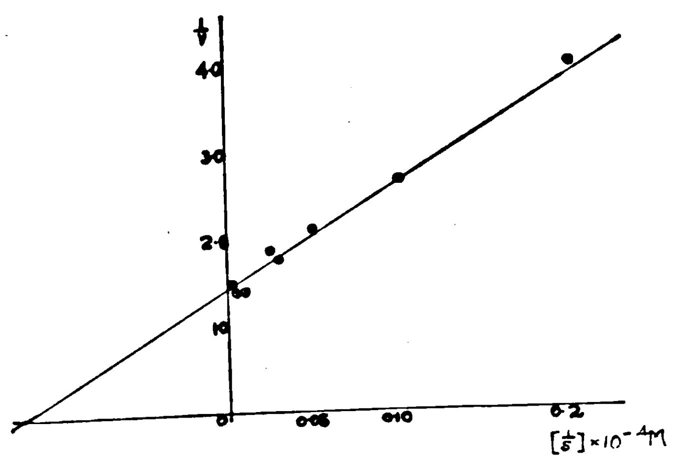




(d)



(e)(i)



(e)(ii)

TABLE XXI. KINETIC CONSTANTS FOR THE EFFECTS OF SUBSTRATE CONCENTRATION ON AMINE/INT REDUCTASE ACTIVITY OF MAO PREPARATIONS FROM GUINEA PIG LIVER.

		Km(x10^{-4}M)		Vmax.	
Tissue preparation	Substrate	From reciprocal ($\frac{1}{s}/\frac{1}{v}$ plots	From SXV plots	From reciprocal plots	From SXV plots
S.MAO	Tyramine	38.5 (NAD^+)	8 (NAD^+)	0.31	
P.MAO	Tyramine	58.8		0.27	
S.S.MAO	Tyramine	400	270	0.46	.41
S.MAO	Tryptamine	400	100	0.278	0.112
P.MAO	Tryptamine	Variable			
		0.741		0.556	
		1.0		0.597	
		4.54	4.5	0.67	0.60
S.S.MAO	Tryptamine	8.51	10	0.69	0.71

B: Amine/NAD reductase activity of S.S.MAO and SS-pH5 preparations from sonicated homogenates.

It was found in the first section of this thesis that, generally, those amines which required added NAD to reduce INT enzymically were also the best substrates for the NAD assays. Conversely, those amines which were the best substrates for the amine/INT reductase system in the absence of NAD were also the poorest substrates for NAD reduction (see tables IX, XI, XIII). The soluble enzymes, S.MAO and its acid fraction, it may be recalled, were prepared from unsonicated homogenates (cf. under Methods).

So far, in this present section, it has been learned from the data already presented (cf. tables XVIII-XXI), that the S.S.MAO derived from sonically treated homogenates no longer required NAD for effective INT reduction by amines. It has also been noted so far that the INT-reductase activity of the acid fraction of S.S.MAO was completely unaffected by added NAD. If the hypothesis that the NAD stimulated amine/INT reductase activity paralleled the amine/NAD reductase activity was correct, then the soluble enzymes derived from sonically treated homogenates would reduce NAD very poorly in the presence of amines. It was, therefore, desirable to test this.

Amine/NAD reductase assays were carried out with the standard reaction mixtures described in the first section of this thesis (see section 1, No. 5, para. 3). Table XXII shows data obtained for soluble MAO preparations from a typically sonicated homogenate.

From table XXII, it is clear that both soluble fractions

TABLE XXII (A & B). AMINE/NAD REDUCTASE ACTIVITY OF SOLUBLE (S.S. & S.S-pH5) MAO PREPARATIONS FROM SONICATED HOMOGENATE.

[illegible]: Guinea pig liver homogenate was sonicated as described in the text. For details of extraction, etc. see the Methods section. For reaction mixtures, etc. see text.

Substrate	μmoles NADH/100mg protein/hour (A) S.S.MAO	(B) S.S-pH5 MAO
1 Tryptamine	15.2	40.6 (9.27)
2 β-Phenylethylamine	5.2	5.3 (33.2)
3 Tyramine	3.48	3.48 (16.2)
4 Serotonin	3.48	
5 Isoamylamine	2.61	2.3 (32.5)
6 Benzylamine	0.87	

Data in brackets are for control unsonicated preparations of a similar fraction.

were very active in reducing NAD in the presence of added amines. Furthermore, it is clear that the substrate specificities of the acid fractions, i.e. the SpH5 and S.SpH5 derived from unsonicated and sonicated homogenates respectively, had been altered markedly by sonication. Thus isoamylamine, β-phenylethylamine and tyramine, which were the best substrates for NAD reduction by the S-pH5 MAO (see tables XII and XIII), were no longer the best for the S.SpH5 system, as shown above. Conversely, tryptamine, the poorest substrate (apart from benzylamine) in the former system was persistently the best substrate for the S.SpH5 enzyme derived from sonicated homogenates.

It may be recalled that it was impossible to follow NAD reduction in sodium phosphate buffer, (pH 8.2) with the S-MAO derived from non-sonicated homogenates because of rapid reoxidation of reduced NAD by the system. It may be further recalled that in order to follow NAD reduction by amines with the S.MAO, sodium pyrophosphate buffer, (pH 8.8) was used to establish a more favourable pH equilibrium and/or system for NAD reduction. The S.S-MAO derived from sonicated homogenate, on the contrary, was suitable for reducing NAD by added amines in sodium phosphate buffer, pH 8.2. In fact, the data presented in table XXII were obtained in this buffer at pH 8.2.

Occasionally, however, some of the S.SMAO preparations were found to reoxidise NADH, though much less rapidly than S-MAO preparations derived from unsonicated homogenates. These S.S-MAO preparations also were found to show substrate

specificities intermediate between those obtained for the S.S-MAO and the S.MAO. It was, however, possible to follow NAD reduction at 340 nm with such preparations. Furthermore, they were found to be partly stimulated by added NAD in the amine/INT reductase system. The conditions of sonication were tested as follows. Liver homogenate was divided into two parts. One part was sonicated for 30 minutes and the other for half the time, as already described. The soluble MAO (S.S-MAO) prepared from these fractions were assayed for NAD reduction and INT reduction by amines. Data obtained in one such experiment for the NAD reduction assays are shown in table XXIII. From these data it is clear that effective sonication appeared to preferentially release an enzyme or enzyme systems from the particles which have greater affinity for tryptamine while the system(s) favourable for the oxidation of the other amines, e.g. isoamylamine and tyramine, were inactivated. This then would suggest that we might be dealing with two enzyme systems or with different isoenzymes of MAO, which differ in their sensitivity to sonication.

TABLE XXIII. THE EFFECTS OF THE TIME OF SONICATION OF HOMOGENATE ON AMINE/NAD REDUCTASE ACTIVITIES OF THE DERIVED SOLUBLE(S.S) MAO PREPARATIONS.

Sonication time	Substrate	μmoles NADH/100mg. protein/hr.
30 minutes	Tryptamine	18.5
"	Serotonin	12.2
"	Tyramine	4.2
"	Isoamylamine	3.5
15 minutes	Tryptamine	10.2
"	Serotonin	11.0
"	Tyramine	14.6
"	Isoamylamine	12.5

C: NAD reduction by S.S-MAO preparations by amines in sodium pyrophosphate buffer, pH 8.3.

The S.S-MAO preparations (from fully or partially sonicated homogenates) were also assayed in sodium pyrophosphate buffer, pH 8.8 for comparison. Table XXIV summarises data compiled from a typical experiment. From the table, it is obvious that the high pH neither affected the substrate specificities nor the enzyme activities. The only advantage of assays in pyrophosphate buffer (pH 8.8) was that the values obtained with partially sonicated homogenate# derivatives were more accurate since enzymic NADH oxidation at pH 8.8 was practically non-existent in such preparations and NAD reduction was quite linear as a function of time.

TABLE XXIV (A & B). NAD REDUCTION BY S.S-MAO PREPARATIONS BY AMINES IN SODIUM PYROPHOSPHATE BUFFER, pH 8.8.

μmoles NADH/100mg protein/hr.

Exp.	Substrate	State of sonication (A) Complete	(B) Incomplete	Buffer, pH
1	Tyramine	5.6	8.9	8.8
	Tryptamine	21.3	7.0	(Sodium pyro-phosphate)
	Serotonin	5.6	8.0	
2	Tyramine	5.58	8.7	8.2
	Tryptamine	21.4	7.05	(Sodium phosphate Na_2HPO_4/ NaH_2PO_4)
	Serotonin	5.5	8.0	

D: <u>The effects of sonication of the homogenate on NAD reduction by the derived S.S.MAO preparation in the presence of acetaldehyde.</u>

Aldehyde oxidase activity was shown in the previous section which dealt with non-sonicated enzyme extracts (cf. table IX). The acid fraction derived from unsonicated homogenate was relatively active in reducing NAD in the presence of acetaldehyde. It was found that soluble enzymes prepared from sonicated homogenates, however, were rather poor in aldehyde oxidase activity as judged from NAD reduction experiments. Aldehyde oxidase activity was detected in the first ten minutes only, within which a plateau was reached. Whether or not this observation was due to the equilibrium of the reaction:- (Aldehyde + NAD^+ $\leftrightarrow$ Acid + NADH)(Aldehyde + NADH $\leftrightarrow$ Alcohol + NAD^+), was not investigated. The absorbance read at 340mμ never exceeded 0.335 OD, even if measurements were continued for 60 minutes. This was almost half what was obtained for soluble enzymes prepared from control (non-sonicated) homogenates. Calculations based on not less than six experiments in each case, gave on the average 3.24 μmoles NADH and 5.41μ moles NADH per 100mg protein per hour for the S.S.MAO and the S-pH5 MAO preparations respectively. It may perhaps be of some interest to state that phenylacetaldehyde was a very poor substrate for both preparations in the NAD and INT reduction assays. These findings partly confirm the findings of Weissbach <u>et al</u>.(35) who observed very little aldehyde oxidase activity in the S-pH5 MAO of guinea pig liver. These authors, however, did not extend their studies to soluble

fractions derived from sonicated homogenates.

E: Oxygen uptake by monoamine oxidase preparations derived from sonicated homogenates.

Soluble (S.S) enzyme preparations derived from sonically treated homogenates were considerably enriched in MAO when the activity was estimated manometrically; see data in table XXV.

The effect of sonication was restricted to the activity of the derived soluble preparation as shown. However, a slight and reproducible stimulation of tryptamine oxidation in the homogenate and the high speed residue was observed after sonication. From preliminary experiments, oxygen consumption by the first residue was found to be slightly decreased after sonication.

These observations confirm the view that sonication caused a redistribution of enzyme activity, resulting in the subsequent enrichment of the MAO present in the soluble fraction. This may explain the increased specific activity (S.A.) after sonication. Table XXVI summarises the observations made on the S.A. ratios for the oxidation of tyramine and tryptamine by monoamine oxidase preparations before and after sonicating the homogenate. It is apparent from the table that the S.A. ratio for the homogenate is considerably less than that of the derived fractions.

TABLE XXV. THE EFFECTS OF SONICATION ON THE AMINE:O_2 OXIDOREDUCTASE ACTIVITY OF THE SONICATED HOMOGENATE AND ITS DERIVATIVES.

Tissue preparation	Substrate	μmoles oxygen absorbed/ 100/mg protein/hr. SONICATION (-)	(+)
Homogenate	Tyramine	15.4	16.3
	Tryptamine	12.1	13.6
High speed residue	Tyramine	6.70	6.47
	Tryptamine	2.68	2.95
Soluble fraction	Tyramine	11.4 (34.1)	38.0 (67.4)
	Tryptamine	4.69 (31.5)	24.3 (65.2)

Data in brackets were obtained in the absence of 0.016M semicarbazide (final concentration).

The homogenate was sonicated and fractions prepared from it.

For further details, see the Methods section.

TABLE XXVI. THE SPECIFIC ACTIVITY (S.A) RATIOS FOR OXYGEN UPTAKE BY MAO PREPARATIONS BEFORE AND AFTER SONICATION OF THE HOMOGENATE.

Tissue preparation	Tyramine/Tryptamine S.A. ratio	
	SONICATION	
	(-)	(+)
Homogenate	1.28	1.20
High-speed residue	2.50	2.18
Soluble fraction	2.43	1.56

SECTION III: ATTEMPTS TO FURTHER CHARACTERISE THE ENZYMIC REDUCTION OF INT, NAD AND OXYGEN BY MONOAMINES.

A: Introduction.

At this point, perhaps it will interest the reader to know some of the reasons why the following course of research soon to be described was pursued.

The results obtained for the various MAO preparations in the three oxido-reductase systems (tetrazolium, NAD and oxygen) with tyramine and tryptamine as substrates, have so far revealed that the methods involving enzymic INT and/or NAD reduction by amines are surprisingly sensitive; even more sensitive than the manometric method, measuring oxygen uptake by tissue preparations in the presence of tyramine or tryptamine.

Thus though it was repeatedly shown spectrophotometrically by enzymic NAD reduction by amines that the sonication of the homogenate from which the soluble (S.S and S.S-pH5) MAO fractions were derived resulted in a substrate specificity different from that obtained for the S.MAO (see table XXII), this important fact could not be detected manometrically. Further still, though the tetrazolium assays revealed that effective sonication of the homogenate either greatly decreased or practically abolished the NAD dependence of the derived soluble fractions for oxidising tyramine and isoamylamine in the presence of INT (cf. tables XIX and XX), this could not be demonstrated manometrically. Tyramine was persistently oxidised at a faster rate than tryptamine by

all the enzyme preparations when they were assayed manometrically. Table XXVII compares tyramine and tryptamine oxidation by the two soluble (S-MAO and S.S-MAO) preparations as determined by the three methods of assay as follows:-

TABLE XXVII. THE EFFECTS OF SONICATION OF THE HOMOGENATE ON THE SUBSTRATE SPECIFICITIES OF THE DERIVED SOLUBLE MAO IN THREE OXIDOREDUCTASE SYSTEMS.

Tissue preparation	Activity/100mg protein/hour.								
	μ moles oxygen			μ moles NADH			μ moles formazan		
	Tryp.	Tyr.	Tryp/Tyr.	Tryp.	Tyr.	Tryp/Tyr.	Tryp.	Tyr.	Tryp/Tyr.
(-)S-MAO	6.7	10.7	0.63	9.27	16.2	0.57	39.7	11.4	3.49
(+)S-MAO	25.7	41.8	0.62	31.1	3.45	9.02	139	66.7	2.08
(-)S.S-MAO	4.19	6.83	0.61	10.1	16.8	0.60	25	8.2	3.05
(+)S.S-MAO	23.0	39.1	0.59	21.5	3.6	5.97	106	43.6	2.43

(-) Derived from non sonicated homogenate

(+) Derived from sonicated homogenate

From table XXVII, it is clear that tyramine was a better substrate for NAD and oxygen reduction assays with the soluble enzyme prepared from unsonicated homogenates. It was still a better substrate than tryptamine for oxygen uptake with soluble preparations (S.S-MAO) derived from sonicated homogenates but a definitely poor substrate for NAD reduction in the S.S.MAO system. Tryptamine remained a better substrate for the tetrazolium system.

Since these two apparently sensitive assay methods (NAD and tetrazolium reduction) were not characterised, it was felt justified to attempt doing precisely this. Some of the questions arising out of this were:- (1) the possible site of NAD or tetrazolium reduction (2) the role of oxidation products such as aldehyde and (3) how far oxygen requirement would affect these assay methods.

Kynuramine appeared to be an ideal substrate for determining the role of an aldehyde intermediate in enzymic NAD or INT reduction by amines. The kynuramine aldehyde formed during oxidative deamination by MAO is known to spontaneously cyclize to 4-hydroxyquinoline (116,117). This means that the aldehyde would not be available for convertion to the acid by aldehyde dehydrogenase(117). Indeed, very little of the aldehyde formed by kynuramine is known to be further oxidised to the acid(116). This lead the author to include kynuramine in the research as a possible good tool for investigation. But before assaying for kynuramine destruction in the NAD and INT reduction methods it was necessary to first demonstrate either manometrically or spectrophotometrically

that it was a good substrate for the guinea pig liver enzyme extracts.

1. Monoamine oxidase activity measurement by the method of kynuramine disappearance.

MAO activity was estimated by this method as already described (cf. Methods) by measuring the OD change at 360 mμ caused by kynuramine disappearance through MAO activity.

Table XXVIII shows the specific activity and total activity per fraction of MAO in various preparations of guinea pig liver.

TABLE XXVIII. KYNURAMINE OXIDATION BY SONICATED GUINEA PIG LIVER MAO PREPARATIONS.

For details of reaction mixtures and procedure of assay see the Methods section.

Tissue preparation	Total volume (ml)	Total activity (Δ OD^{360} /hr.)	Specific activity. (ΔOD^{360}/hr/ 100 mg protein)
Homogenate	70	871.5	42.1
2nd residue (high speed residue)	9	380.7	129.0
High speed supernatant	62	421.6	24.4
S.S.MAO	30	276.0	41.4

2. The effects of anaerobiosis on enzymic INT reduction by amines.

Hare(1) Pugh and Quastel(13) Philpot(14) and Kohn(19) showed that oxygen was necessary for MAO activity. These authors, however, assayed the enzyme manometrically measuring oxygen uptake. Attempts by Hare to use other electron acceptors instead of oxygen were unsuccessful because of the excessive endogenous enzyme activity. It was, therefore, worthwhile to investigate the effects of oxygen on the amine/INT reductase reaction in the presence and absence of added NAD.

Thunberg tubes containing the appropriate reaction mixtures for normal INT reduction assays (see Methods and also table IX legend), were evacuated of air. The tubes were preincubated at 37°C for 30 minutes after tipping the substrate from the side arm. Control experiments were carried out in air. Preincubation in the case of the control experiments was for 2 minutes and incubation with the complete system in air was 10 minutes.

In the case of the anaerobic experiments after 30 minutes anaerobic preincubation, the tubes were opened and INT was added, with further aerobic incubation for 10 minutes.

It was observed that when INT was added to the tubes containing NAD in the complete system (containing added substrate, tryptamine) spontaneous INT reduction occurred. The endogenous INT reduction in the presence of added NAD of this experiment was very slow. It is necessary to note that it was found from preliminary experiments that if the dye was

preincubated with the enzyme under anaerobic conditions, it was rapidly reduced to formazan thus masking the value due to added amine. If however it was preincubated with the substrate there was little endogenous tetrazolium reductase activity. The activity due to added amine was correspondingly much decreased by anaerobiosis.

From this observation and the results presented in table XXIX it is apparent oxygen is necessary for enzymic INT reduction.

TABLE XXIX. THE EFFECTS OF ANAEROBIOSIS ON NAD-AMINE/INT REDUCTASE ACTIVITY OF S-pH5 MAO.

Added Substrate	Additive	Treatment	Δ OD^{520}/ml enzyme/hr.	ΔOD^{520}/ml enzyme/hr.
None	-	Aerobic pre-incubation	0.210	-
Tryptamine	-	"	0.540	.330
None	0.15mM NAD	"	0.420	-
Tryptamine	"	"	0.780	0.360
None	-	Anaerobic pre-incubation	0.180	
		Aerobic incubation		
Tryptamine	-	"	0.420	0.240
None	0.15mM NAD	"	0.420	
Tryptamine	"	"	0.600	0.180

B: Inhibition studies.

1. Inhibition of oxygen uptake of S.S.MAO.

(i) Semicarbazide.

Oxidation of amines by S.S.MAO was at least about 50 per cent inhibited by 0.016M (final concn) semicarbazide (table XXX).

The inhibition of kynuramine oxidation by semicarbazide is of special notice since kynuramine aldehyde is known to spontaneous cyclize to form 4-hydroxyquinoline which requires no oxygen(116). The strong inhibition of kynuramine oxidation (i.e. more than 50 per cent: see table XXX), may mean either a direct action of semicarbazide on the enzyme or a chemical reaction between semicarbazide and kynuramine resulting in the removal of available substrate for enzyme action. No obvious interaction was apparent by spectroscopic examination. Youdim had earlier found inhibition of kynuramine oxidation by semicarbazide(121).

Isoamylamine oxidation was also inhibited as previously observed with the S.MAO.

In the case of tyramine, tryptamine and serotonin, dye formation was also abolished. This was in conformity with the observations of other workers(120).

Semicarbazide inhibition of oxygen uptake is interpreted to mean that the aldehydes were not further oxidised to the corresponding acids(15,16).

TABLE XXX. THE EFFECTS OF SEMICARBAZIDE ON OXYGEN CONSUMPTION BY S.S.MAO PREPARATION FROM GUINEA PIG LIVER.

