THE EFFECTS OF IONS ON MONOAMINE OXIDASE ACTIVITY OF RAT LIVER

- by -

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THESIS

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FOREWORD

The present investigation is part of a research programme in progress at the Allan Memorial Institute of Psychiatry, under the direction of Dr. R.A. Cleghorn. In our laboratory, we are particularly interested in the metabolism of physiologically occurring pressor amines such as adrenaline, nor-adrenaline and serotonin. Oxidation by monoamine oxidase (MO) constitutes one of the important ways by which these biogenic amines are inactivated in the body. Little is known, however, on the natute of the MO "system".

Search of theliterature indicates that virtually no investigation has been done on the effects of ions, particularly the metals, on MO. In view of the requirement for metals by many enzymes, a study of the effects of ions on MO has been undertaken in our laboratory and the results of this investigation are reported in this thesis. Furthermore, there is evidence reported in the literature indicating that amine oxidation is catalysed by a riboflavin-enzyme. It has recently been found that a large proportion of the yellow enzymes also bear a metal (iron, copper or molybdenum) in their prosthetic group. The possibility that the oxidation of monoamines is catalysed by a metallo-flavoprotein enzyme has been briefly investigated.

Concurrently with this work, other studies bearing on the mechanism of action and on the nature of the MO system have been carried out.

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INTRODUCTION

A. HISTORICAL

The first indications that amines are broken down in the body by deamination are to be found in a paper published by Schmiedeberg in 1877 (1). Schmiedeberg demonstrated that in the dog, benzylamine given orally was excreted as hippuric acid, and he assumed that free benzoic acid was first formed prior to conjugation. A little later, Schmiedeberg and Minkowski (2, 3) were both able to isolate benzoic acid from minced rabbit liver incubated with benzylamine. This demonstrated the deamination reaction *in vitro*. Other amines were later shown to be deaminated in perfusion experiments as well as *in vivo* (4-6). Ewins and Laidlaw, in 1910, were able to show that a numberof amines, when added to the perfusion fluid of intact organs or given to intact animals, were broken down to give corresponding acid (4, 5). These authors found, for example, that tyramine was metabolized to p-hydroxyphenylacetic acid. It was believed at that time that this reaction took place with the intermediary formation of an alcohol. Guggenheim and Löffler (6), had isolated tyrosol, as well as the acids derived from *iso*-amylamine[1] and tryptamine.

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1 In 1915, Bain (7) reported that *iso*-amylamine, which is the decarboxylation product of leucine, is found in the urine of man. This finding which would imply that *iso*-amylamine is not deaminated in the body by monoamine oxidase prior to its excretion, could not be confirmed by later investigators (8).

In all the early feeding and perfusion experiments, the aldehyde primary product of amine oxidation was not detected. This is not surprizing, since in such experiments, the aldehyde formed would be readily oxidized by aldehyde oxidase and mutase to its corresponding acid and alcohol.

The early work reviewed above, as well as more recent studies using radio-isotope techniques (9, 10, 11), fully confirm the general concept propounded by Schmiedeberg (1877!) concerning the fate of amines in the body, in which it is suggested that,

> "probably all the mono-amino bases in which the nitrogen is not directly linked to the benzene ring, i.e. all bases which contain the atomic grouping - CH_2NH_2 are broken down in the organism with the formation of ammonia". (1).

In 1928, Hare discovered "tyramine oxidase, an enzyme found in mammalian liver capable to catalyzing the oxidative deamination of tyramine (12). In her second paper, Hare-Bernheim suggested that the first reaction product of amine oxidation was an aldehyde (13). This was quite in contrast with the earlier work, where deamination was thought of as a hydrolytic process, giving rise to the formation of alcohol, according to reaction (1),

$$R\text{-}CH_2NH_2 + H_2O \rightarrow R\text{-}CH_2OH + NH_3 \qquad (1)$$

It was in 1937 that the first experimental evidence for the formation of an aldehyde as primary reaction product of amine oxidation was presented (14, 15). Pugh and Quastel (14) studied the oxidation of aliphatic amines by an enzyme present in brain and in other tissues which they named "aliphatic amine oxidase". These authors isolated the aldehyde formed in the enzymic oxidation of iso-amylamine[2]. They were able to show, moreover, that oxidative deamination occurred both in brain cortex slices and extracts, although the amine oxidase activity of these preparations was less active than that of liver tissue. They also reported that higher amines such as iso-amylamine depressed oxygen uptake of brain cortex slices, although deamination did take place.