Substrate	Inhibitor	Final molar concn.	μ moles O_2/100mg protein/hr.	Per cent inhibition
1. Kynuramine	-		42.9	-
"	semicarbazide	1.6×10^{-2}	17.1	60
2. Isoamylamine	-		45.2	-
"	semicarbazide	1.6×10^{-2}	23.4	48
3. Tyramine	-		64.3	-
"	semicarbazide	1.6×10^{-2}	29.5	54
4. 5-Hydroxy-tryptamine	-		68.0	-
"	semicarbazide	1.6×10^{-2}	29.5	57
5. Tryptamine	-		56.3	-
"	semicarbazide	1.6×10^{-2}	27.9	50

(ii) Sulphydryl compounds.

Lagnado and Sourkes(71) and a number of other authors (73,74) have shown that MAO activity is inhibited by SH-inhibitors such as PCMB and $HgCl_2$. These compounds were therefore tested in the amine-S.S.MAO system by manometric assay. Table XXXI shows that these findings were confirmed by the present author, for soluble enzyme preparations.

TABLE XXXI. THE EFFECTS OF SULPHYDRYL AGENTS ON OXYGEN REDUCTION BY S.S.MAO PREPARATION FROM GUINEA PIG LIVER.

Exp.	Substrate	Additive	Final molar con^{c}.	μmoles O_2/100mg. protein/ hour	% inhibition
1.	Tyramine	-		26.80	-
		$HgCl_2$	2.5×10^{-4}	20.1	25
	Tryptamine	-		20.1	-
		$HgCl_2$	2.5×10^{-4}	15.0	25
	Histamine	-		2.83	-
		$HgCl_2$	2.5×10^{-4}	2.83	0
2.	Tyramine	-		24.0	-
		PCMB	5×10^{-4}	20.0	25
	Tryptamine	-		20.0	-
		PCMB	5×10^{-4}	15.0	25
	Histamine	-		2.7	-
		PCMB	5×10^{-4}	2.72	0

(iii) The effects of semicarbazide on oxygen uptake by mitochondrial MAO.

Oxygen uptake by thawed guinea pig liver mitochondrial suspension was about 50 per cent inhibited when tyramine, tryptamine or serotonin were tested as substrates. Semicarbazide 0.016M (final conc^{n}) did not affect the reaction when isoamylamine was substrate. These findings were in good agreement with the findings of other workers(15,16). The result was, however, in contrast with that seen for isoamylamine when S.MAO and S.S.MAO were assayed for oxygen uptake. With these preparations semicarbazide strongly inhibited the oxygen consumption in the presence of isoamylamine.

Table XXXII shows data compiled for oxygen uptake by mitochondrial suspensions in phosphate buffer, pH 7.6.

TABLE XXXII. THE EFFECTS OF SEMICARBAZIDE ON OXYGEN UPTAKE BY MITOCHONDRIAL SUSPENSIONS IN THE PRESENCE OF MONOAMINES.

Substrate	Additive	Final molar conc^{n}. (M)	μ moles oxygen/ 100 mg protein/ hr.	% inhibition
Isoamylamine	-		48.5	-
	Semicarbazide	1.6×10^{-2}	49.0	0
Tyramine	-		67.6	-
	Semicarbazide	1.6×10^{-2}	28.2	58
Serotonin	-		69.7	-
	Semicarbazide	1.6×10^{-2}	27.9	60
Tryptamine	-		58.2	-
	Semicarbazide	1.6×10^{-2}	27.9	52

(iv) The effects of tetrazolium salts on oxygen consumption by S.S-MAO.

Lagnado and Sourkes(79) and Weissbach et al.(99) showed that there was a lagphase when MAO was assayed by the tetrazolium technique. Both groups also demonstrated independently that carbonyl reagents, e.g. cyanide and semicarbazide, inhibited the amine/tetrazolium reductase reaction. These reagents, however, are not inhibitors of MAO when it is assayed manometrically(15,16,74). Since the rate of serotonin disappearance was linear as a function of time and it was not affected by the carbonyl reagents, these authors(99) postulated that the amine/tetrazolium reductase reaction depends on the presence of an aldehyde. The lagphase could then be explained in terms of either the time for the accumulation of the aldehyde or on the formation of an electron carrier from the aldehyde, necessary for tetrazolium reduction. These authors(99) also claimed that indoleamines were the best substrates for the amine/INT reductase assay of lyophilised rat liver mitochondria. Weissbach et al. did not test the effects of tetrazolium salts (INT) on oxygen uptake stimulated by MAO substrates. Lagnado and Sourkes, however, showed that rat brain MAO was strongly inhibited by TTC and NTC when it was assayed manometrically; there was no reduction of these tetrazoles during oxygen uptake. More recently, Lallemant et Baron(138) extended these findings by demonstrating that a number of other tetrazoles, including INT and nitro-BT (NBT), were competitive inhibitors of mitochondrial MAO. These authors did not report whether or not any

of these salts were reduced in their experiments. The present author was led, therefore, to investigate the contribution of aldehyde to tetrazolium reduction by MAO and also to find if, in fact, the S.S.MAO could catalyse INT and NBT reduction during oxygen uptake. Since semicarbazide had been already shown to prevent further oxidation of the aldehyde by trapping it (cf. tables XV, XXX, XXXII), this meant that there would be no aldehyde available to reduce the tetrazolium salt. Furthermore, since the formation of the postulated carrier is claimed to depend on an aldehyde, the trapping of the aldehyde would eliminate the possibility of its formation(99). The tetrazolium salts tested were INT and in a few cases NBT also.

MAO activity was estimated by the Warburg technique with air as the gas phase, and in the presence of 0.016M semicarbazide (final molar conc^{n}).

The results summarised in table XXXIII below clearly show that INT is a potent inhibitor of S.S.MAO and that the tetrazolium salt is readily reduced under the experimental conditions. The amount of formazan produced was directly proportional to the amount of dye added. It is also apparent that the usual inhibition of oxygen uptake by semicarbazide due to the suppression of further oxidation of the aldehyde intermediate to the acid was abolished in the presence of the dye.

The inhibition by the dye of oxygen consumption was less marked when it was preincubated in the side arm with the substrate (cf. table XXXIII) (Exp. 3c). Similar findings were made with NBT when tryptamine was substrate. It can also

be seen from the data presented (Exp. 2) that the dye (INT) inhibited oxygen uptake in the presence of isoamylamine more strongly at a lower concentration than at a higher one, as was the case for all the other substrates tested. This anomaly may conceivably be related to the behaviour of this substrate (isoamylamine) towards semicarbazide inhibition of oxygen consumption(15,16). Generally, tyramine and isoamylamine reduced INT very poorly but tryptamine was a very good substrate for dye reduction during oxygen uptake as shown in table XXXIII. This was in keeping with data on the amine/INT reductase activity in the conventional assay system (see e.g. tables IX, X, XVIII). It is apparent from the data presented that the inhibition by INT of respiration by S.S.MAO was greater in the absence of semicarbazide than it was when this trapping agent was added. The amount of formazan produced in the absence of semicarbazide was practically the same as it was in the presence of semicarbazide. Identical results were obtained when NBT was used as a dye.

TABLE XXXIII. THE EFFECTS OF INT ON OXYGEN UPTAKE BY S.S.MAO.

Legend: The enzyme was preincubated for 25 mins. with 0.015M semicarbazide and INT. The reaction was started by tipping the substrate from the side arm. Control experiments were included in which water replaced INT, semicarbazide and substrate. The amount of dye reduced was calculated on the basis of a molar extinction coefficient for the corresponding formazan of 13,300 in ethylacetate solution at 520 $m\mu$.

Exp.	Substrate	INT (μ moles/ flask.	Oxygen (μ moles/ hr/ml enzyme.	% of control	Formazan μ moles/ flask.
1.	Tyramine	-	3.5	100	-
		0.79	1.9	54	0.176
		1.58	1.3	37	0.24
		2.37	1.0	29	0.44
2.	Isoamylamine	-	1.39	100	-
		0.79	0.43	31	0.176
		1.58	0.78	56	0.235
3. (a)	Tryptamine	-	5.6	100	-
		0.79	4.8	85	0.74
		1.58	2.6	44	1.33
		2.37	1.88	33	1.8
(b)	Tryptamine *	-	10.7	100	-
		0.79	5.14	48	0.67
		1.58	3.1	29	1.45
		2.37	1.49	14	2.11
(c)	Tryptamine**	-	5.6	100	-
		0.79	4.7	84	0.65
		1.58	4.1	73	1.47
		2.37	3.75	67	2.02

In (a) and (c) semicarbazide 0.016M (final conc^{n}) was present;

in (b) there was no semicarbazide;

in (c) INT was tipped from the side arm together with the substrate at zero time. In all other experiments the dye was preincubated with the enzyme.

(v) The effects of INT on oxygen uptake by S.S.MAO in the presence of kynuramine.

Kynuramine was found to be a good substrate for MAO in manometric and substrate disappearance determinations (cf. tables XXIX, XXX).

In preliminary experiments with kynuramine, it was found to be a very poor substrate for enzymic tetrazolium reduction. However, in a mixed substrate experiment involving tryptamine and kynuramine, kynuramine was found to strongly inhibit enzymic reduction by tryptamine. As kynuramine is known not to form a free aldehyde which would be further oxidised to the acid, because of the spontaneous cyclisation of the aldehyde to 4-hydroxyquinoline(116,117), it was thought to be an ideal substrate for further investigation. Table XXXIV shows that kynuramine oxidation by S.S.MAO was strongly inhibited by INT during oxygen uptake just as it was for all the other amines tested.

According to this table (XXXIV), semicarbazide caused relatively slight inhibition of oxygen reduction in the absence of added INT, as would be expected since the aldehyde formed in the reaction would cyclize to 4-hydroxyquinoline which does not involve oxygen consumption(116,117). However, INT and NBT reduction did occur to a small extent at the lowest concentrations tested. Under these conditions, semicarbazide appeared to stimulate dye reduction and simultaneously inhibit respiration in the presence of kynuramine by at least 50 per cent.

At higher dye concentrations there was a strong inhibition

of oxygen absorption, and a small inhibition of respiration by semicarbazide, which also caused an apparent stimulation of dye reduction. Dye reduction in all cases was slight.

The strong inhibition by INT of kynuramine oxidation by S.S.MAO in the absence of high aldehyde oxidase activity suggests a direct inhibition of the enzyme itself by the dye (INT). This may explain the inhibition of MAO activity by INT with all the amines tested. It would also explain the apparent weak inhibition of tryptamine metabolism when the dye was preincubated in the side arm with the substrate, instead of the normal preincubation with the enzyme (cf. table XXXIII exp. 3 a-c). The poor kynuramine/INT reductase activity may not necessarily mean the involvement of aldehyde in INT reduction: after all, tyramine or isoamylamine which form a free aldehyde are equally poor substrates for enzymic tetrazolium reduction.

TABLE XXXIV. THE EFFECTS OF INT ON OXYGEN CONSUMPTION BY S.S.MAO IN THE PRESENCE OF KYNURAMINE.

Kynuramine (3mM)	INT (μ mole/ flask)	μ moles oxygen per ml enzyme/hr.	% of control	Formazan (μ moles/ flask
(a)	-	3.86	100	-
	0.99	2.19	57	0.117
	1.98	1.11	29	0.0
	2.97	1.11	29	0.0
(b)	-	3.11	100	-
	0.99	0.89	30	0.176
	1.98	0.89	30	0.073
	2.97	0.89	30	0.073

In (a) semicarbazide was excluded.

In (b) 0.016M semicarbazide (final conc.) was added.

(vi) The combined effects of INT and tranylcypromine (TCP) on oxygen abscrption by S.S.MAO in the presence of tryptamine.

Tranylcypromine (TCP) inhibited MAO activity about 75 per cent whether INT was present or not. INT reduction was inhibited about 30 per cent by TCP. The presence of TCP enhanced the respiratory inhibition by INT as shown in table XXXV.

TABLE XXXV. THE COMBINED EFFECTS OF TRANYLCYPROMINE (TCP) AND INT ON TRYPTAMINE OXIDATION BY S.S.MAO*.

INT (μ moles flask)	T.C.P. final molar concn (M)	μ moles oxygen/ hr/ml enzyme	% of control	Formazan (μ mole/ flask)
-	-	9.64	100	-
-	10^{-5}M (TCP)	2.32	24	-
0.99	-	6.56	68	0.602
0.99	10^{-5}M (TCP)	0.89	9	0.391

* Semicarbazide was omitted in this experiment.

FIG. 8. TIME CURVE FOR INT REDUCTION BY S.S-MAO DURING OXYGEN UPTAKE IN THE PRESENCE OF TRYPTAMINE.

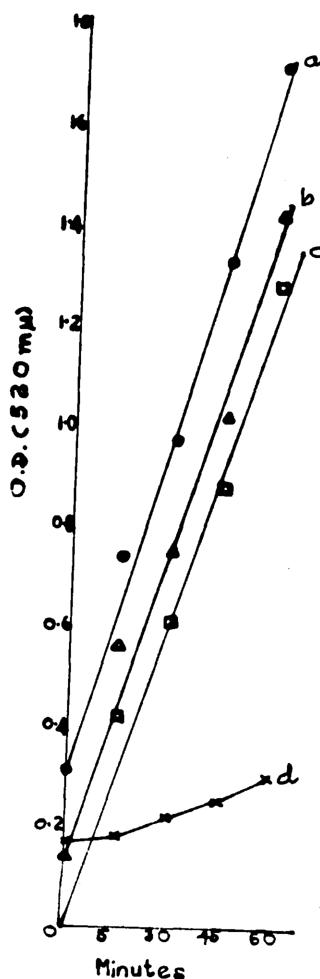
The reaction mixture was the same as for the legend of table XXXIII except that only one concentration of INT was used i.e. 7.92 mM (final conc.).

a = Total formazan produced in the presence of substrate (tryptamine).

b = Excess formazan produced over that of the endogenous substrates.

c = Correction for zero time.

d = Formazan produced by INT reduction by endogenous substrates.



2. <u>The inhibition of kynuramine disappearance by semicarbazide in the presence of NAD-nicotinamide mixture.</u>

Kynuramine disappearance (as measured by spectrophotometric assay at 360 mμ) was strongly inhibited by 0.016M neutralised semicarbazide in the presence of a mixture consisting (in final concentrations) of 0.35 mM NAD and 1.67mM nicotinamide. Kynuramine oxidation, however, was not affected by either semicarbazide alone or by the NAD-nicotinamide mixture in the absence of semicarbazide. If anything, kynuramine destruction was slightly enhanced by the NAD-nicotinamide mixture as compared to the control. The reason for the semicarbazide inhibition of kynuramine metabolism in the presence of the mixture is obscure since kynuramine aldehyde is not expected to be available for trapping by semicarbazide(116,117). Whether this inhibition was caused by a direct inhibition of the enzyme by the reaction mixture or through the formation of an anti metabolite is not clear. It was possible semicarbazide was inhibiting a pathway involving kynuramine and NAD. However, it may be stated that attempts to demonstrate enzymic NAD reduction by kynuramine at 340 mμ was unsuccessful. This, however, might be due to Quenching since 360 mμ the wavelength at which kynuramine disappearance is measured is too close to 340 mμ where NADH formation is assayed. Youdim(120) claimed to have demonstrated the inhibition of kynuramine oxidation by semicarbazide at acid pH. Table XXXVI sums up the results described above.

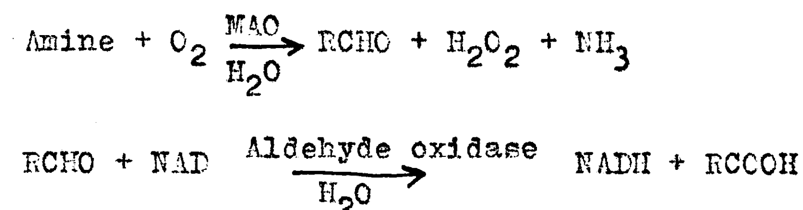
TABLE XXXVI. THE EFFECTS OF SEMICARBAZIDE AND NAD-NICOTINAMIDE MIXTURE ON KYNURAMINE DISAPPEARANCE.

Addition	Trapping agent	Final molar conc^{n} (M)	ΔOD^{360}/ml enzyme/ hr.	% Inhibition
None	-		3.00	
NAD-nicotin-amide mixture	-		3.72	- 24*
None	semicarbazide	0.016	3.00	
NAD-nicotin-amide mixture	-	"	1.44	52

* stimulation

3. Effects of semicarbazide on amine/NAD reductase activity of S.S.MAO.

Weissbach *et al.*(35) postulated the following reaction mechanism to show that NAD reduction during MAO activity is mediated through the aldehyde intermediate in the presence of aldehyde oxidase:-



$$\text{Amine} + O_2 \xrightarrow[H_2O]{\text{MAO}} \text{RCHO} + H_2O_2 + NH_3$$

$$\text{RCHO} + \text{NAD} \xrightarrow[H_2O]{\text{Aldehyde oxidase}} \text{NADH} + \text{RCOOH}$$

Indeed, these authors claimed to have stimulated 5-hydroxyindole acetic acid (5HIAA) formation from the aldehyde in the presence of added excess aldehyde oxidase to various fractions of soluble MAO preparations from guinea pig liver. The acid fraction appeared to be the most dependent on this treatment in the presence of added NAD. The authors, however, did not demonstrate the effects of a carbonyl reagent such as semicarbazide on the reaction. Furthermore, the availability of kynuramine lead us to re-investigate the role of aldehyde on amine/NAD reductase activity. Six monoamines were tested. These were: tryptamine, serotonin, tyramine, isoamylamine, benzylamine and kynuramine. A typical result obtained for kynuramine was shown in table XXXVI. The enzymic NAD reduction by kynuramine could not be readily assayed at 340 mμ for the reason already stated, i.e. the possibility of quenching.

It was repeatedly found in the case of the enzymic NAD reduction by the other amines tested (tryptamine, serotonin

and tyramine) that the addition of excess aldehyde oxidase to the reaction mixture as suggested by Weissbach et al.(35) did not result in the stimulation of NAD reduction by the acid fraction (S-pH5). In order to determine the role of aldehyde in NAD reduction assays during amine oxidation, the effects of various concentrations of semicarbazide on the reaction rate were tested. Acetaldehyde was used as a control substrate.

Indeed, NAD reduction by the S.S MAO system with acetaldehyde as substrate was stopped within 2 minutes by 0.0133M semicarbazide (final conc^{n}) (see fig. 9). NAD reduction by sucrose mitochondrial suspensions with tyramine as substrate was hardly affected by an identical concentration of this trapping agent (see fig. 5b). This then was in contrast with the result obtained for the reaction involving acetaldehyde.

Furthermore, with the S.S.MAO preparations, semicarbazide concentrations (all final conc^{n}) ranging from 0.0033M to 0.0133M were found to stimulate amine/NAD reductase activity when serotonin and tryptamine were tested as substrates (fig. 9). In other experiments involving these substrates the stimulation of serotonin/NAD reductase activity by semicarbazide (0.0133M) was confirmed. In the case of tryptamine there was occasional contradiction in that semicarbazide appeared to inhibit the reaction; such results were obtained at the highest semicarbazide concentration tested (0.0133M). This inhibition, however, was especially noticeable after 20 minutes of NADH formation. The maximal inhibition in this case was about 30 per cent. The inhibition by semicarbazide

of oxygen uptake was, however, about 50 per cent for these substrates (tryptamine, serotonin and tyramine) (see tables XXX and XXXII).

The enzymic NAD reduction by benzylamine, the weakest substrate, was abolished by the lowest semicarbazide concentration (0.0033M). When tyramine and isoamylamine were tested as substrates for NAD reduction with the S.S.MAO, it was found that 0.0066M semicarbazide inhibited the oxidation of isoamylamine by 40 per cent after 20 minutes and also that of tyramine (see fig. 9).

From these observations it can be inferred that an aldehyde may be somehow involved in NAD reduction by some amines in the presence of MAO preparations, assuming semicarbazide acts solely by trapping the aldehyde. The contribution of the aldehyde to the reaction rate, however, appeared not to be the main factor. It is apparent from these results that other pathways, yet unknown, do exist for electron transfer in which MAO can participate during oxidative deamination of monoamines, when NAD is used as final electron acceptor.

FIG. 9. THE EFFECTS OF SEMICARBAZIDE CONCENTRATION ON NAD REDUCTION BY S.S.MAO IN THE PRESENCE OF MONO-AMINES OR ACETALDEHYDE.

The reaction mixture contained in a final volume of 3.0ml, 6.7μmoles substrate (neutralised); 106μmoles NAD; 12μmoles nicotinamide; 150μmoles sodium pyrophosphate phosphate buffer pH 8.8; enzyme: 0.25ml (4mg protein); water was added as required to make up the final volume. Water replaced the substrate and semicarbazide in blanks and controls respectively.

Fig. 9A represents tryptamine as substrate

1 = control (semicarbazide was ommitted)

2 = effect of 0.0033M semicarbazide

3 = effect of 0.0066M semicarbazide

4 = effect of 0.0133M semicarbazide

Fig. 9B represents serotonin as substrate

1A = control (semicarbazide absent)

2A = effect of 0.0066M semicarbazide

3A = effect of 0.0133M semicarbazide

Fig. 9C represents the following substrates with and without semicarbazide:-

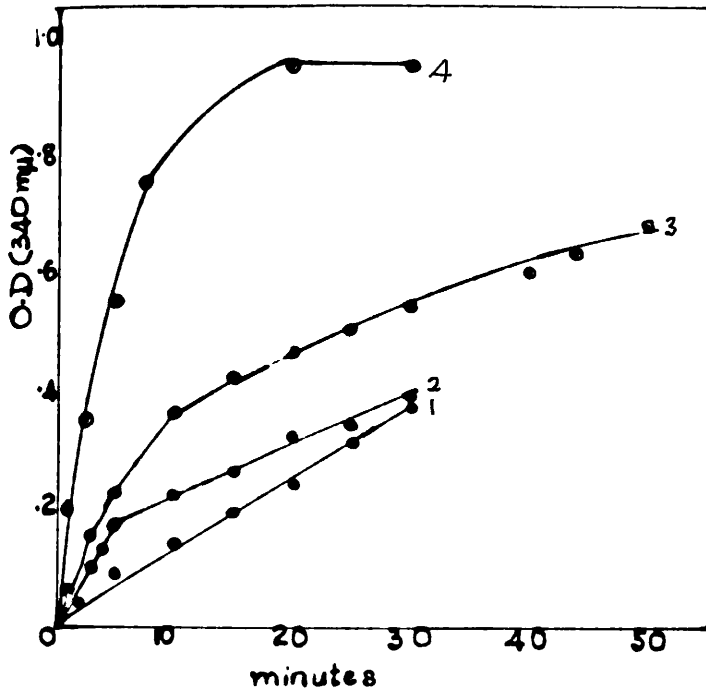
1b,2b benzylamine with and without 0.0033M semicarbazide respectively;

3b,5b isoamylamine with and without 0.0033M semicarbazide respectively;

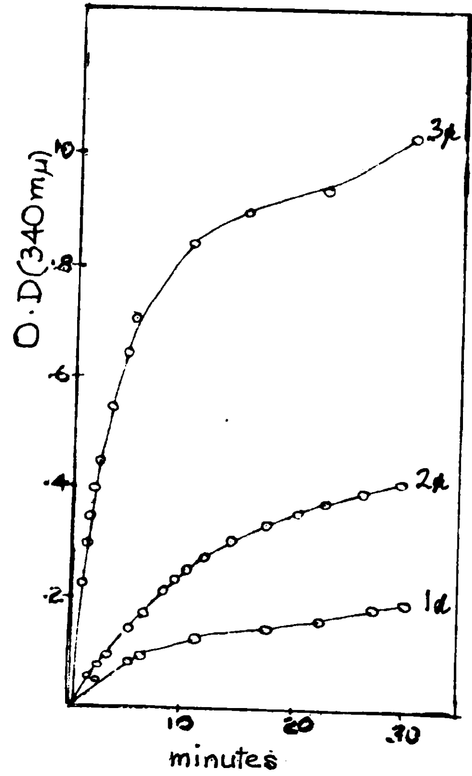
4b and 6 tyramine with and without 0.0066M semicarbazide respectively.

Fig. 9D represents acetaldehyde as substrate.

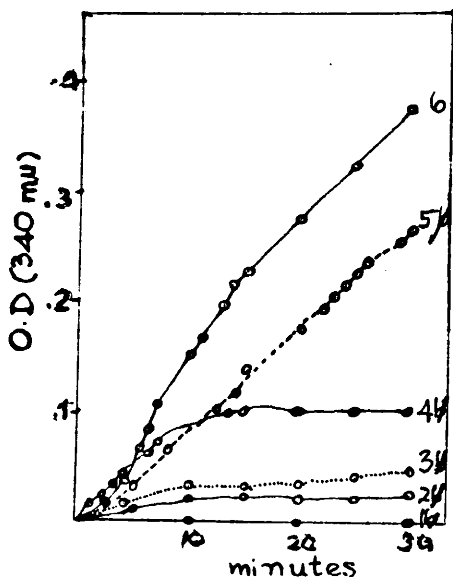
+ and - indicate result with and without 0.0133M semicarbazide respectively. All concentrations of semicarbazide were final.



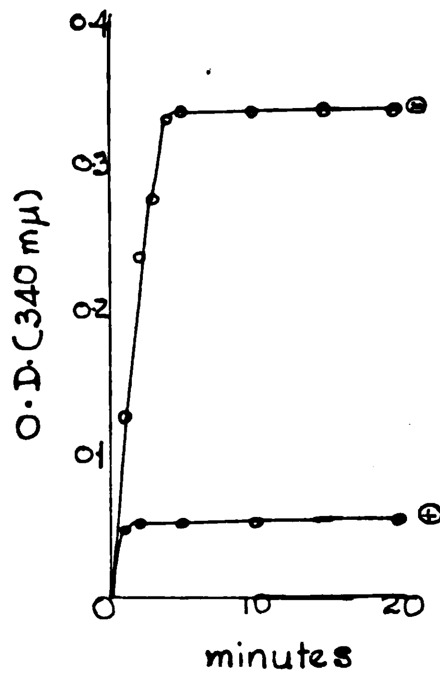
A



B



C



D

4. Amine/INT reductase activity of soluble (S.S.MAO) and particulate (mitochondrial suspensions) preparations from guinea pig liver.

The effects of:

(i) semicarbazide.

Lagnado and Sourkes(79) and Weissbach et al.(99) demonstrated that tetrazolium reduction by rat tissue particulate MAO was inhibited by semicarbazide. The degree of inhibition depended on the substrate (amine) tested. Thus Weissbach et al. found 11 per cent and 70 per cent inhibition of INT reduction by 0.04M semicarbazide (final conc) in the presence of tyramine and serotonin, respectively. These workers(99) suggested aldehyde as the reductant. The availability of kynuramine which does not form a free aldehyde prompted us to re-study the possible reductant of tetrazolium in soluble and particulate MAO systems. The substrates tested included succinate and acetaldehyde, in addition to the amines.

It was found that neutralised semicarbazide at a concentration known to exclude all aldehyde without interfering with other enzyme systems(49), abolished the enzyme activity when isoamylamine was tested for INT reduction by the soluble and the particulate enzymes. Tryptamine was the most resistant amine to semicarbazide inhibition when either enzyme preparation was assayed for tetrazolium reduction. The concentrations of semicarbazide producing 50 per cent inhibition of the reaction in the soluble (S.S) enzyme system were as follows:-

Serotonin:	0.009M
Tyramine:	0.0113M
Tryptamine:	0.0225M

It was also found that while acetaldehyde/INT reductase activity was abolished by 0.008M semicarbazide, succinate/INT reductase activity was not more than 30 per cent inhibited by 0.03M semicarbazide in the S.S.MAO system. The percentage inhibition of the particulate enzyme activity was generally slightly higher for all the substrates tested.

Kynuramine was a rather poor substrate in the INT-reductase assay and it was not possible to obtain reliable inhibition data with this substrate. Nevertheless, it is apparent that semicarbazide inhibition of tetrazolium reductase activity does not necessarily reflect its effect on an aldehyde intermediate.

The case of kynuramine is a complex one, the very fact that of all the known MAO substrates it alone should have a unique pathway for the metabolism of its end product merits special attention. It is also important to recall that semicarbazide very strongly inhibited oxygen uptake by S.S.MAO in the presence of kynuramine (table XXX). Similarly, it was kynuramine oxidation which was most inhibited by INT (table XXXIV) and finally the disappearance of kynuramine as assayed spectrophotometrically was strongly inhibited by semicarbazide under certain conditions (table XXXVI). Special attention may also be paid to the fact that even at four-fold the concentration of semicarbazide known to exclude all aldehyde (49), Weissbach _et al._ did not succeed in inhibiting tyramine/

INT reductase activity by more than 11 per cent. Table XXXVII shows the effects of semicarbazide on enzymic INT reduction by amines, acetaldehyde and succinate.

TABLE XXXVII (A). THE EFFECTS OF SEMICARBAZIDE ON INT REDUCTION BY S.S.MAO IN THE PRESENCE OF MISCELLANEOUS SUBSTRATES.

Exp.	Substrate	Additive	Final molar conc^n (M)	μ moles formazan /100mg protein/ hr.	Per cent of control.
1.	Tyramine	-	-	47.4	100
	"	Semicarbazide	7.5×10^{-3}	39.5	83
	"	"	15.0×10^{-3}	22.3	48
	"	"	30.0×10^{-3}	16.9	36
2.	Serotonin	-	-	77.8	100
	"	Semicarbazide	7.5×10^{-3}	46.7	60
	"	"	15.0×10^{-3}	21.4	28
	"	"	30.0×10^{-3}	13.5	17
3.	Tryptamine	-	-	95.9	100
	"	Semicarbazide	7.5×10^{-3}	65.2	68
	"	"	15.0×10^{-3}	32.4	55
	"	"	30.0×10^{-3}	42.3	44
4.	Isoamylamine	-	-	20.3	100
		Semicarbazide	7.5×10^{-3}	0.00	000
5.*	Acetaldehyde	-	-	20.9	100
		Semicarbazide	13.3×10^{-3}	0.00	000
6.	Succinic acid	-		45.1	100
	"	Semicarbazide	7.5×10^{-3}	38.4	85
	"	"	15.0×10^{-3}	36.1	80
			30.0×10^{-3}	33.8	75

* NAD (0.5mM final conc.) and nicotinamide (0.0025M final conc.) were incorporated in the reaction mixture.

Note: Preincubation of enzyme and semicarbazide was 10 minutes in all cases. Controls (in the absence of semicarbazide) were similarly treated.

TABLE XXXVII (B). THE EFFECTS OF SEMICARBAZIDE ON INT REDUCTION BY THAWED SUCROSE MITOCHONDRIAL SUSPENSION IN THE PRESENCE OF MISCELLANEOUS SUBSTRATES.

Exp.	Substrate	Additive	Final molar conc. (M)	μ moles formazan 100 mg protein/ hr.	% of control
1.	Succinic acid	-		109.2	100
	"	semicarbazide	$5x10^{-3}$	79.6	73
		"	$10x10^{-3}$	60.1	55
2.	Isoamylamine	-		54.6	100
	"	semicarbazide	$5x10^{-3}$	00.0	000
3.	Tryptamine	-		98.1	100
	"	semicarbazide	$5x10^{-3}$	39.3	40
	"	"	$10x10^{-3}$	6.87	7

(ii) The effects of sulphydryl agents and metal chelators on amine/INT reductase activity of S.S.MAO.

There is much controversy as to whether MAO requires Cu or Fe for full activity or not(74,82). This prompted our investigating the effects of their chelators on the system. An attempt was also made to compare the effects of thiol reagents on the amine-tetrazolium (INT) reductase system of the soluble enzyme with that of mitochondrial MAO. The results obtained are presented in table XXXVIII (A and B).

It was found that $AgNO_3$, PCMB and $HgCl_2$ were effective inhibitors, the degree of inhibition being proportional to the concentration of the inhibitors cf. table XXXVIII (A). These compounds are known to inhibit enzyme systems mainly by inhibiting the SH groups(71,73,74).

4-hydroxyquinoline and 8-hydroxyquinoline were very poor inhibitors. 4-hydroxyquinoline at 0.05M (final conc^n) was not inhibitory. This was in agreement with other workers(74). At 0.025M (final conc^n), 8-hydroxyquinoline inhibited the tyramine INT-reductase reaction by only 4 per cent while that of tryptamine was 10 per cent stimulated. This was in contrast to the results of Erwin and Hellerman(74) for bovine kidney mitochondrial MAO. Similarly, the strong inhibition of the reactions by o-phenanthroline at 0.005M (final conc^n) was in contrast with the results of these authors. These authors obtained greater inhibition of benzylamine and kynuramine oxidation by their enzyme with 8-hydroxyquinoline.

α-Naphthol 0.013M (final conc^n) strongly inhibited tryptamine/INT reductase reaction when it was preincubated

TABLE XXXVIII (A). THE EFFECTS OF SH-INHIBITORS ON ENZYMIC/INT REDUCTION BY AMINES. ENZYME: S.S.MAO PREPARATION FROM GUINEA PIG LIVER.

Exp.	Substrate	Inhibitor	Final molar conc^n.	μ moles Formazan/ 100mg protein/ hr.	Per cent inhibition
1.	N-Butylamine	-		12.50	-
		PCMB	2×10^{-4}	7.50	40
		"	5×10^{-4}	0.00	100
	Tyramine	-		43.00	-
		PCMB	2×10^{-4}	27.20	37
		"	5×10^{-4}	0.00	100
	Tryptamine	-		80.00	-
		PCMB	2×10^{-4}	44.90	44
		"	5×10^{-4}	0.00	100
2.	Tyramine	-		42.00	-
		$HgCl_2$	5×10^{-5}	34.44	18
		"	10^{-4}	26.9	36
		"	2×10^{-4}	10.1	76
	Tryptamine	-		72.0	-
		$HgCl_2$	5×10^{-5}	57.6	20
			10^{-4}	43.0	40
			2×10^{-4}	14.6	80
3.	Tryptamine	-		75.0	-
		$AgNO_3$	1.25×10^{-4}	58.0	23
			2.5×10^{-4}	41.0	45
			5×10^{-4}	7.23	94

Preincubation of enzyme and inhibitor at 37°C was for 15 minutes in all cases.

TABLE XXXVIII (B). THE EFFECTS OF METAL CHELATORS ON TETRAZOLIUM (INT) REDUCTION BY S.S-MAO PREPARATION FROM GUINEA PIG LIVER.

Exp.	Substrate	Chelator	Final molar concn.	μ moles formazan/ 100 mg protein/ hr.	Per cent inhibition
1.	Tyramine	-		14.2	-
		4-Hydroxyquinoline	5×10^{-2}	14.2	0
	Tryptamine	-		30.4	-
		4-Hydroxyquinoline	5×10^{-2}	30.4	0
2.	Tyramine	-		14.0	-
		8-Hydroxyquinoline	5×10^{-3}	13.4	4
	Tryptamine	-		30.6	-
		8-Hydroxyquinoline	5×10^{-3}	33.7	- 10 *
3.	Tryptamine	-		30.5	-
		α-Naphthol	6×10^{-3}	25.3	17
		"	1.3×10^{-2}	12.2	60
4.	Tyramine	-		15.5	-
		o-phenanthroline	10^{-3}	12.7	18
		"	5×10^{-3}	2.3	86
	Tryptamine	-		36.0	-
		o-phenanthroline	10^{-3}	29.2	19
		"	5×10^{-3}	1.4	96

* Mean values for three experiments, each of which was activated by the chelator.

for 30 minutes with the enzyme. Inhibition was complete and irreversible when the mixture was kept in the cold overnight. Naphthols are claimed to inhibit enzyme systems by forming charge transfer complexes with FAD(74). This might suggest that the guinea pig liver enzyme contained a flavin, probably FAD.

(iii) The effects of some respiratory chain inhibitors on INT reduction by guinea pig liver MAO preparations in the presence of tyramine.

It was reported earlier in this thesis (see section 1 Nos. 6 and 7) that both mitochondrial suspensions and S.MAO oxidised NADH. This was more marked below pH 8.2. The potent respiratory chain inhibitors, antimycin A and sodium amytal were effective in blocking the activity of the mitochondrial NADH oxidases but they were ineffective in the case of NADH oxidation by S-MAO. The NADH oxidation by S.MAO was most probably a reflection of the pH of the buffer, since at higher pH, the NADH oxidation was abolished. NAD was found to stimulate INT reduction by S-MAO in the presence of tyramine and some other amines (see table IX). All the amine/INT reductase assays were, however, carried out at pH 7.6. This would mean that the NADH formed in such assays would be rapidly oxidised by the tissue, probably so that very little would be available for reducing INT during the amine/INT reductase reaction of S.MAO. Nevertheless, there was considerable NAD stimulation of enzymic tetrazolium reduction by amines. This would imply that the two systems are not

equivalent. A comparison of the amine/INT and amine/NAD reductases of S-pH5 MAO (see table XIV) shows that this is apparently correct. This was confirmed by the results of sonication from which it was found that though the substrate specificities of the amine/NAD reductases of the soluble MAO preparations had been altered as the result of sonication of the homogenate, those of the amine/INT reductases remained unchanged (see table XXVII).

It was possible, therefore, that though NADH oxidation could not be blocked in the amine/NAD reductase system of S-MAO by the respiratory inhibitors, they might do so in the amine/INT reductase reactions of the soluble MAO preparations. It was, therefore, decided to test the effects of the respiratory chain inhibitors, antimycin A and amytal on the amine/INT reductase systems of soluble and mitochondrial MAO preparations from guinea pig liver.

Table XXXIX summarises the results obtained when mitochondrial suspensions (in phosphate buffer) and soluble MAO preparations were assayed for INT reduction under specified conditions.

Tetrazolium reduction by mitochondria with tyramine as substrate was about 50-70 per cent stimulated by 0.15mM NAD (final conc.) alone.

The tyramine/INT reductase reaction was 11 per cent inhibited by 0.025mM nicotinamide (final conc^{n}). Though nicotinamide does not inhibit the respiratory chain a mixture of NAD and nicotinamide at the concentrations stated strongly inhibited (62 per cent) INT reduction by mitochondria in the

presence of tyramine; the endogenous INT reduction was, however, strongly stimulated by the mixture. Antimycin A (2 μg/ml.) was found to stimulate the mitochondrial catalysed INT reduction by tyramine (8 per cent). The presence of antimycin A, however, enhanced the nicotinamide inhibition of INT reduction. On the contrary, antimycin A partially supressed the strong inhibition effect of NAD/nicotinamide mixture. The endogenous reaction was greatly stimulated by the combined mixture of antimycin A, NAD and nicotinamide.

These observations were in contrast with those had when amytal was used. The mitochondrial amine/INT reductase reaction was about 60 per cent stimulated by 3.3 mM amytal (final conc). As stated earlier, nicotinamide inhibited the reaction about 11 per cent. The presence of nicotinamide, however, was to enhance amytal stimulation of enzymic INT reduction by mitochondria by about 140 per cent. Though NAD-nicotinamide mixture strongly inhibited enzymic INT reduction by tyramine about 60 per cent, the effect of amytal at the specified concentration was to stimulate the reaction by about 140 per cent. In all experiments involving amytal, the endogenous INT reduction was not stimulated by this chemical.

With the soluble enzyme preparations (S.MAO S-pH5 MAO S.S.MAO and S.S-pH5 MAO) there was no observable inhibition of enzymic INT reduction by nicotinamide or by NAD-nicotinamide mixture. Antimycin A alone was, however, found to slightly inhibit the reaction in the S.S.MAO preparations. In the presence of NAD nicotinamide mixture, antimycin A strongly stimulated INT reduction by S.MAO and S-pH5 MAO in the pre-

sence of tyramine.

These observations show that the amine/INT reductase systems of mitochondria and the soluble MAO preparations are very complex. The complexity is emphasised in their behaviour towards the respiratory chain inhibitors.

Furthermore, it appeared the effects of antimycin A and amytal on the mitochondrial system in the presence of INT were not equivalent. The behaviour of the inhibitors towards nicotinamide in the mitochondrial system were also different. These observations suggest that separate pathways operate in mitochondria for INT reduction by tyramine in the presence of the respiratory chain inhibitors.

TABLE XXXIX. THE EFFECTS OF SOME RESPIRATORY CHAIN INHIBITORS OF INT REDUCTION BY GUINEA PIG LIVER MAO PREPARATIONS IN THE PRESENCE OF TYRAMINE.