In the same year, Blaschko et al (18, 19, 20) described an enzyme present in a variety of tissues, capable of inactivating adrenaline by deamination, and suggested that their "adrenaline oxidase" was identical with Hare's tyramine oxidase (12), and Pugh and Quastel's "aliphatic amine oxidase" (16). At this time, Kohn (21) proposed the following sequence

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2 It was found by these authors (116) that the aldehyde formed from butylamine or iso-amylamine could undergo a Cannizzarro reaction, catalyzed by aldehyde mutase, to form the corresponding alcohol and acid. Iso-amylalcohol is characterized by its odour. It may be mentioned here that Holtz et al (17), in the next year, isolated the aldehyde formed during the enzymic oxidation of dopamine.

of reactions in the oxidation of amines by amine oxidase, shown in reaction (2),

$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CHO + NH_3 + H_2O_2 \qquad (2)$$

Soon afterwards, Zeller proposed a common name for these enzymes, "monoamine oxidase" (subsequently referred to as MO), to distinguish it from "diamine oxidase" (DO) (22, 23). Thence arose the concept of MO which was not to be challenged until 1943, when Alles and Heegaard (24), on the basis of extensive studies on the substrate and inhibitor specificity of MO, suggested that MO was in fact, composed of a group of several closely related enzymes. Later work, which shall be discussed subsequently, lends support to this point of view. It is of interest, in this respect, to quote Zeller et al (25) who state in a recent paper that,

> "The differentiating characteristics between monoamine-oxidase (MO) and diamine-oxidase (DO) have been lessened... The question arises as to whether the amine oxidases are distinct entities or are homologous enzymes".

In other words, not only is the homogeneity of MO questioned, per se, but there is also some question as to the validity of the MO-DO dichotomy which has long been regarded as an established fact.

B. PROPERTIES OF AMINE OXIDASE

1. Distribution of Monoamine Oxidase

Monoamine oxidase activity has been found in animal and plant tissues (19, to 27) as well as in some bacteria (28). The distribution of MO has been reviewed in detail by Blaschko (29) and Zeller (30); Werle (27) has reviewed more in particular the distribution of the enzyme in plants. Table I briefly summarizes what is known on the distribution of MO.

Table I : Distribution of Monoamine-oxidase

Source		Reference
Chordates	mammals, birds reptiles, amphibians, teleosts, elasmobranchs (Torpedo marmorata)	19, 26, 29
Invertebrates	molluscs, echinoderms, annelids	19, 31
Plants	higher plants such as: Salvia ulginosa, Colchicum bornmuelleri, Cannabis indica, Momordica balsamina and Pisum sativum L.	27, 32, 33
Bacteria	B. aeruginosa, E. coli	28

The richest source of MO hitherto found is the hepatopancreas of the molluscs Sepia and Octopus (34, 35). In mammalian organisms, a wide variety of tissues have been studied for MO activity, liver intestine and kidney[3] being the richest sources for the enzyme. Until recently, plasma has been regarded as devoid of MO activity (29). In 1954, however, Tabor et al (36) described a purified amine oxidase from beef plasma, thus confirming some earlier findings reported by Hirsch (37) and by Werle et al (27). Although the plasma enzyme differs in some respects from the "classical" enzyme, it was considered by Tabor et al as a monoamine oxidase (36).

Zeller et al have reported that the hepatic MO in male rats is higher than in female rats (38). It has also been found, by Langemann, that there exists an inverse relationship between MO and cholinesterase activity in a wide variety of tissue, and that this is especially evident in brain tissue (39).

In way of summary, it may be said that the mammalian enzyme has been found principally in glands (40 to 43), plain muscle (29) and nerve (14, 29, 44). The possible implications of the distribution of the enzyme as regards

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[3] Exceptionally it has been found that rat kidney is devoid of MO activity (17).

its physiological role shall be discussed subsequently.

Table II illustrates the distribution of MO in a number of tissues of man and pig.

Table II[4] : Distribution of Monoamine Oxidase in Man and Pig

Values given represent the number of microliters of oxygen taken up/hr./gm. of tissue, using tyramine as substrate.

Organ	Man	Pig
Liver	1027	257
	138 (metastasis)	
Kidneys	567 (cortex)	453
	252 (medulla)	
Intestine		94
Pancreas		75
Brain	323 (thalamus)	
	246 (putamen)	
Heart	552	33
Muscle	155	
Uterus	111	
Bladder	92	
Thymus	46	
Thyroid		6
Lung	253	
	80 (pneumonia)	
	212 (tuberculosis)	
	99 (metastasis)	

[4] Modified from Zeller (30).

2. Substrates for Amine Oxidase (General refs.: 19,29,30, 45 to 47)

A number of amines in which the amino group is on the end of the carbon chain and where there is no other substitution on this carbon atom (e.g., carboxyl, methyl groups, etc.) are oxidized by MO (45). The introduction of a methyl group in the alpha-position, the replacement of one beta-H atom by an hydroxyl group, or the replacement of both beta-H atoms by some other radical causes characteristic changes in the behaviour of these substrates towards MO (45, 46).