Exp.	Tissue preparation	NAD*	Nicotinamide	Inhibitor	Final concn.	µmoles formazan /100mg protein/ hr.	µmoles "extra" formazan /100mg protein/ hr.
1.	Mitochondrial suspension	-	-	-		110.88	109.0
		+	-	-		166.71	164.0
		-	-	AntimycinA	2ug/ml	119.4	117.5
		-	+	-		99.16	97.0
		-	+	AntimycinA	2ug/ml	67.26	63.5
		+	+	-		48.19	41.05
		+	+	AntimycinA	2ug/ml	80.78	76.5
2.	Mitochondrial suspension	-	-	-		76.00	74.1
		+	-	-		127.16	125.0
		-	-	Amytal	$3.3x10^{-3}$M	121.00	118.2
		+	-	Amytal	$3.3x10^{-3}$M	178.16	176.0
		-	+	-		68.18	66.0
		+	+	-		32.7	28.9
		+	+	Amytal	$3.3x10^{-3}$M	176.9	174.1
3.	Soluble S.S.MAO	-	-	-		43.8	41.8
		-	-	AntimycinA	2µg/ml	46.6	44.8
		-	+	-		43.8	41.8
		-	+	AntimycinA	2µg/ml	39.2	36.0
		+	+	-		43.8	41.8
		+	+	AntimycinA	2µg/ml	40.0	37.4
4.	Soluble (sonic) (S.S. pH5)MAO	-	-	-		56.9	55.7
		-	-	AntimycinA	2µg/ml	56.9	55.7
		-	+	-		56.9	55.7
		-	+	AntimycinA	2µg/ml	51.7	49.3
		+	+	-		57.0	55.7
		+	+	AntimycinA	2µg/ml	50.0	48.4
5.	Soluble S.MAO	-	-	-		12.3	9.0
		-	+	-		12.1	9.0
		-	-	AntimycinA	2µg/ml	12.2	9.0
		+	+	-		38.55	30.3
		-	+	AntimycinA	2µg/ml	12.0	9.0
		+	+	"	"	38.2	34.9
6.	Soluble (S-pH5) MAO	-	-	-		14.3	12.3
		-	+	-		14.3	12.3
		-	-	AntimycinA	2µg/ml	14.3	12.3
		+	+	-		30.0	27.0
		-	+	AntimycinA	2µg/ml	14.3	12.3
		+	+	"	"	33.0	30.2

* Final molar concentration of NAD: $1.5x10^{-4}$M

** " " " " nicotinamide: $2.5x10^{-3}$M

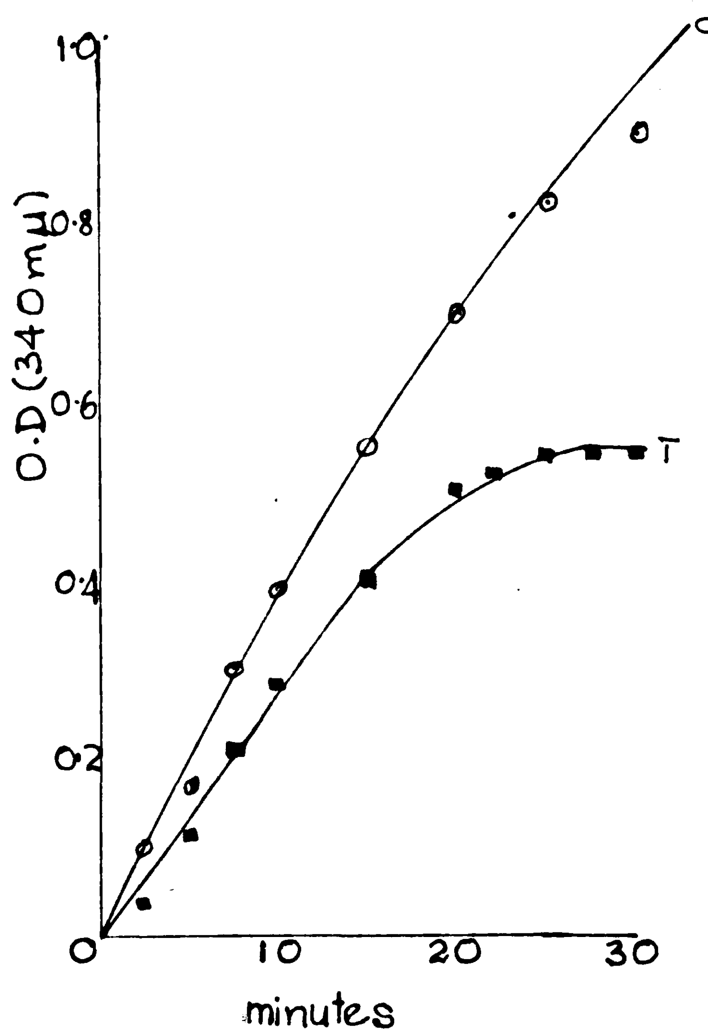
5. The effects of Arsenite on NAD reduction by S.S.MAO.

Arsenite (0.001M final conc.), a typical inhibitor of the system, S - S ⇌ SH SH was found to inhibit amine/NAD reductase activity after 10 minutes preincubation with the enzyme in the absence of added substrate (tryptamine) (Fig.10). The inhibition was progressive as a function of time and was complete within 30 minutes. This was suggestive of the requirement for a longer preincubation time. The inhibition of the reaction by arsenite also suggest the requirement of SH and -S-S-groups for full enzyme activity. This was in partial agreement with the findings of Friendenwald and Herrmann(121) and Singer and Barron(123) who first demonstrated the requirement of SH groups by MAO for full activity.

FIG. 10. THE EFFECTS OF ARSENITE ON TRYPTAMINE/NAD REDUCTASE ACTIVITY OF S.S MAO.

C = Control.

T = Arsenite 10^{-3} M (final $conc^{n}$) was present.



6. INT and NAD reduction by highly purified solubilised mitochondrial MAO.

Aldehyde oxidase activity in rat liver mitochondria is said to be negligible(124). If the postulate of Weissbach et al.(35,99) that INT and NAD reduction by MAO preparations is mediated by aldehyde oxidase is correct, then highly purified rat liver mitochondrial MAO could not catalyse INT and NAD reduction by amines. Highly purified and solubilised rat liver mitochondrial MAO shown to have no aldehyde oxidase activity(120) was generously supplied by Dr. Youdim. Preliminary experiments showed that the enzyme contained no aldehyde oxidase activity when it was tested for tetrazolium or NAD reduction with acetaldehyde. This was in agreement with the findings of Youdim.

On the contrary, when the enzyme was assayed for INT or NAD reduction by tryptamine, there was a definite enzyme activity in both oxidoreductase systems. This then was a direct contradiction of what was expected on the basis of the postulate(35,99). The only plausible explanation for this would be that aldehyde oxidase is not the only pathway for NAD or INT reduction during MAO activity. This view was further strengthened by the findings that semicarbazide (0.016M final conc.), a well known aldehyde trapping agent did not appear to have much effect on the enzyme activity in both reductase systems.

Table XL briefly summarises typical results in the two reductase systems.

From the table it is clear that the enzyme activity was

generally much less than that observed for the guinea pig liver enzyme (cf. tables IX, XIII, XIX, XXII). This was, however, in agreement with what is known about the relative activities of the enzyme in these two species(16,36,116). The inhibition of the enzyme activity by semicarbazide in the absence of aldehyde oxidase activity may mean a direct inhibition of the enzyme by semicarbazide.

TABLE XI. (A,B) INT AND NAD REDUCTION

BY HIGHLY PURIFIED AND SOLUBILISED

RAT LIVER MITOCHONDRIAL MAO.

Reductase system	Substrate	Inhibitor	Final molar conc. (M)	Activity in μmoles/100mg protein/hr. ж
INT reductase (A)	1. Acetaldehyde	-		0.000
	2. Tryptamine	-		30.01
	"	+	semi-carbazide (0.016M)	24.0
NAD reductase (B)	1. Acetaldehyde	-		0.00
	2. Tryptamine	-		7.5
	"	+	semi-carbazide (0.016M)	4.9

ж Activity in system A was expressed in μmoles formazen.

Activity in system B was expressed in μmoles NADH.

CHAPTER FOUR

THE SEARCH FOR THE POSSIBLE ISOENZYMES OF GUINEA PIG LIVER MONOAMINE OXIDASE PREPARATIONS.

A: Introduction.

It has been shown unequivocally that a number of enzymes derived from a tissue or even a single cell can exist in multiple molecular forms, with distinct electrical, chemical and immunological properties. Thus Vesell and Bearn(125) and Markert and Møller(126) demonstrated by electrophoresis that crude tissue preparations could resolve five distinct forms of lactic dehydrogenases (LDHs). Markert and Møller suggested the term "Isozyme" for such multiple forms. "Isoenzyme" and "I s o z y m e" are synonyms. Isoenzymes may be distinguished by such properties as their mobilities during electrophoresis, their cofactor requirements and their susceptibilities to inhibitors. Thus Kaplan and collaborators(127,128) were able to differentiate LDH isoenzymes by means of their catalytic properties towards NAD and its analogues. Thorne(129) showed that two malate dehydrogenases (MDHs) of rat liver may be either separated physically by DEAE cellulose chromatography or by their catalytic properties. One of the isoenzymes is mitochondrial and the other is found in the supernatant fraction, in other words, it is soluble.

Similarly, isoenzymes have been demonstrated in spermatozoa, thus establishing unequivocally that a single cell

type may have its own isoenzymes(130). Further still, Blanco and Zinkham(131) and Goldberg(132) have shown that of the five LDH isoenzymes found in human tissues and sperms, one is unique to the sperm cell and is not found elsewhere in any other human tissues or cells.

Although monoamine oxidase is generally believed to show a broad substrate specificity(20), Oswald and Strittmatter(36) showed by differences in temperature stabilities that the enzyme present in rat and guinea pig tissues may be more than one. Similarly, Gorkin(133) and Gorkin and collaborators(38) showed by partial separation by chromatography of rat liver mitochondria and by inhibitor sensitivities respectively that the mitochondrial enzyme may exist in two enzyme forms. The availability of a soluble MAO from guinea pig liver, therefore, led the author to investigate further if the soluble enzyme is identical to or different from the mitochondrial enzyme.

Since one of the most powerful, relatively cheap and convenient methods currently used for demonstrating isoenzymes is electrophoresis, this was the method applied to the study to be reported.

It may be stated from the outset that when this research was started, there was no literature anywhere on the electrophoresis of MAO. The methods adopted were therefore based on trial and error.

B: Methods.

1. Preparation of tissue: Soluble enzymes were prepared as already described in Part I under Methods. Any other modifications will therefore be described alongside the experimental results.

2. Starch gel electrophoresis of MAO.

Horizontal starch gels were prepared in perspex trays (23 cm x 9 cm x 4 mm) by the method of Smithies as described by Ivor Smith(134), using 10-12.8 g hydrolysed starch per 100 ml buffer solution.

(a) Rapid method: Modified Wieme's agar gel method(135).

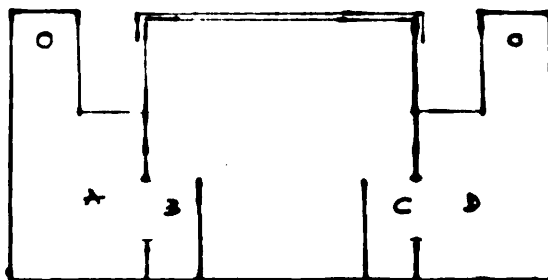
Gels cut from perspex trays were transferred onto thin glass plates (11 cm x 8 cm x 2mm). Contact with electrode vessels was by agar gel forming a bridge between the electrode and buffer compartments as seen in fig. 11.

FIG. 11. SCHEME OF WIEME'S AGAR-GEL ELECTROPHORESIS TANK (SIDE VIEW)

Tanks A and D are buffer compartments and tanks B and C are filled with buffered agar gel.

The large holes AB and CD ensure good electric continuity.

The central tank is filled with petroleum ether to cool the gel which is placed on the bridge BC.



Cooling was by petrol ether (40-60°C bp) poured onto the gel.

Samples were applied by inserting six 3 MM Whatman filter paper wicks soaked in the appropriate protein solution into vertical slots made in the gel. Electrophoresis was at a potential gradient of about 20 volts/cm for about 3-5 hours.

(b) Slow method: This was performed by the method of Smithies as described by Ivor Smith(134). Gels prepared in perspex trays as already described were used.

Samples were applied as already described in (a) but were sealed in with a microscopic slide smeared with vaseline. The gels were covered with parafilm to prevent evaporation. Contact with the electrode compartment was made by buffer soaked 3 MM Whatman filter paper strips. Electrophoresis was overnight (18-24 hours) at a constant voltage of 120 volts or 3 mA/gel.

After the electrophoresis, the gels were sliced horizontally and stained as follows:-

(i) Protein bands were detected by staining for 5 minutes in a 1 per cent solution of amidoschwarz 10B in methanol/water/acetic acid (50: 50: 20v/v) and washed for several days with 10 per cent acetic acid in methanol. Alternatively, they were stained for an hour in 1 per cent amidoschwarz 10B in glycerol/water/acetic acid solution (50: 50: 20 v/v).

The gels were cleared in glycerol/acetic acid/water mixture.

(ii) For the detection of MAO activity on gels, the following reaction mixture was generally used:-

The gel was covered in a solution containing 0.005M amine

substrate (neutralised), 10 mg NBT, 45 ml 0.1M sodium phosphate buffer pH 7.6 and water to make a final volume of 50 ml. Water replaced the substrate in controls. For inhibition studies, 0.002-0.005M iproniazid (final conc^{n}) was preincubated with the gel for 30 minutes at 37°C before adding the substrate and dye mixture. With the complete system, the time of incubation was not more than 90 minutes. Depending on the enzyme activity, the incubation could be 20 minutes or even less.

(c) <u>Buffers used for starch gel electrophoresis</u>.

i. Phosphate buffer, pH 6.8-8.0
   gel buffer: 0.04M
   reservoir: 0.06M

ii. Borate buffer, pH 8.6
   gel: 0.03M
   reservoir: 0.30M

the pH was adjusted with NaOH (1.0M).

iii. Discontinuous system: Method of Poulik
   Tris/citrate/borate buffer system, pH 8.6
   gel: tris (hydroxymethyl) aminomethane (tris) 0.076M and 0.005M citric acid.
   reservoir: 0.30 borate

the pH was adjusted with 1.0M NaOH.

(ii). <u>Thin layer agar gel electrophoresis (polyvinylpyrrolidone, PVP/agarose)</u>.

The electrophoresis was conducted by the method of Ogita(136). The excessive endoelectrosmotic flow of agar gel was reduced by adding PVP to the agar gel. Since all

other types of agar (e.g. agar Noble) tested gave very plastic gels, agarose was preferred.

The buffer used was either

(a) Tris/glycine buffer, pH 9.6

gel: 0.094M

reservoir: 0.376M

or

(b) Veronal buffer pH 8.6

gel: 0.025M

reservoir: 0.050M

Procedure: The gels were generally prepared in batches as follows:-

Agarose powder (0.5-0.7g) was mixed with an equal amount of PVP. The mixture was poured into 100 ml of the appropriate buffer and the resulting mixture was left at room temperature for 24 hours. The mixture was then boiled until it was completely clear and it was then poured into boiling tubes in 50 ml lots. The stocks were covered and kept at 5°C in the dark until required for electrophoresis.

Thin layer gels were prepared by pouring molten gels onto horizontal thin plates (11 cm x 8 cm x 2 mm). Samples were applied by pouring the enzyme and molten gel mixture at 35-40°C into long thin slots made in the horizontal gel. More molten gel was poured over the sample to seal it in. Connection between buffer and electrode compartments was by 2 per cent agar gels prepared in bridge (reservoir) buffer. Cooling was by pouring petrol ether (40-60°C b.p) on the gel, i.e. as described for fast horizontal starch gel method. The electrophoresis was for 30-45 minutes at 20 volts/cm.

The enzyme activity on gels was detected by the tetrazolium technique using the standard mixture described under starch gel electrophoresis (see 2b, ii).

Protein bands were similarly detected by staining with amido schwarz (amidoblack) 10B solution (see b, i).

(iii). Cellogel Electrophoresis.

This was conducted as directed by the manufacturers(137) and in 0.05M veronal buffer, pH 8.6.

Procedure:

Cellogel strips (17 cm x 4 cm), were submerged in veronal buffer for 10 minutes. The strips were then laid between two sheets of clean filter paper to remove excess buffer. The strips were then placed in position on the electrophoretic bridge with the opaque side facing up. Samples were applied by dipping a coverslip into the enzyme solution and then carefully applying it to the strips.

Electrophoresis was at 0.09 mA/strip for 90 minutes. Each strip was cut into 4 thin longitudinal striplets which were stained as follows:-

(a) Protein bands: were stained for 5 minutes in a reaction mixture consisting of 0.5 g Lissamine green; 50 ml methanol; 40 ml water and 10 ml acetic acid. The excess stain was removed by washing with changes of 5 per cent acetic solution in water. The strips were made transparent by dipping for a minute in a solution comprising 50 ml water; 37 ml methanol; 5 ml acetic acid; 5 ml lactic acid and one or two drops of glycerol. (Lactic acid may be replaced with 8 ml diacetonalcohol or 6 ml diacetin). The strips were then placed on glass plates and incubated in the oven at 60°C until they were completely transparent.

(b) The enzyme activity was detected as described for starch gel.

(iv). Polyacrylamide gel electrophoresis.

The polyacrylamide gel combines the molecular sieving effect of starch gel with a unique property of being able to provide gels the concentrations of which can be varied over a wide range. This simply means that the molecular size of the gel matrix can be controlled at will to suit the conditions likely to result in a successful resolution of protein bands. The separation of bands of the test material (the enzyme preparations) were shown to be poor in starch, PVP agar gel and cellogel supporting media.

The versatility of acrylamide gel led the author to extend the study to polyacrylamide gels. 5 per cent gels were prepared from either cyanogum 41 or acrylamide/bis acrylamide for the purpose.

Procedure:

(a) Five per cent gels were prepared from cyanogum 41 by dissolving 5g cyanogum 41 in 95 ml buffer. 0.1g of ammonium persulphate was dissolved in the solution to serve as a catalyst for polymerization. 0.1 ml of N:N:N':N': - tetramethyl methylene diamine was then well stirred in the solution to initiate the reaction. The solution was carefully poured, with a syringe, down the sides of thin vertical glass tubes (diameter: 0.5 cm; length: 7.5 cm), marked about 1.2 cm from the top and capped at the bottom. Filling was up to the mark. A thin film of water was layered on the gel meniscus to keep it flat and exclude air which might otherwise interfere with the polymerisation. Gelling was within 20 minutes. The best gels were selected for the electrophoresis. The caps were removed and the tubes were placed vertically in position in the upper reservoir with the lower ends dipping in the buffer in the lower eservoir. 0.1 - 0.2 ml test samples were applied to the meniscus of the gels by means of a pipette, under the buffer surface to avoid introducing air bubbles. Bromphenol blue in crystalline bovine serum albumin solution served as a marker.

Electrophoresis was between 30 minutes and 3 hours depending on the buffer, with a current of 3mA/tube. Initially the current was one mA/tube but when albumin entered the gel the current was increased to 3mA/tube.

Buffers:

(i) Tris (hydroxymethyl) aminomethane (tris) 0.05M or 0.1M pH 8.9. The pH was adjusted by adding 2N HCl.

(ii) Tris/glycine pH 9.5.

i.e. 0.094M tris buffer pH 9.5. The pH was adjusted with saturated glycine solution.

(b) Acrylamide/bis acrylamide gel electrophoresis.

Stock solutions:

(i) 0.376M tris (hydroxymethyl) aminomethane (tris) buffer pH 9.5 adjusted to the correct pH with saturated glycine.
(ii) the same buffer containing 0.6 ml N:N:N':N' - tetramethyl methylene diamine ("temed") per 500 ml buffer (for gels).
(iii) 19g acrylamide and 1 g N:N' - methylene bis-acrylamide all dissolved in 80 ml water.
(iv) 0.2 per cent ammonium persulphate solution in water. The solution was kept frozen until required and was freshly prepared every week.

Preparation of 5 per cent gels.

5 per cent gels were prepared from stock solutions by mixing equal volumes of gel buffer (solution ii) and acrylamide bis-acrylamide solution (iii) with two volumes of solution (iv) at room temperature.

The capped vertical tubes were filled to the mark as already described in the text for cyanogum 41. The running buffer (solution (i)) was diluted three times with cold distilled water.

The electrophoresis was carried out for 30 minutes.

Sample and spacer gels were excluded for both gel types. Before the actual electrophoresis of either gel type, there were 30 minute pre runs for stabilising the medium and for removing any traces of impurities such as the decomposition products of persulphate or excess persulphâte from the gels.

The stock solutions and the method of gel preparation just described were used in the initial stages of this work.

It was found that polymerization time was erratic and very unreliable, similarly the electrophoretic patterns were equally variable when the stock solutions were used. Experience showed that better results were obtained if all the solutions except solution (i) were freshly prepared in the amount required for preparing gels for a daily run. Under such conditions polymerization was within 20 minutes and reproducible results were also obtained. The method for preparing such gels is as follows:-

Acrylamide: 4.75 g)
bis-acrylamide: 0.25 g) dissolved in running buffer (solution i); 0.1 g ammonium per sulphate was dissolved in the solution; 0.1 ml "temed" was finally stirred in the solution to initiate the reaction (polymerization). Filling of tubes was as already described. This method of preparing the gels was the same whether the running buffer was tris/HCl or tris/glycine.

Generally it took 2-3 hours to conduct the electrophoresis in tris-HCl buffer pH 8.5 (0.1 0.05M), with a current of 4mA/tube.

After the electrophoresis, the gels were stained in test tubes with the various reaction mixtures, i.e. the enzyme activity was detected by the tetrazolium technique while protein bands were detected with Lissamine green solution as was described for cellogel.

RESULTS

1. Starch gel electrophoresis of guinea pig liver MAO preparations.

Generally the results of the electrophoresis were rather poor. It was, however, possible to detect enzyme activity by the tetrazolium technique using either NBT or INT. Fig. 12 shows a typical gel. The electrophoresis was in borate

buffer, pH 8.6 (see ii, buffers). The dye was NBT (10 mg in 50 ml of reaction mixture consisting of 0.005M amine substrate, 45 ml 0.1 M phosphate buffer, pH 7.6 and water). From the figure, two bands can be seen apart from the stain at the origin. The enzyme activity on the other half of the gel (left) was suppressed by 0.002M iproniazid (final conc). The iproniazid was run with the enzyme during the electrophoresis by the rapid method.

Similar results were also obtained by preincubating the gel. with iproniazid in the absence of the dyes (tetrazolium salts) for 30 minutes. The substrates used were tryptamine and tyramine. The result was the same for both substrates.

The effects of buffer on the electrophoresis.

Generally, borate buffer (continuous system) gave better results than either phosphate buffer or tris-citrate/borate buffer. With phosphate buffer, the separation was very poor due to excessive trailing. Fig. 13 shows a typical run in this buffer, pH 8.0.

From the figure, it can be seen that no bands were separated during electrophoresis. The results described were obtained with 50 per cent aqueous liver homogenates. The only enzyme preparation which gave similar results was sonicated mitochondrial supernatant. None of the soluble enzyme preparations gave any detectable enzyme activity on the gel after electrophoresis.

In all acases, however, the paper wicks were stained weakly after the electrophoresis. Attempts to concentrate the enzyme did not improve the result. Whether the enzyme

preparations were inactivated on the gel or not was difficult to tell.

Apart from one or two cases in which cathodic bands were detected with the homogenates after electrophoresis, all the bands were anodic in movement.

Because of the poor resolution in starch gel supporting medium, the study was extended to other electrophoretic supporting media.

FIG. 12. STARCH GEL ELECTROPHORESIS OF 50 PER CENT AQUEOUS GUINEA PIG LIVER HOMOGENATE.

The elctrophoresis was in borate buffer pH 8.6 (see text) for 3-5 hours at a potential gradient of 20 volts/cm.

For the staining mixtures and the method of staining see the Methods section of Part II.

The blank part of the gel(left) indicates the effect of 0.002M iproniazid.

The part showing at least 2 bands (right) indicates the result of staining with monoamine oxidase substrate, tyramine hydrochloride.

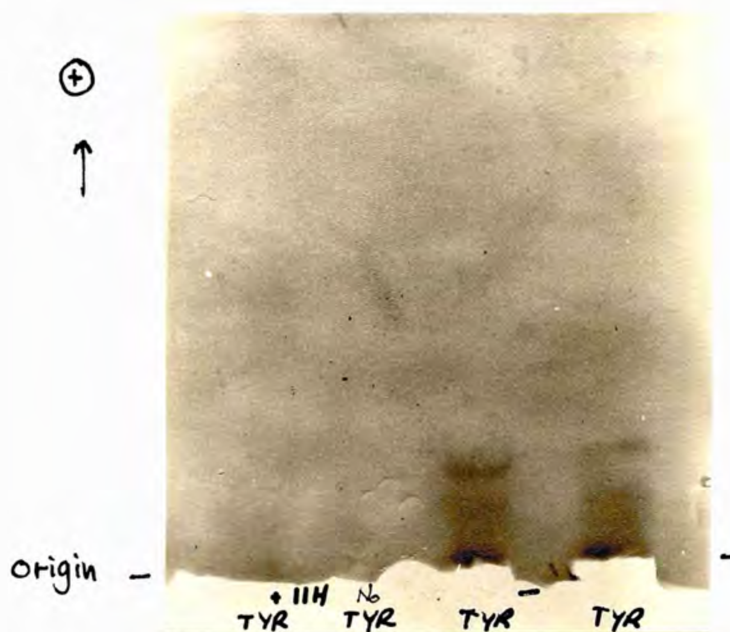
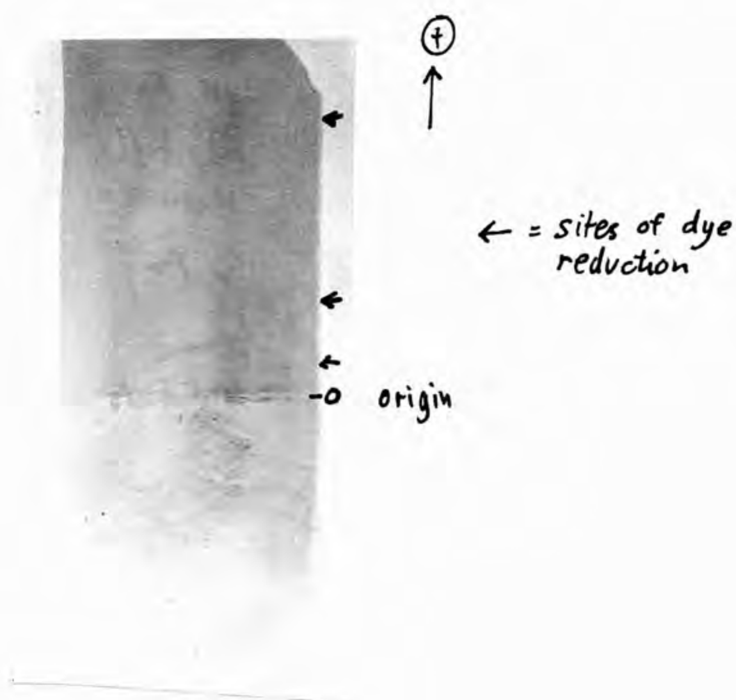


FIG. 13. THE EFFECTS OF BUFFER ON STARCH GEL ELECTROPHORESIS OF 50 PER CENT AQUEOUS GUINEA PIG LIVER HOMOGENATE.

The electrophoresis was conducted in sodium phosphate buffer pH 7.5 (0.04M gel buffer and 0.06M bridge buffer), at a potential gradient of 20 volts/cm for 3-5 hours.

Staining was by the tetrazolium technique already described in this section.



2. <u>Results of thin layer agar gel</u>.

The electrophoretic separation of bands in the PVP agar medium with any of the buffers was very poor. In other words, the PVP agar gel medium provided no great advantage over the starch gel supporting medium as far as the resolution of the bands of the enzyme were concerned. Fig. 14 shows typical results obtained with a 45 minute run in veronal buffer, pH 8.6 (gel buffer 0.025M; bridge buffer 0.05M) using S.MAO (fig. 14A) and S.S.MAO (Fig. 14B) preparations. In fig. 14A, tryptamine was tested as substrate and semicarbazide (0.01M final conc^{n}) was tested as a trapping agent. It is seen that in the presence of tryptamine the staining of the gel was rather weak (gel on the right) while the staining in the presence of tryptamine and the trapping agent (semicarbazide) was relatively more intense (gel on the left). The result was contrary to what was expected on the basis of the postulate of Weissbach *et al*.(99) already stated in detail. It is also contrary to what was expected on the basis of what is known on the nature of MAO substrates. Similar results were obtained with S.S.MAO runs as shown in Fig. 14B. Tyramine was the substrate (gel on the right) and sodium azide (0.01M final conc^{n}) was the inhibitor. It will be observed that semicarbazide stimulated dye reduction while the enzyme activity in the presence of tyramine was weak.

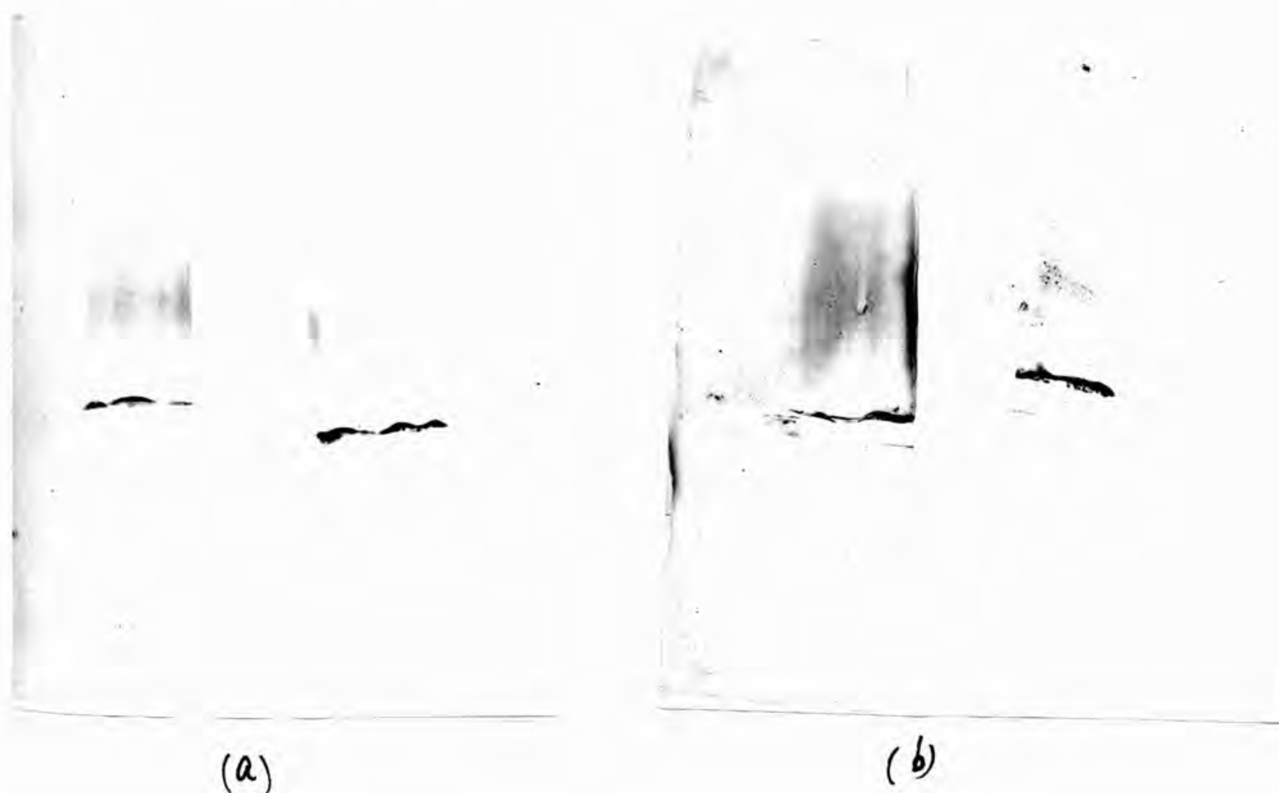
In attempts to characterise further the properties of the enzyme on PVP agar gels, iproniazid, a well known MAO inhibitor was tested. This was found to act as a substrate for the enzyme. A sharp cathodic band was detected by the tetrazolium

FIG. 14. THE EFFECTS OF CARBONYL AGENTS ON THE DETECTION OF ENZYME ACTIVITY AFTER PVP AGAROSE GEL ELECTROPHORESIS OF S.S.MAO.

The thin layer PVP agarose gel electrophoresis was conducted in veronal buffer pH 8.6 (gel buffer 0.025M and resevoir buffer 0.05M) at 20 volts/cm for 30-45 minutes.

In Fig. 14a semicarbazide 0.01M (final conc^{n}) was the carbonyl agent (gel on the right) and tryptamine was the substrate (gel on the left). The stain was NBT. Incubation was for 45 minutes.

In Fib. 14b sodium cyanide was the carbonyl agent (gel on the right) and tyramine was the substrate (gel on the left). NBT was the tetrazolium salt.



technique in one case.

It was not possible to detect the enzyme activity on such gels with homogenates. Similarly, it was impossible to use agar alone as a supporting gel medium for electrophoresis. The gels were very plastic. The reason was not apparent.

The electrophoresis was extended further to gels including cellulose acetate paper and cellogel (a gelatinised cellulose acetate paper).

3. Results of Cellogel.

A typical result is shown in fig. 15. It would be seen that iproniazid (IIH) suppressed the enzyme activity (fig. 15A). In B both the origin and a distance about 15 mm from the origin were stained by the tetrazolium method with NBT as dye and tryptamine as substrate. C and D show a broad protein band spreading from the origin and forming a peak at about 15 mm from the origin. The peak corresponds to the area in B which showed enzyme activity. The protein was detected with Lissamine green solution as described under procedure. In all cases, the movement of the sample was anodic. This was in contrast with what Tipton(73) demonstrated with his pig brain enzyme which moved cathodically in tris-HCl buffer, pH 8.9. Whether these differences in movement were caused by differences in the method of enzyme extractions or by species differences is uncertain. Species differences in MAO activity are well known(16,22,36,38). It is also well known that many factors such as the method of homogenisation, the concentration of reaction mixtures, the period of storage of tissue or enzyme may affect the isoenzymes of a particular enzyme(139).

FIG. 15. CELLOGEL ELECTROPHORESIS OF D.S.MAO.

The electrophoresis was conducted in 0.05M veronal buffer at a constant current of 0.09 MA/strip of 17cm. x 4 cm.

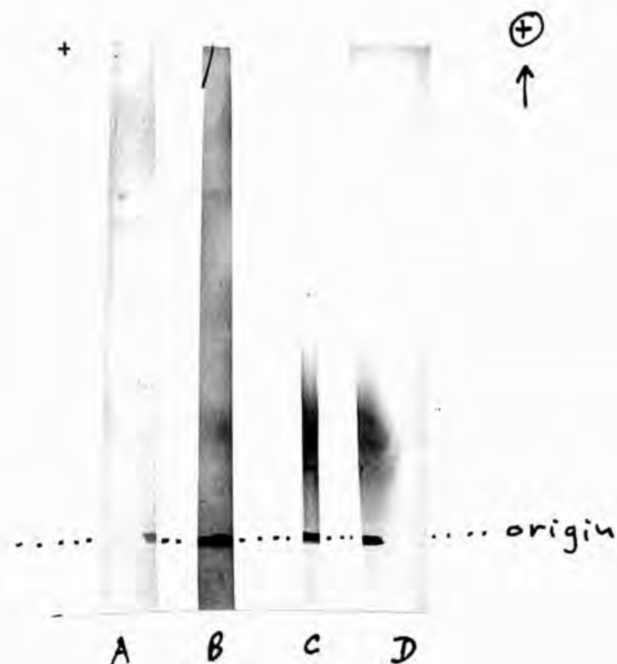
The strips were stained as follows:

A: staining in the presence of iproniazid.

B: staining in the presence of tryptamine hydrochloride (Neutralised).

C: staining in the presence of Lissamine green 0.5 g in 50 methanol.

D: staining in the presence of lissamine green in 40 ml water and 10 ml acetic acid.



It was not possible to demonstrate the enzyme activity by the electrophoresis of homogenates. Attempts with cellulose acetate paper in preliminary studies were also unsuccessful.

Such preliminary experiments were also conducted with homogenates. It was possible that the failure was due to dilute sample. The result just presented was obtained from a typical S.S.MAO preparation.

4. Results of Polyacrylamide gels.

Fig. 16 shows typical results obtained with cyanogum 41 electrophoresis under specific conditions. Fig. 16A shows the electrophoretic patterns obtained with 50 per cent sonicated aqueous guinea pig liver homogenate preparation. The substrate was tryptamine. The electrophoretic buffer was 0.094M tris glycine, pH 9.5. Starting from the left to the right, it can be seen that the enzyme activity was abolished by parnate 0.0002M (final conc.) both in the control (substrate blank) and in the presence of added substrate, tryptamine. Two bands can be seen, one at about 2.5-3 cm and the other at about 6.5 cm from the origin. The bands were somewhat diffuse. The stained bands and the stain at the origin represented the enzyme activity in the presence of added substrate. In the extreme right, only the stain at the origin can be seen, this was obtained with substrate blank.

Fig.16B compares the results obtained under similar conditions of electrophoresis when S.S.MAO and highly purified solubilized rat liver mitochondrial MAO, generously supplied by Dr. Youdim, were tested in the same experiment. As in

Fig. 16A, the electrophoresis was 25 minutes in 0.094M tris/ glycine buffer, pH 9.5 with 3mA/tube. Starting from the left to the right, 4 gels can be seen. The first and the last gels were obtained with S.S.MAO when the enzyme activity was detected by the tetrazolium technique with tyramine and tryptamine as substrates, respectively. The second and the third gels show the zymogram patterns of the rat liver mitochondrial enzyme. The weaker stained gel showing two diffused and closely associated bands represented the result with tryptamine as substrate. The deeply stained, in fact, the most intensely stained gel represented the result of an attempt to inhibit the enzyme activity of the rat liver particulate (purified) enzyme with iproniazid 0.002M (final concn). Similar results were obtained with the S.S.MAO when iproniazid was tested as an inhibitor. In each case, iproniazid caused intense dye reduction at or near the site of activity seen with the substrate alone.

The results of Fig. 16B indicate clearly that the mobilities of the S.S.MAO and the rat liver particulate enzyme were very different. When the homogenate and its derived S.S.MAO zymograms are compared (Figs. 16 A and B) it is obvious that the extra band shown in A, i.e. the slow band, was not represented in the S.S.MAO derived from the homogenate.

FIG. 16. POLYACRYLAMIDE GEL ELECTROPHORESIS OF GUINEA PIG LIVER AND RAT LIVER MAO PREPARATIONS.

The electrophoresis was in 0.094M tris glycine pH 9.5. The gel was 5 per cent cyanogum 41.

In fig. 16A 50 per cent aqueous guinea pig liver homogenate was used. The staining for fig. 16A was as follows from left to right.

1st gel: the effect of 0.0002M parnate as inhibitor

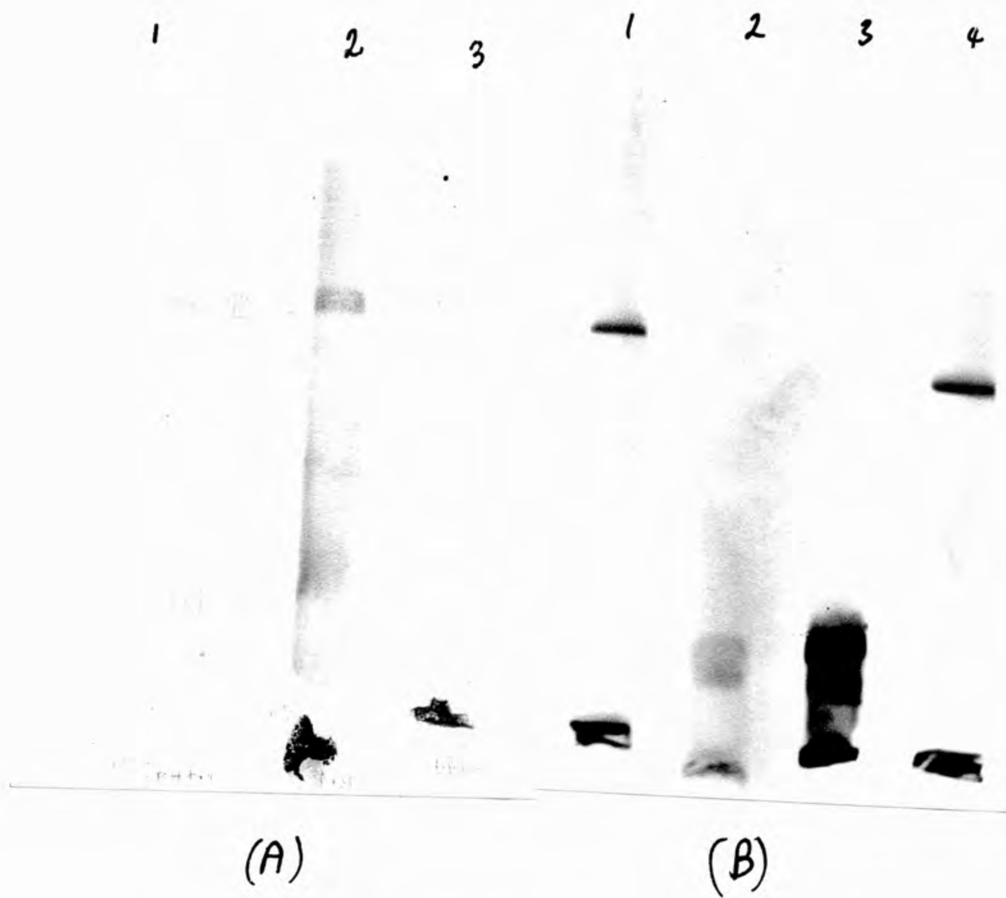
2nd gel: i.e. middle gel: staining in the presence of tryptamine hydrochloride

last gel: endogenous enzyme activity, i.e. substrate blank.

In fig. 16B S.S.MAO and rat liver MAO (provided by Dr. Youdim) were used.

The 1st and last gels were for S.S.MAO with tyramine and tryptamine respectively as substrate.

The 2nd and 3rd gels were of rat liver mitochondrial MAO. The 2nd gel was stained in the presence of tryptamine and the most intensely stained gel of all (3rd gel) was stained in the presence of tryptamine and iproniazid 0.002M (final conc^{n}).


1
2
3
1
2
3
4
(A)
(B)

The effects of the brand of dye on the pattern of the zymogram.

Fig. 17 shows the zymogram patterns when S.S.MAO symograms were stained after electrophoresis in tris-glycine buffer, pH 9.5. The gels were prepared from acrylamide/bis-acrylamide and the tetrazolium salt was NBT purchased from Koch and Light Ltd. In all, 5 gels can be seen. Starting from left to right are gels a - e in that order. (a) was obtained in the absence of added substrate. In gel (b), two clear bands about 2.5 cm and 8 cm from the origin can be seen. The bands were obtained in the presence of 0.0002M (final $conc^n$) parnate. In (c) - (e) three bands about 2.5, 8 and 9 cm. from the origin can be seen. The patterns were arrived at as follows:-

in (c) 0.0002M parnate (TCP) and tryptamine were present,
in (d) staining was caused by the tryptamine alone and
in (e) IIH and tryptamine were combined. Thus it can be seen that none of the inhibitors, TCP and IIH, had any effect on the enzyme activity when the Koch Light brand of NBT was tested as dye. In fact, there was not much difference between the substrate blank and the other gels since the blank also showed at least a band.

FIG. 17. THE EFFECTS OF THE BRAND OF TETRAZOLIUM SALT ON THE ZYMOGRAM PATTERN.

The electrophoresis was in 5 per cent acrylamide bis acrylamide gel with trisglycine buffer as in Fig. 18.

Staining was as follows with Koch Light brand of NBT.

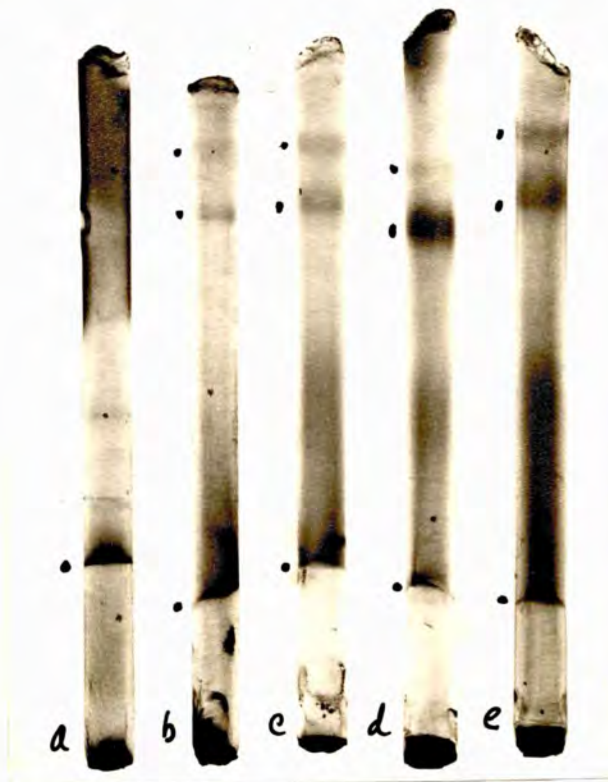
Gel a   substrate blank.

Gel b   stained in the presence of 0.0002M parnate.

Gel c   stained in the presence of 0.0002M parnate and tryptamine.

Gel d   stained in the presence of tryptamine.

Gel e   stained in the presence of tryptamine and iproniazid 0.002M.



The effects of the age of the solutions on electrophoretic patterns.

Fig. 18 shows typical results obtained when stock solutions were used in preparing acrylamide/bis acrylamide gels. It was found that not only did the age of the solutions used in preparing the gels affect the time of polymerization of the gels but it also affected the electrical mobilities of the test materials during electrophoresis. Thus a single enzyme preparation could give very variable results in a single run. In fig. 17 the 4 gels shown were prepared from solutions stocked for a fortnight. The gels were prepared at a sitting and were electrophoresed simultaneously in a single run in 0.094M tris glycine buffer, pH 9.5 at 3mA/tube. The enzyme extract was sonicated mitochondrial MAO prepared by the method of Youdim(81,90). The mitochondrial suspension was sonicated at 20 kc/second for 60 minutes in the presence of 0.003M benzylamine at 0-4°C. All other steps of the enzyme preparation were as described under "Methods" in chapter 2, No. 1.

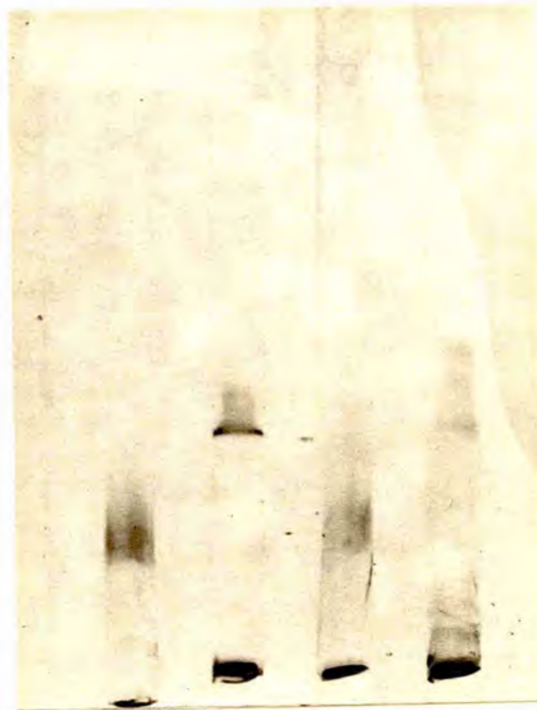
It can be seen from the data presented (fig. 18) that the zymogram patterns were not uniform. In other words, the electrical mobility of the enzyme differed from gel to gel. All the four gels were stained with Sigma type III NBT. The substrate for the first two gels (left to right) was tryptamine and tyramine was the substrate for the last two gels. The results were contrary to what would be expected when the conditions of electrophoresis, enzyme preparation and substrate were identical. One would expect the zymogram patterns to be

shown by different enzyme preparations and not by the same preparation under identical conditions. These inconsistent zymogram patterns resulting from preparing gels from stock solutions no more occurred when gels were obtained from freshly prepared solutions. Fig. 17 shows a typical result when the gels were prepared from fresh solutions. A comparison of figs. 17 and 18 will show that the pattern shown by gels in fig. 17 was very reproducible and consistent while the result of gels in fig. 18 was erratic. The reason for the relatively slow mobility of the mitochondrial enzyme from guinea pig liver as compared to the fast movement of the S.S.MAO derived from a sonicated homogenate could be attributed to the age of the solutions.

FIG. 18. THE EFFECTS OF THE AGE OF THE SOLUTION ON THE ELECTROPHORETIC PATTERNS.

The zymogram patterns were obtained from a single enzyme preparation (sonicated mitochondria). The first two gels were stained in the presence of tryptamine hydrochloride and the last two gels were stained in the presence of tyramine hydrochloride.

The polyacrylamide gels were prepared from stock solutions of more than one week old.



CHAPTER FIVE

DISCUSSION

Monoamine oxidase (MAO) is a mitochondrial enzyme(32-42). The presence therefore of soluble MAO in the high speed supernatant fraction of guinea pig liver homogenate raises the question as to whether the soluble enzyme is identical to or different from the classical particulate MAO. However, before attempting an answer it is proposed to define clearly the meaning of the term "soluble" as applied to MAO. The term "soluble" refers to the enzyme derived from the high speed supernatant fraction after spinning it at 100,000g for at least two hours and finally precipitating the enzyme from the final supernatant fraction withgraded ammonium sulphate solution. The criterion for solubility was therefore based mainly on the centrifugation speed. By this method, Lehninger, Sudduth and Wise(144) referred to preparations of D- β-hydroxybutyric dehydrogenase from mitochondria as soluble. These authors obtained their enzyme after high speed centrifugation for an hour at 76,000g. Similarly, Oswald and Strittmatter(36) obtained their "soluble" MAO from guinea pig liver after spinning the homogenate at 100,000g for an hour. The original soluble MAO of Weissbach _et al_.(35) was prepared after high speed centrifugation at 100,000 for 30 minutes only. According to these workers, about 10 per cent of the total activity of the enzyme present in the homogenate was recovered as soluble MAO. Oswald and Strittmatter found a very much lower percentage for their preparations. From our preliminary experiments, it was found that at least 5 per cent of the total

MAO activity of guinea pig liver homogenate was recoverable in the high speed supernatant fraction while about a third was microsomal and the bulk was mitochondrial. This was in reasonably good agreement with the results of Oswald and Strittmatter.

This leads us back to the identity of the soluble and particulate fractions. The possibility that the soluble enzyme might differ from the classical particulate MAO is supported at first sight, by the fact that semicarbazide strongly inhibited the oxygen uptake of the soluble enzyme when isoamylamine was tested as substrate (see tables XV and XXXII) though the oxidation of this substrate by the particulate MAO from guinea pig liver was resistant to 0.01M semicarbazide (c.f. also refs. 15-16). It should be noted however that semicarbazide did not only affect the oxygen consumption by all the soluble MAO preparations tested with isoamylamine but it also abolished the endogenous respiration of all types of MAO preparations including homogenate and mitochondria. This effect of the chemical on the endogenous respiration of MAO was reported previously by other workers using MAO from different mammalian species(15,16,94). Furthermore, the chemical was found to strongly inhibit (i.e. 60 per cent) kynuramine oxidation by the S.S.MAO (c.f. table xxx) in the absence of any aldehyde oxidase activity since kynuramine aldehyde is known to spontaneously and nonenzymically cyclize to 4-hydroxyquinoline(116,117). It is therefore possible that semicarbazide had a direct inhibitory effect on the enzyme itself, at least in the case of isoamylamine and kynuramine. This then may suggest that there is present in guinea pig liver

homogenate and mitochondria a factor antagonistic to semicarbazide, this factor is either removed or is very much diluted during the preparation of the soluble MAO, thus making it more prone to the effects of semicarbazide. The possibility of an alternative pathway operating in the different fractions of MAO for the metabolism of isoamylamine may not however be excluded. Any suggestion that the soluble MAO might be identical or similar to the plasma enzyme is ruled out by the fact that the plasma enzyme is pyridoxalphosphate dependent and is incapable of metabolizing N-substituted amines(23,147). Moreover, the soluble MAO prepared from guinea pig liver displayed the substrate specificity typical of the classical MAO. Further still, the view that the various soluble MAO preparations (i.e. S.MAO and S.S.MAO and their corresponding acid derivatives) are identical to the classical enzyme is supported by their identical inhibitor sensitivities and catalytic properties apart from their different sensitivities to semicarbazide. Thus both types are sensitive to the typical MAO inhibitors: iproniazid and parnate to the same degree (c.f. table XIV A and B). Furthermore, the stoichiometry as judged from manometric experiments with and without semicarbazide indicated that both types of MAO catalyze the characteristic MAO reaction:- $RCH_2NH_2+\frac{1}{2}O_2+H_2O \rightarrow RCHO+H_2O_2+NH_3$. Further still, the substrate concentration curves for the particulate and soluble MAO preparations in the tetrazolium assay system yielded K_m values which were of the same order and comparable to the values obtained for the "classical" MAO using oxygen as final acceptor (tables XXI, see also refs. 19,22 & 36).

Further still, the effects of SH reagents and metal chelators on both types of enzyme preparations are similar (table XXXVIII; refs. 73,74,94). The similarity of the soluble enzymes derived from non-sonicated and sonicated homogenates in their catalytic properties in the tetrazolium and Warburg assay systems stands out as a plain evidence that the classical MAO is identical to the soluble MAO.

According to evidence from recent work on the subcellular localization of MAO(41,42,73), the enzyme is more or less exclusively located in the outer membrane of mitochondria. Since this membrane is easily ruptured, enzymes attached to it are easily solubilized. This may suggest that sonication merely ruptured the membrane thus releasing the free enzyme into solution. However this does not preclude the existence of soluble MAO in the supernatant fraction with very similar or identical properties to the mitochondrial enzyme. The results of the electrophoresis of the various soluble and particulate MAO and homogenate preparations also support the view that the soluble and "classical" MAO are either the same enzyme or are at least very similar as demonstrated by the tetrazolium and other techniques (see eg. chapter 4). This leads us to the very vexed controversy of whether or not MAO exists in isoenzymic forms.

As it was stated in the introductory part of chapter 4, there are claims that MAO exists in isoenzymic or multiple forms. This argument is based on the following observations:-

1. The substrate specificty of MAO is determined by the method of extraction. Thus Werle and Roewer(29) claimed to

have separated two types of MAO by acetone fractionation, one fraction of which was found to be very active in oxidising aliphatic monoamines (butylamine) while the other oxidised almost exclusively aromatic monoamines.

2. The properties of a given preparation of MAO vary greatly with the substrate used to measure its activity(16,22,38). These properties include sensitivities to various inhibitors(38), pH optima(22) and the effects of temperature on anion inhibition as has been claimed recently by van Woert and Cotzias(140).

3. The substrate specificity of MAO preparations has been observed to be determined by the origin of tissue or organ i.e. the substrate specificity has been found to be organo-specific(16,92,141,142). Thus Weiner(16) showed that the substrate specificity of brain and liver MAO of man, dog, cat, etc. varied greatly with the source of the enzyme. Similarly, Hope and Smith(92) showed that substrate specificities of MAO from different organs of mouse differed widely. Furthermore, Johnston(141) has claimed the existence of MAO inhibitor capable of discriminating between the two MAO activities in rat brain. This evidence has been further strengthened by the report by Squires(142) of the existence of three distinct mitochondrial MAO in mouse capable of deaminating kynuramine. These forms have been shown to be separable by their sensitivities to harmine and N-benzyl-N-methylpropagylamine (pargyline) and also by their thermal stabilities. While such evidence may be indirect, Gorkin(133) has claimed to separate, by Sephadex chromatography, multiple forms of rat liver MAO. Youdim and Sandler(143) have similarly claimed to have sep-

arated by acrylamide gel electrophoresis at least two forms of MAO from rat liver and three types from human placenta. This situation is further complicated by some of my own electrophoretic results (see chapter 4). In this connection it may be seen from figs. 12 and 16a that at least two bands were detected with guinea pig liver MAO on starch and acrylamide gels respectively. Only a single band was demonstrable by cellogel electrophoresis (fig. 15) and by acrylamide electrophoresis (fig. 16b) using S.S.MAO preparations. The preparations which gave two bands were homogenates. Furthermore, it can be seen from table XXVII that the substrate specificity of the S.MAO or S-pH5 MAO in the NAD assay system was reversed on sonicating the parent homogenate. In the case of the reversal of substrate specificity just stated, it may be recalled that Vesell and Brody(139) have pointed out that there are some factors which may lead to obtaining results suggestive of isoenzymes. Some of these factors include the method of homogenization etc. It is possible that sonication produced a comformational change in the enzyme molecule resulting in a changed substrate specificity. The reversal of substrate specificity after sonicating the homogenate may not therefore be a conclusive evidence that isoenzymes of MAO have been separated. The possibility however, that sonication may produce conformational changes in an enzyme molecule does not exclude the likelihood that sonication resulted in the differential inactivation or activation of substrate specific enzyme fractions though this did not show up by the zymogram technique in the case of MAO tested.

An argument in support of the possibility of preferential inactivation of different types (i.e. isoenzymes) of MAO by sonication, was the parallel observation in preliminary experiments that freezing and thawing preferentially affected the enzyme's ability to catalyse the tetrazolium reaction by amines without any bad effects however, on oxygen consumption and kynuramine disappearance. Thus, though it was for example impossible to measure its activity by this technique with isoamylamine and difficult with tyramine as substrates, it was quite easy to demonstrate it with tryptamine as substrate. It must be stated that the endogenous tetrazolium reductase activity was equally markedly diminished by the treatment. This phenomenon is obviously similar to the effects of sonication on the endogenous tetrazolium reductase activity where formazan formed by the S.MAO and S.S.MAO were respectively 3.68 and 1.64 moles/100mg protein/hour (see table XVIII).

Monoamine oxidase is a flavoprotein(71,73,74,80,83). It is inhibited by o-pheranthroline(73,74,94, see also table XXXVIIIB) and also by tiron(73). Werle and Roewer(29) claimed that the enzyme was inhibited by an iron-binding beta-globulin of human plasma. These observations indicate that the enzyme (MAO) is a metalloflavoprotein. Considerable evidence is accumulating to show that when metalloflavoproteins are prepared in a soluble form, changes do occur in their properties especially in relation to their acceptor specificity, particularly where NAD is involved(145). This fact has been described for a number of NAD-linked dehydrogenases(145). These changes are known to be caused by conformational and catalytic changes.

From this reasoning therefore, it may appear that the alteration of the NAD acceptor specificity of the soluble enzyme by sonication was most probably a direct consequence of conformational changes rather than the liberation of isoenzymes of MAO by the process.

By similar reasoning, it can be argued that the apparent detection or separation of different forms of MAO by heat treatment coupled with pH changes(36,38,81) may be referrable to conformational and catalytic changes in the enzyme molecule by the treatment. In this connection, Squires' claim of demonstrating the presence of at least three forms of mitochondrial MAO in different organs of rat and guinea pig by inhibitor sensitivity and thermal stability merits further experimentation. It is worthy to note that the author himself made the following comment: "It must be emphasized that the magnitude of the half-lives of the different forms of MAO are <u>very sensitive to changes in the experimental conditions</u>, including washing of the mitochondria, pH, and type of buffer used (sodium borate buffer, for example, gives results different from those obtained with tris-sulfate). In addition there may be other <u>critical</u> variables such as mitochondrial swelling[1] which were not controlled in the present work". These comments clearly call for caution before putting any interpretations on the results.

Recently, Tipton and Spires(146) and McEwen, Sasaki and Lenz(147) have produced kinetic evidence to indicate that pig brain or human liver mitochondrial MAO is a single enzyme species. None of these authors obtained any kinetic data

on the inhibition of the mitochondrial MAOs in question by pargyline to suggest otherwise. In the work of Tipton and Spires, the effects of harmine on the enzyme also confirmed their results with pargyline. While the findings of these two groups indicate that the tissues of the species studied do not seem to have isoenzymes of MAO, they cannot be taken as an absolute declaration that MAO cannot or does not exist in such forms elsewhere. It may be recalled that Tipton(73) demonstrated the presence of two forms of the pig brain MAO by sephadex chromatography, one of which had a molecular weight of 102,000 and was more active than the other fraction with a higher molecular weight. Furthermore, it may be recalled that Tipton(42) reported differences in the submitochondrial distribution of rat brain and liver MAO. So far, it is apparent that the only safe conclusion is that MAO is a heteroenzyme. The final decision whether MAO exists in isoenzyme forms or not cannot be taken unequivocally without further research. At present the experimental conditions are such that it is easy to mistake an artifact for the real. For example, it is easy to obtain several bands instead of one depending on the purity of the dye (see fig. 17). Similarly, the brand of dye used can determine whether or not the specific MAO inhibitors, iproniazid and parnate can effectively inhibit the enzyme on a gel. Furthermore, the age of the solution can also determine the number of bands (see fig. 18).

At this point we turn to the effects of sonication and semicarbazide on oxygen, tetrazolium and NAD acceptor systems of soluble and particulate MAO preparations with a view to

speculating on the mechanism of electron transfer.

That the classical MAO can in addition to oxygen, reduce tetrazolium salts also was clearly demonstrated by Lianzani(96), Francis(97,98), Blaschko(27), Lagnado and Sourkes(71,72,79), Lagnado(94) and Weissbach et al.(99). Weissbach et al.(35) also showed that not only oxygen and tetrazolium salts (INT) were reduced by the soluble enzyme (S.MAO) but also NAD. Table XXVII shows that the soluble enzyme preparations can reduce all the three electron acceptors: oxygen, tetrazolium and NAD by amines. It also shows that oxygen consumption increased 4-6 fold by sonication in two separate experiments using tyramine and tryptamine as substrates. Similarly, a 2-3 fold increase was also seen in NAD reductase assay involving tryptamine as a substrate but a 5-fold decrease occurred in the enzyme activity when tyramine was tested as a substrate. It can be seen that in the INT assay system there was a 3-4 fold stimulation with tryptamine as substrate but with a 5-6 times increase when tyramine was tested while the dependence of tyramine on NAD for reducing tetrazolium enzymically was greatly diminished by sonication. Similarly, table XVIII shows that tyramine/INT reductase activity was 6-fold stimulated by sonication while tryptamine/INT reductase activity was more than 3-fold stimulated by this treatment. The great dependence of tyramine/INT reductase activity on NAD was drastically diminished by sonication as shown. Sonication however had no effect on tryptamine/INT reductase affinity for NAD since the unsonicated enzyme did not require NAD. The endogenous tetrazolium reductase activity was reduced to less than half

the value of the unsonicated preparation. This suggests a preferential inactivation of different amine/tetrazolium reductases. This observation reported in table XVIII calls to mind the effects of freezing and thawing on the enzyme. Similarly, table XXII(B) shows that while sonication markedly increased the activity of the amine/NAD reductase system for tryptamine oxidation by the acid fraction (S.S.pH5 MAO), that for tyramine, β-phenylethylamine or isoamylamine exidation by the enzyme was correspondingly much decreased.

An examination of tables X-XIV, XVIII-XX, XXII and fig. 5b establishes it beyond doubt that though the soluble MAO preparations could reduce either NAD or INT by amines, NAD reduction by mitochondrial suspensions in the presence of amine (tyramine) was rather difficult to demonstrate since the reduced pyridine nucleotide (NADH) was rapidly oxidised by the tissue.

While NAD does not stimulate aerobic MAO activity as measured by manometry, the amine/tetrazolium reductase system can be strongly stimulated by NAD (ref. 94 cf. also tables IX and XI). In fact, it is possible to make the system completely dependent on NAD (table XI). In all cases involving NAD stimulation of tetrazolium reductase activity, the reaction with isoamylamine as substrate appeared to be the most stimulated. Under this condition the enzyme activity in the presence of isoamylamine is also the highest (tables IX and XI).

A comparison of the substrate specificities of the aerobic system i.e. oxygen consumption on one hand and the tetrazolium acceptor system on the other, with tyramine and tryptamine as

substrates, shows that the two systems are very similar if not identical (table XXVII). However, unlike the aerobic system which is insensitive to carbonyl agents such as cyanide and semicarbazide, the tetrazolium acceptor system is carbonyl reagent sensitive. Similarly, while there is no lag phase when the enzyme activity is measured manometrically, there is a definite lag phase when the assay is carried out in the tetrazolium reductase system (see refs. 94,99 and also this thesis). It was these two observations that led Weissbach <u>et al</u>.(99) to conclude that the site of tetrazolium reduction was at the level of aldehyde oxidation. As seen in this thesis however, the strong inhibition by semicarbazide of kynuramine oxidation in the absence of aldehyde oxidase activity (tables XXX, XXXVI) argues strongly against the interpretation of semicarbazide inhibition of the tetrazolium system simply to mean the inhibition of aldehyde oxidation(99). Furthermore, the demonstration by the present author that strong semicarbazide concentrations known to exclude all aldehyde(49) may have practically no effect on tetrazolium reduction during oxygen uptake (tables XXXIII, XXXIV) necessitates the reinvestigation of all the factors involved in the enzymic tetrazolium reduction by amines.

The demonstration of a lagphase during tetrazolium reduction in other enzyme systems such as succinate oxidase is also known(148). The occurrence of the lag phase depends on a number of factors; for example the type of tissue preparation (102), the presence or the absence of cofactors(94,102,103,148) and on the substrate(99). Thus while Weissbach <u>et al</u>.(99)

demonstrated a lag phase for the reaction involving tyramine and serotonin oxidation during tetrazolium reduction, they also showed that the reaction was spontaneous when adrenaline (epinephrine) was tested as substrate. Similarly, though Slater(102) demonstrated a lag phase with rat tissue homogenates using succinic acid as substrate for tetrazolium reduction, in the presence of vitamin C and menadione as cofactors(102, 103,148), the lag phase was abolished. Furthermore, this author(102) showed that beef heart muscle preparations could catalyze the same reaction without showing any lagphase and requiring no cofactors. Further still, in connection with cofactor requirements, Lagnado and Sourkes(94) and Sourkes and Lagnado(149) showed unequivocally that a number of purines were effective in abolishing the lag phase. In a parallel manner, the degree of the inhibition of the amine/tetrazolium reductase system by semicarbazide depends on the substrate tested (ref. 94,99 see also table XXXVIA) and also on the type of preparation as shown in this thesis (see tables XXXVII A & B). Thus the mitochondrial system was more strongly inhibited than the soluble enzyme by semicarbazide. The point being raised here is that it is not justified to extrapolate the results of one system to that of another since there is such overwhelming evidence against so doing(94,99-103,148).

The inhibition of the amine/tetrazolium reductase system by carbonyl reagents may be an indication that a metal such as iron is involved in electron transfer. This view is supported by the fact that o-phenanthroline(73,74,89,94) and tiron(73) two powerful and perhaps the most sensitive iron

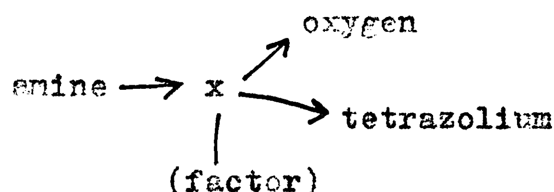
chelators are known to inhibit aerobic dehydrogenation of monoamines. O-phenanthroline was shown by Lagnado(94) to strongly inhibit the amine/tetrazolium reductase reaction. This finding was confirmed in this research (table XXXVIII B No. 4).

Furthermore, Youdim and Sourkes(90) have demonstrated the presence of iron in highly purified and solubilized rat liver mitochondrial MAO preparations. Similarly, Werle and Roewer(29) reported the inhibition of MAO by iron-binding beta-globulin of human serum. Further still, Lagnado and Sourkes(48) showed that low concentrations of iron stimulated the aerobic dehydrogenation of amines in the presence of rat tissues. Perhaps, one of the greatest setbacks in elucidating the properties of MAO is the different interpretations that various groups have put to their findings. For example, because 8-hydroxyquinoline poorly inhibited the bovine kidney enzyme, Erwin and Hellerman(74) concluded Cu was absent but their finding that o-phenanthroline (an iron chelator) strongly inhibited the enzyme was not much commented upon since their main aim appeared to have been either to prove or disprove the presence of Cu as an important component of MAO. Further still, various groups deal with a few substrates and assume the result might be the same for all other substrates. In this connection, the group just cited is important. These authors studied mostly the reaction involving the oxidation of benzylamine and kynuramine both of which are somewhat untypical tissue MAO substrates(23,65,116,117).

At this point it is proposed to suggest the possible

mechanism for the enzymic tetrazolium reduction by amines with special emphasis on the results obtained during oxygen uptake (tables XXXIII & XXXIV), sonication (tables XVIII-XX) and on the effects of semicarbazide (tables XXXVII & XLI). As seen from the data presented (table XXXIII), it is apparent that at least two pathways were involved in tetrazolium reduction during oxygen uptake. One pathway operated in the absence of inhibition of oxygen uptake (no preincubation of enzyme with dye, i.e. dye in the side arm) and the other operated when the enzyme was preincubated with the dye, i.e. when oxygen uptake was inhibited by the dye. The amount of dye reduced along either pathway was considerable. In the case when the dye was added at zerotime with the substrate (uninhibited reaction), however, there was little inhibition of oxygen uptake. Along the inhibited pathway (by preincubation of enzyme with dye) and in the absence of semicarbazide, the inhibition by the dye of oxygen uptake was greater than when semicarbazide was present. The presence or absence of semicarbazide in the system had practically no effect on the amount of dye reduced along either pathway. These results appeared to suggest that both aerobic dehydrogenation (oxygen consumption) of amines and the amine/INT reductase activity whether or not accompanied by oxygen consumption occurred simultaneously. When this was occurring the inhibition of oxygen uptake resulted. The question therefore arises whether the reaction was competitive or not. It appeared the reaction was most certainly competitive as it had been shown by Lagnado(94) and Lallement et Barron(138). It is therefore

certain that the reaction

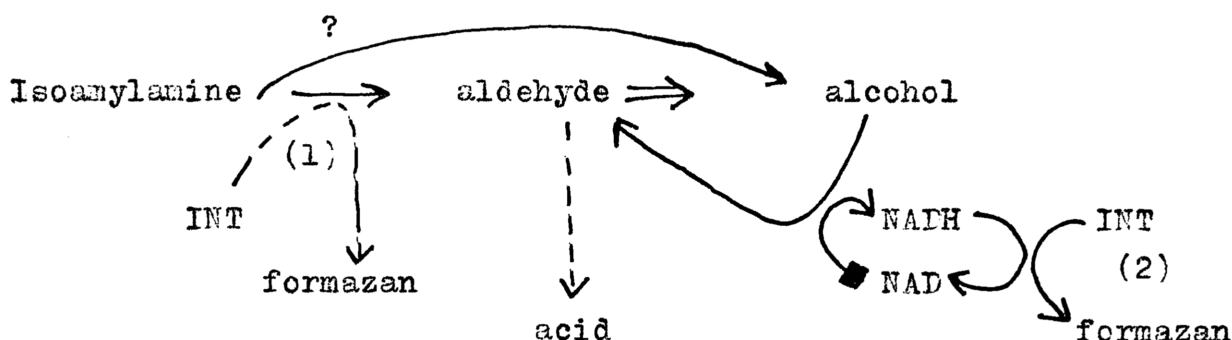


is a probability. Under these conditions semicarbazide does not inhibit oxygen reduction in the presence of tetrazolium (INT), suggesting that the dye inhibits aldehyde oxidising systems. The factor x shown above may be in a totally or partially reduced state of the enzyme. Electron transfer from this factor to oxygen appears to be more efficient than to tetrazolium salt.

The existence of a reduced state of MAO in the presence of amine has been demonstrated by Tipton for the pig brain MAO(51,73,150) in which the flavin component was shown to be in a reduced or partially reduced state. Since H_2O_2 formed during the aerobic dehydrogenation of amines by MAO corresponds to the substrate destroyed or the oxygen consumed, the inhibition of the reaction by tetrazolium salt would mean a decrease in the amount of H_2O_2 formed. The question therefore arises as to what the reductant of tetrazolium salt is if the supposed reductant aldehyde(99) is inhibited by semicarbazide as shown, eg. in table XXXIII. Can H_2O_2 participate in tetrazolium reduction? Since tetrazolium inhibits amine oxidation as already just stated, the quantity of H_2O_2 produced could not account for the amount of dye (INT) reduced. Is it possible then that the dye is reduced at the level of the factor x which in this case will be at the primary dehydro-

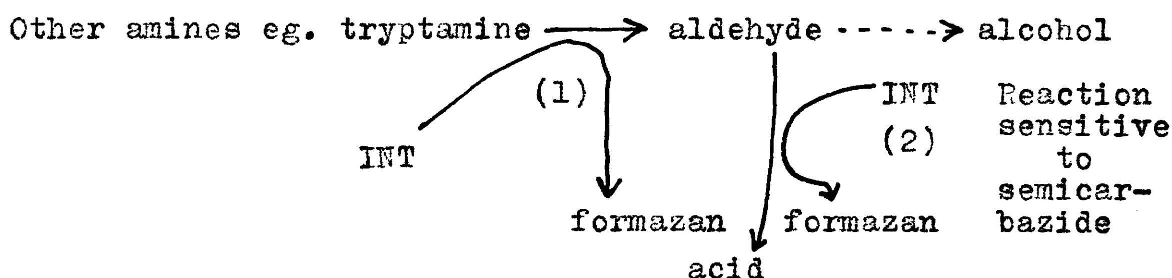
genase or flavin level? So far, it has not been possible to demonstrate that tetrazolium salts accept electrons directly from flavoproteins such as soluble succinic dehydrogenase(100-103). Intermediate carriers such as phenazonium methosulphate are necessary for transferring the electrons from flavins to tetrazolium salts. Nitroblue tetrazolium chloride (NBT) is known to accept electrons at a level near the primary dehydrogenase (100,101,103). In preliminary experiments, phenazonium methosulphate was found to slightly enhance the reduction of this dye (NBT) by MAO preparations in the presence of monoamines. What are the intermediate carriers and what is their nature? Why should semicarbazide have any effect whatsoever on the amine/tetrazolium reductase or for that matter on the succinate/tetrazolium reductase system? The difference in the degree of semicarbazide inhibition of the mitochondrial and soluble enzyme preparations for typtamine or succinate oxidation may be due to the firmer binding of semicarbazide to the particulate than the soluble enzyme and therefore more strongly inhibiting the particulate enzyme system.

In all INT reductase assays involving amines, the maximum semicarbazide effect occurred with isoamylamine as substrate. Could it be that under the conditions of this assay formazan production was partly or mainly due to the reoxidation of isoamylalcohol (in the presence or absence of NAD) by either aldehyde dehydrogenase or alcohol dehydrogenase as shown below?



In this case semicarbazide should cause complete inactivation, especially if alcohol can be formed, bypassing site 1. It is of interest to note that NAD stimulation of the tetrazolium reaction is strongest for this amine, being almost obligatory. The ready formation of isoamylalcohol is also well documented (13,16).

For the other amines the possible sites of formazan production may be represented thus:-



In this case the maximum inhibition by semicarbazide should be about 60 per cent which indeed fits nicely with the experimental results (table XXXVII A & B).

It should be stressed that the data of Weissbach et al.(99) pertained to the mitochondrial system of rat tissue only. Since they presented no detailed data on the soluble enzyme system(35) on tetrazolium reduction by amines, their results on rat tissue may not apply to the soluble system. And even for the mitochondrial system, these authors remarked that other

enzymic pathways for tetrazolium reduction by amines could exist. This remark was based on these facts that (1) though a lagphase was observed for the oxidation of some amines by the INT reductase system the reaction was spontaneous in the case of epinephrine and (2) that even at 40 mmolar final concentration of semicarbazide, the tyramine/INT reductase activity was not more than 11 per cent inhibited while serotonin oxidation was very strongly inhibited (about 70 per cent).

In this thesis it was found that though INT strongly inhibited oxygen consumption by the S.S.MAO preparations in presence of amines, twice such concentrations had practically no inhibitory effect on the conventional tetrazolium assay system in certain cases. Table XLI compares the effects of tetrazolium (INT) concentration on oxygen uptake and on the conventional tetrazolium assay system in the presence of tyramine or tryptamine.

TABLE XLI. COMPARISON OF THE EFFECTS OF TETRAZOLIUM CONCENTRATION ON OXYGEN UPTAKE AND ON AMINE/TETRAZOLIUM REDUCTASE ACTIVITY.

Oxidoreductase system		Oxygen oxidoreductase	Amine/INT reductase	
Substrate	INT concentration in μmoles/ml	% inhibition	INT concentration in μmoles/ml	% inhibition
Tyramine	0.316	46	0.742	1
	0.632	63	1.48	8
	0.948	71	2.22	17
Tryptamine	0.316	15 (52)	0.742	13
	0.632	56 (71)	1.48	30
	0.948	67 (86)	2.22	49

Data in brackets represent result in the absence of semicarbazide.

From the data just seen, it is very clear that while tyramine oxidation was more strongly inhibited than that of tryptamine by INT during oxygen consumption the result was completely reversed in the conventional INT reduction assay system. It is also clear that just about the same amount of INT which caused more than 50 per cent inhibition of oxygen consumption for the oxidation of either substrate inhibited tyramine and tryptamine oxidation by 1 per cent and 13 per cent respectively in the tetrazolium assay system.

Does it then mean that the two systems are inequivalent in the presence of INT? Again why should semicarbazide appear primarily to inhibit oxygen consumption and not tetrazolium reduction (see table XXXIV)? Further still, why is it necessary to preincubate the enzyme and the dye together before producing reasonable inhibition of oxygen uptake? From the results of time curve (see fig.8), it appears as if the tetrazolium salt was functioning as a substrate for the system which it was simultaneously inhibiting. From table XXXIII it appears it could be possible to abolish oxygen-utilising reactions by increasing tetrazolium concentration. If this is indeed the case can it be possible that the concentration of the dye coupled with sufficient time of preincubation with dye can be such that any effects of oxygen on the amine/tetrazolium reductase system would be abolished? Since this line of research was not pursued not much could be said about this point.

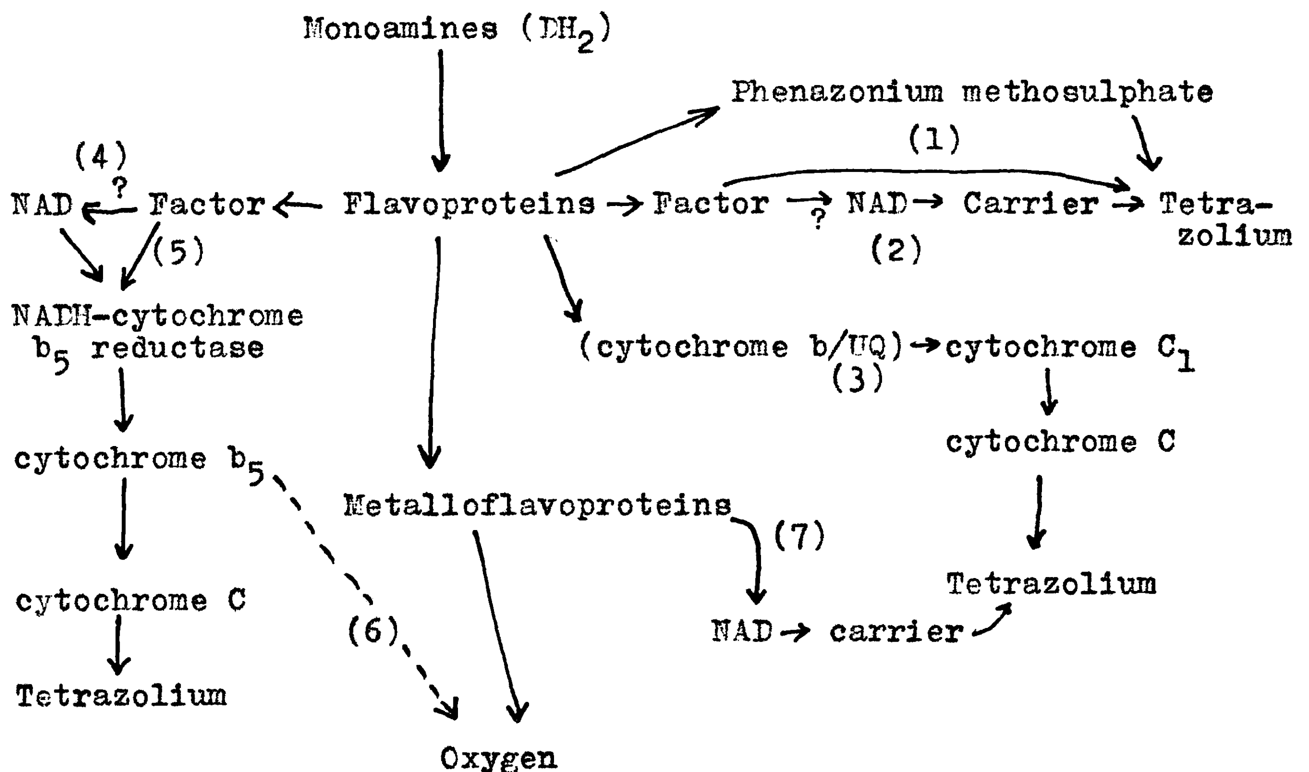
Another important fact to note is that Lagnado(94) demonstrated that though neotetrazolium chloride NTC and triphenyl-

tetrazolium chloride (TTC) strongly inhibited oxygen uptake, no dye reduction was observed. Is it then possible that these salts accept electrons at a level in the electron transport chain which is different from that of INT and NBT? That NTC and TTC actually tap the electrons at a site different from that by NBT and INT was demonstrated for succic dehydrogenase (100-103). That this is also possible for MAO is supported by the fact that the S.MAO and S.S.MAO preparations failed to reduce NTC and TTC in the presence of amines. As already stated, though Lagnado and Sourkes(94) remedied the inability of NTC and TTC to accept electrons from washed mitochondria or the supernatant fraction in the presence of monoamines by incorporating simple purines (NAD inclusive) into their reaction mixture, the present author found that only NAD was effective as cofactor in the amine/INT reductase system of the soluble MAO preparations. Further still, it may be recalled that the NAD stimulation of amine/INT reductase activity and that of the endogenous INT reduction (see tables IX-XI, XVII and fig. 6) were either greatly decreased or suppressed by sonication (see eg. table XIX). This supports the view that there are at least two pathways present in guinea pig liver MAO preparations for the amine/tetrazolium (INT) reductase system, one of which is NAD dependent and can be separated by sonication, from the non NAD dependent system. The results obtained in the experiments with respiratory chain inhibitors (table XL) also supports this view. What then are the intermediate electron carriers in the two types of tetrazolium reductase systems? According to recent findings on the sub-

cellular localisation of MAO, the two main enzyme systems almost exclusively located in the outer mitochondrial membrane of rat liver are MAO and amytal-, antimycin A-, and rotenone-insensitive NADH-cytochrome C reductase. This electron transfer system is closely linked with cytochrome b_5(42,88, 89) in that cytochrome b_5 is known to reduce cytochrome C by NADH(151,152).

Is it possible therefore that the amine/INT reductase system may operate along a pathway involving NADH-cytochrome b_5 reductase electron chain system as illustrated below?

Scheme (1) showing the possible sites of tetrazolium reduction by monoamine oxidase substrates.



Meaning of figures:-

(1) indicates pathway not requiring NAD to stimulate tetrazolium reduction. This pathway could operate either through phena-

zonium methosulphate or some other carrier which would transfer the electrons from the reduced flavoproteins to the dye or through a factor which would then donate the electrons to the dye.

(2) represents NAD dependent pathway, which though it appears reasonable theoretically has not as yet any experimental foundation since to our present state of knowledge flavins are not known to reduce NAD directly.

(3) Pathways sensitive to cyanide and antimycin A.

(4) Both pathways (4) and (5) involve NADH cytochrome b_5 reductase but pathway (4) differs from (5) in that (4) involves NAD as well.

(6) This pathway involves cytochrome b_5 with oxygen as final electron acceptor.

The reduction of tetrazolium salts by MAO preparations in the presence of monoamines along a pathway involving cytochrome b_5 or a still yet unidentified electron acceptor(s) is not unreasonable to suppose in the light of the recent findings of Muraoka and his associates(26) that MAO purified from Aspergillus niger can catalyse cytochrome C reduction both aerobically and anaerobically by MAO substrates. In this guise, the findings of Igaue, Gomes and Yasunobu(153) of the presence of a flavin-like component in beef mitochondrial MAO is important. One may imagine the presence of a chain of a number of flavins of various types (known or still yet unknown) taking part in the catalysis of MAO, and having a special and unique electron transport system of its own.

Another mechanism which might operate leading to tetra-

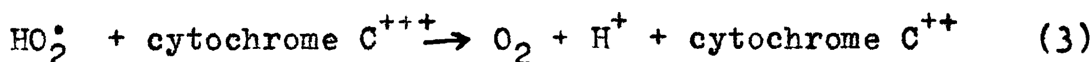
zolium reduction by MAO in the presence of its substrates may be similar to the one described by McCord and Fridovich(154) for milk xanthine oxidase. In this reaction, under highly aerobic states oxygen can exist as an unstable superoxide ($O_2^{\bullet}$) free radical. The free radical is probably formed by the dismutation of perhydroxyl ($HO_2^{\bullet}$) radicals formed by the reduced enzyme-substrate complex (Enz H_2) in the presence of adequate oxygen supply by the following steps:-

$$\text{Enz } H_2 + O_2 \longrightarrow HO_2^{\bullet} + \text{EnzH}^{\bullet} \qquad (1)$$

(divalently reduced enzyme) perhydroxyl radical univalently reduced enzyme

$$HO_2^{\bullet} + HO_2^{\bullet} \xrightarrow{\text{dismutation}} H_2O_2 + O_2 \qquad (2)$$

or alternatively the perhydroxyl radical may reduce cytochrome C thus:



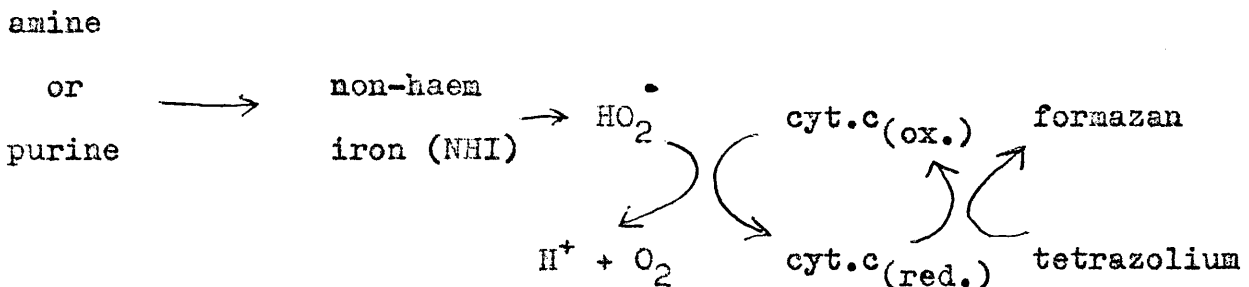
$$HO_2^{\bullet} + \text{cytochrome } C^{+++} \rightarrow O_2 + H^{+} + \text{cytochrome } C^{++} \qquad (3)$$

In other words, it is the superoxide ($O_2^{\bullet}$) free radical which initiates the chain of reactions resulting finally in the reduction of cytochrome C by oxygen.

According to Handler and Fridovich(156) this oxygen dependent reduction of cytochrome C by milk xanthine oxidase is strongly and competitively (competitive with respect to cytochrome C) inhibited by Tiron, which has no effect however on the aerobic reduction of xanthine by the enzyme to uric acid. These authors found that non-haem iron was involved in the reaction. Later Handler, Rajagopalan and Aleman(157) tentatively concluded that only non-haem iron-containing flavoproteins give any evidence of superoxide free radical production. Similarly, Bray *et al.*(158) have recently demon-

strated the superoxide radical production and concluded that both iron and alkaline pH are necessary for its production. The possibility that MAO is a non-haem iron-containing flavoprotein has been discussed in this chapter. Similarly, the relevance of Youdim and Sourkes (90) finding that iron is present in highly purified MAO preparations has been discussed. Before citing further evidence in support of the view that MAO may reduce tetrazolium and most probably NAD in the presence of monoamines through the formation of superoxide anion, the scheme itself must be represented.

Scheme (2) showing the possible site of tetrazolium by monoamine oxidase substrates or xanthine oxidase substrates.



in this scheme, cyt.c can be replaced by ferricyanide.

Clearly, the reaction relates to enzymes e.g. milk xanthine oxidase which act as oxidases to generate H_2O_2 and require partial pressure of oxygen. In other words the enzyme is in a "modified state" similar to the type proposed by Tipton (51) for monoamine oxidase. The requirement of high oxygen tension by MAO is very well documented (1,2,13,14,

19,51). Very recently, Tipton(51) demonstrated by means of more sophisticated instruments the need for high oxygen supply for amine oxidation by the pig brain MAO. This enzyme has also been shown to be strongly inhibited by Tiron(73) which inhibits the milk xanthine oxidase. Lagnado and Sourkes(94) demonstrated the requirement of purines for abolishing the lagphase during amine/tetrazolium reductase activity. ~~The requirement of purines by the milk xanthine oxidase is shown in the scheme~~. Further still, sulphydryl groups or disulphide groups are also known to be involved in the electron transfer system of the xanthine dehydrogenases or oxidases(145). Similarly, sulphydryl groups are known to participate in the aerobic dehydrogenation of monoamine oxidase substrates(23, 48,73,74,121,123). Lagnado and Sourkes(94) demonstrated the requirement of SH groups and purines for the amine/tetrazolium reductase system. More recently, Vesco and Giuditta(110) have tentatively identified two types of tetrazolium reduction pathways for rat brain mitochondria and microsomes. The microsomal pathway is sensitive to SH reagents while the mitochondrial system is independent of SH groups for reducing tetrazoles. Tables XXXI and XXXVIII show that the S.S MAO of guinea pig liver was sensitive to SH agents when it was assayed for oxygen and tetrazolium reduction by amines. In the face of such evidence, it will not be unreasonable to suppose that MAO may also catalyse similar reactions as the milk xanthine oxidases.

We will now turn our attention to the results obtained in the amine/NAD reductase assay system. It may be recalled

that the S.MAO and its acid derivative (S-pH_5 MAO) were active in reducing NAD by amines and that the substrate specificity generally parallelled that of the NAD-dependent INT reductase system (see table XIV). It may also be recalled that though the S.S MAO obtained from sonically treated homogenate and its acid derivative, the S.S-pH5 MAO were equally active in catalysing NAD reduction by monoamines (table XXII) the acceptor specificity was reversed. Since the possible cause of this reversal has already been dealt with earlier in this chapter, it will not be repeated here. Instead, we will attempt to determine the possible reductant and the site(s) of interaction of NAD with the electron transport chain. The role of aldehyde intermediate will be further discussed together with the significance of the effects of semicarbazide and some specific MAO inhibitors on the amine/NAD reductase system.

Weissbach and his associates(35) who first studied the soluble (S-pH5) MAO of guinea pig liver tentatively assumed that the reductant of NAD was the aldehyde produced by the aerobic dehydrogenation of MAO substrates. They reported that senotonin oxidation by their assay system was accelerated by excess exogenous aldehyde oxidase. Since these authors did not investigate the effects of carbonyl agents eg. semicarbazide on amine/NAD reductase activity of their system it was thought of interest to investigate this.

Fig. 7b shows that NAD reduction by guinea pig liver mitochondrial suspension in the presence of tyramine was hardly affected by 0.013 M (final concentration) semicarbazide. In contrast however, NAD reduction by S.S.MAO in the presence

of acetaldehyde was arrested within two minutes by similar concentration of semicarbazide (see fig. 11). In other experiments with this same enzyme (i.e. S.S.MAO) preparation, semicarbazide inhibition of the oxidation of certain substrates was not more than 30 per cent. Further still, this chemical appeared to actually stimulate amine/NAD reductase activity in certain cases (see fig. 11). It is also to be noted that this chemical (semicarbazide) inhibited the aerobic dehydrogenation of amines by about 50 per cent. These results would therefore indicate that aldehyde oxidation alone could not account for amine/NAD reductase activity.

Tyramine/NAD reductase activity was very strongly inhibited by the specific MAO inhibitors, Parnate and Iproniazid at low concentrations (see fig. 8); in contrast concentrations of semicarbazide higher than those normally used to effectively trap aldehydes (ref. 49) did not inhibit NAD reduction by MAO substrates. Though there was no apparent NAD reduction by kynuramine known not to form any free aldehyde as the result of MAO activity(116,117), it should be noted that the case of kynuramine is a very complex one. The wave length at which it absorbs maximally (360mμ) is too close to that at which NADH formation is demonstrated (i.e. 340mμ). The possibility therefore that the OD fall caused by kynuramine disappearance and the simultaneous OD rise due to NADH formation would neutralize each other cannot be dismissed lightly. In any case, not all monoamines capable of forming free aldehydes were good substrates for reducing NAD enzymically (see eg. table XXII). Further still, since the NAD acceptor

specificity of the MAO preparation was reversed by sonication while that of the aerobic dehydrogenase system remained unchanged (table XXVII), it is apparent that aldehyde may not be the only reductant of NAD. The question therefore arises as to where NAD reduction occurs and by what? Can amines as such themselves serve as the substrate for NAD reduction by MAO, in other words, could NAD reduction be coupled to the primary dehydrogenation of the amine substrates by MAO or is there an "amine dehydrogenase"?

If "amine dehydrogenase" exists, then even if aldehyde oxidation is prevented or is very low, NAD reduction could still occur. As already indicated, there was good NAD reduction by tryptamine and other substrates even in the presence of strong concentrations of aldehyde trapping agent (figs. 7b and 11), whereas little NAD reduction occurred in the presence of MAO inhibitors (fig. 8). This shows plainly that MAO activity was essential for demonstrating the reaction. Furthermore, the observation in certain experiments that NAD reduction by some monoamines eg. tryptamine, serotonin and tyramine occurred without any lagphase makes it very unlikely that the aldehyde intermediate is indeed involved in NAD reduction, since there must necessarily be some lapse of time for the oxidisable aldehyde to be accumulated. This argument is strengthened further by the observation that highly purified and solubilised rat liver mitochondrial MAO, said to contain no aldehyde oxidase activity(120), catalysed NAD reduction by tryptamine while acetaldehyde failed to serve as a substrate for the reaction (table XLI). Incidentally, this preparation

catalysed INT reduction by amines but was ineffective with acetaldehyde as substrate for the system. Similarly, though acetaldehyde could not serve as a substrate for tetrazolium reduction on gels, with tryptamine or tyramine as substrate, two bands were demonstrable with the Youdim enzyme(120) (see fig. 18). The inescapable conclusion arising from these and other observations is that there may be other pathways for amine oxidation in which the NAD and tetrazolium acceptors may participate during electron transfer.

<u>The possible mechanism of NAD reduction by MAO substrates.</u>

Some of the arguments raised for and the mechanisms proposed to explain the possible sites of the enzymic tetrazolium reduction by amines will be useful here also. In other words, it is proposed that MAO may catalyse a reaction similar to that of either scheme 1 and/or 2. In the course of this catalysis NAD may be reduced. The observation that reduced NAD i.e. NADH was oxidised and this oxidation was almost insensitive to amytal and antimycin A (fig. 7), could then be explained by the postulate that NADH-cytochrome b_5 reductase is part of the electron transport chain. By sonication the link with this part of the chain may be affected or the step may be completely by-passed. NAD reduction may occur at the level of the primary dehydrogenase. Should this occur the pathway followed could involve NADH-cytochrome C reductase route. The aldehyde oxidase pathway would occur further down along an alternate route as shown in scheme 3 below. The aldehyde oxidase pathway would explain the partial sensitivity of the reaction to semicarbazide.

The effect of arsenite on the amine/NAD reductase reaction (fig. 10) would be explained by the fact that the presence of S — S ⇌ SH SH system is required as a necessary component of the electron transport chain. This component may probably be associated with the non-haem iron-containing flavoprotein. This disulphide-sulphide exchange system may also participate in the tetrazolium and oxygen electron acceptor systems. The inhibition of these latter systems by organomercurials(48,73, 74,94 and table XXXVIII A) indicates the inhibition of the SH part the systems.

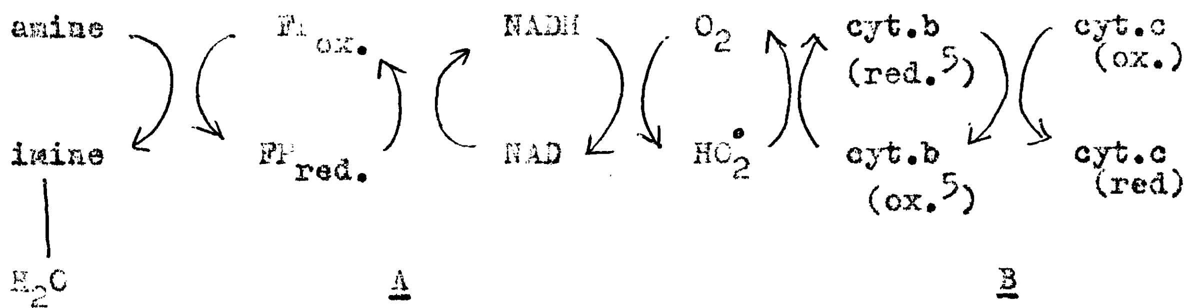
NAD reduction at the level of the primary dehydrogenase could in part at least be effected by the superoxide free radical, the immediate primary reaction product known to have powerful reducing properties(156-158). The involvement of the superoxide anion in the enzymic NAD reduction by amines could then in part at least explain the observation of Weissbach <u>et al</u>.(35) that NAD reduction by their system was not linear as a function of time after 30 minutes. The slowing down of the reaction with increasing time of incubation (which only excess aldehyde oxidase could only partially remedy), could mean that there was little oxygen available for generating more superoxide anion. The requirement of high oxygen tension for the radical production(154,156-158) and for amine oxidation (1,2,13,14,19,51) are well known. If the superoxide radical is the reductant of NAD, the decrease in its concentration would of course slow down the reaction.

The inhibition of the amine/tetrazolium and amine/NAD reductase systems by specific MAO inhibitors could be explained

by postulating that the inhibition occurs at the initial stages of the reaction. This view is consistent with a recently postulated mechanism by Helerman and Erwin (155) for MAO inhibition. According to this postulate, the inhibition occurs as the result of a direction reaction between the inhibitors and flavin groups of MAO.

Scheme 3 shows the possible mechanism and sites of the enzymic NAD reduction by MAO substrates.

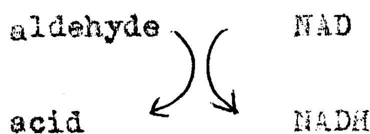
Cebeme 3. The possible mechanisms and the sites of the enzymic NAD reduction by MAO substrates.



NH_3

A = sensitive to Tyron, arsenite organo-mercurials and to high concentrations of semicarbazide.

B = part of NADH-cyt.c reductase system. Insensitive to amytal, rotenone and to Antimycin A.



Reaction type 1 might occur in unsonicated mitochondrial suspensions (see fig. 5).
Reaction 3 may occur in all types of soluble MAO prepations.
Induction 2 is probably responsible for greater proportion of NAD reduction than reaction types 1 and 3.
Where the reaction is strongly oxygen dependent, the superoxide anion may be intermediate.

CHAPTER SIX

SUMMARY, CONCLUSIONS.

1. The possible occurrence of a soluble form of monoamine oxidase in guinea pig liver was investigated, using both manometric and tetrazolium reduction assays.

2. A soluble enzyme preparation, active in both assay systems, was derived from aqueous extracts of guinea pig liver by salt fractionation and acidification. The enzyme was considered soluble as it was not sedimented by 3-6 hours centrifugation at 100,000g.

3. The properties of soluble monoamine oxidase preparations were investigated in some detail, and compared to those of crude particulate and mitochondrial fractions of guinea pig liver.

4. The particulate and soluble enzymes differed in a number of ways, and the differences were particularly evident in the dye-reduction assay system. Thus, for the soluble enzyme preparation it was found that the enzymic reduction of tetrazolium salts (INT in particular), by amines was markedly dependent on added NAD. NAD acted as a cofactor in the tetrazolium reductase system, whereas it had no effect on the amine-stimulated oxygen uptake in the manometric assay. Added NAD had little or no effect on enzyme activity of particulate preparations in both assay systems. The enzymic reduction of INT by amines was abolished by typical monoamine oxidase inhibitors under suitable experimental conditions.

5. NAD could not be replaced by NADP, AMP, ATP, and a number of simple purine derivatives (e.g. purine, inosine) as cofactor in the INT-reductase system.

6. NAD activated both endogeneous and amine-stimulated INT-reductase activity in soluble preparations. The effects of NAD on endogeneous activity could be eliminated by aerobic preincubation of the enzyme preparation in the absence of NAD and dye. Aerobic preincubation under these conditions did not affect the amine-stimulated system.

7. The effects of NAD on the amine-INT reductase system varied according to the amine substrate employed. In general, NAD stimulation was inversely proportional to the relative activity seen with different amine substrates. Thus, NAD had no effect when tryptamine, the best substrate in the INT-reductase assay, was used, but was most effective when isoamylamine, the least active substrate, was employed. Intermediate results were obtained when tyramine was used as substrate.

8. These results led to the discovery of an active system, in soluble enzyme preparations, catalyzing the enzymic reduction of NAD by amines in the absence of tetrazolium dye. The activity of the NAD-reductase system was followed spectrophotometrically. This revealed that the ability of various amine substrates to enzymically reduce NAD was directly related to their dependence on added NAD in the tetrazolium-reductase assay. The enzymic reduction of NAD by amines was strongly inhibited by typical monoamine oxidase inhibitors.

9. Particulate preparations of monoamine oxidase also catalyzed the reduction of NAD by amines when precautions were taken to prevent the re-oxidation of reduced NAD.

10. It was found that sonication of homogenates caused considerable alterations in the properties of the soluble emzyme whose activity was increased in all three assay systems employed, and also produced a loss of NAD-dependence in the amine-INT reductase system and a reversal of the substrate specificity pattern in the NAD-reductase assay.

11. The effects of semicarbazide, SH-inhibitors, metal chelating agents, and respiratory-chain inhibitors were tested in an attempt to analyze the pathways responsible for the enzymic reduction of molecular oxygen, INT and NAD by soluble preparations derived from control and sonicated homogenates.

12. The effects of tetrazolium salts on monoamine oxidase present in soluble enzyme preparations was investigated. Tetrazolium salts act as potent non-competitive inhibitors of the enzyme when oxygen is used as final electron acceptor. Dye reduction under these conditions was directly proportional to the amount of dye added. However, dye reduction, unlike oxygen uptake, was unaffected by relatively high concentrations of semicarbazide, showing that tetrazolium reduction, under these conditions, was not due to the further oxidation of the aldehyde product of amine oxidation.

13. The evidence accumulated points to the existence of novel pathways involved in the coupling of amine oxidation

with the reduction of tetrazolium salts and NAD. It is also apparent that the reduction of electron acceptors such as these cannot be fully accounted for by the further oxidation of the aldehyde primary product of the action of monoamine oxidase on amines. The possible role of non-haem iron and FAD, associated with the enzyme, and hydrogen peroxide, formed during amine oxidation, in tetrazolium and NAD reductase pathways was discussed. The use of several electron-acceptor systems has provided some insight into the mechanism of amine oxidation which was not readily apparent from experiments in which molecular oxygen was used as final electron acceptor.

14. A variety of electrophoretic techniques were studied in an attempt to detect the possible presence of isoenzymes of monoamine oxidase, using tetrazolium salts to detect sites of enzyme activity. In general, only one weak band showing enzyme activity was seen after separation on Cellogel or agar gels. In starch and polyacrylamide gels, one and occasionally two bands of enzyme activity were detected using tryptamine or tyramine as substrate. Sonicated preparations contained a relatively greater amount of enzyme which migrated anodically under the conditions used. In most experiments, some enzyme activity remained at the origin. It was concluded that under the experimental conditions tested, monoamine oxidase behaved as a single enzyme fraction, and that the activity at the origin could be due to active aggregates of the enzyme.

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