MO catalyses the oxidation of a number of aliphatic monoamines (e.g., butylamine, iso-amylamine), phenylalkylamines (e.g., tyramine, tryptamine) as well as diamines of the general formula $NH_2(CH_2)_nNH_2$, where n is greater than 6 (e.g., decamethylenediamine) (48, 49). Diamines of the same general formula, but where n is smaller than 6 (e.g., putrescine and cadaverine) are not substrates for MO. Furthermore, long-chain diamines where n is larger than 13 are not attacked by MO; the rate of oxidation of these amines decreases with increasing chain-length (48, 49). It has been suggested that short-chain diamines are not substrates of MO because of inappropriate spatial configuration of the amine molecule on the enzyme surface (29). On the other hand, it is

possible that the orientation of long-chain diamines (where n is less than 14) on the enzyme surface may resemble that of monoamines, thus insuring the formation of an active enzyme-substrate complex. Blashko (29) suggests moreover, that long-chain diamines where n is greater than 13) may resist attack by MO because of the formation of micelles, which would result in a decreased solubility of the diamines and make them unavailable.

3. Homogeneity of Amine Oxidase

The homogeneity of MO was questioned by Alles and Heegaard (24). The original criteria in support of the existence of a single enzyme for amine oxidation are three-fold (29) :

(1) representatives of different types of substrates are oxidized in all organs in which amines are oxidized;

(2) the rate of oxidation of two typical substrates is intermediary between the rates of oxidation of the two amines when tested separately;

(3) the inhibitor specificity of the enzyme in the presence of different types of substrates is the same.

Alles and Heegaard (24) found marked species differences in the relative rates of oxidation of a wide variety of substrates. Moreover, by studying the inhibition of amine oxidase by substances competing for the enzyme, these authors observed marked variations in the K_i (dissociation constant of enzyme-inhibitor complex, cf. Table IV) dependent upon the competitive substrate. These points are illustrated in Tables III and IV. Table III illustrates the species differences of MO activity, while Table IV illustrates the inhibitory effects of phenisopropylamine on the oxidation of different substrates of MO.

Mescaline is deaminated by rabbit liver amine oxidase, but not by liver of other species (50). This suggests the existence of a special amine oxidase responsible for the oxidation of mescaline which is not identical with MO.

Werle et al (27) have presented evidence to show that there are two distinct amine oxidases in plant and animal tissues, one only attacking aliphatic monoamines, whilst the other only oxidizes aromatic monoamines. These authors succeeded in fractionating liver and plant tissue extracts with acetone obtaining two enzyme preparations, one of which contained a system

capable of oxidizing only aliphatic monoamines, the other, only aromatic monoamines. These authors found, moreover, that fresh rat liver homogenates oxidized butylamine and tyramine at a similar rate, whilst the same preparation obtained from rabbit oxidized tyramine much faster than it did butylamine. This finding was interpreted as further evidence for the existence of two distinct monoamine oxidases in rabbit liver. Comparing the amine oxidase activity of plant and animal tissues, Werle et al (27) found that the former was much more active towards aliphatic than towards aromatic amines, the contrary being true in the case of most animal tissues.

Recently, Tabor et al (36) succeeded in purifying from ox plasma a soluble amine oxidase, "spermine oxidase". This enzyme is considered by Tabor et al to resemble MO, although there are marked substrate and species differences between the two amine oxidases (cf. Tables V and VI). "Spermine oxidase", in contrast with MO, does not attack sympatheticomimetic amines (e.g. adrenaline), though it is active towards a number of other MO substrates. On the other hand, typical diamine oxidase (DO) substrates such as histamine and putrescine are not attacked by the plasma enzyme.

In 1955, Mann (33) obtained a highly purified amine oxidase preparation from pea-seedling extracts whose substrate specificity is less strict than that of the amine oxidases previously described. In fact, this enzyme was shown to oxidatively deaminate putrescine and histamine (both typical DO substrates), aliphatic monoamines (e.g., ethylamine), phenylalkylamines (e.g., tryptamine); unlike all the previously mentioned amine oxidases, it attacked L- and D- lysine (33). Presumably, lysine behaves towards the enzyme as some long-chain diamines do towards MO (cf. p. 8).

Table V compares in a summarized way the substrate specificity of various amine oxidase described in the foregoing section.

Table III[5] : Substrate Specificity of Amine Oxidase

Substrate	Species			
	Rabbit	Guinea-Pig	Cat	Cattle
Ethylamine	0	0	20	5
Butylamine	50	100	90	110
Amylamine	110	140	110	85
Benzylamine	30	10	110	130
Phenethylamine	100	100	100	100
Tyramine (4-hydroxyphenethylamine)	90	200	120	130
Hordenine (4-hydroxyphenethyl-dimethylamine)	30	10	45	80
Dopamine (3,4-dihydroxyphenethylamine)	65	200	80	85
Arterenol	65	180	30	65
DL adrenaline	15	40	20	5

Source of enzyme : liver extracts

Values given as maximum oxidation rate in per cent relative to phenethylamine.

Experimental conditions : substrate: 0.008 M; phosphate buffer, pH 7; 30° C.

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5 Modified from Alles and Heegaard (24).

Table IV[6] : Inhibition of Amine Oxidase

Substrate	Concn. of phenylisopropylamine	1/V max.	K_i (M)
Amylamine	0.000	0.023	
"	0.002	0.036	0.0011
Phenethylamine	0.000	0.25	
"	0.020	0.030	0.0470
Tyramine	0.000	0.031	
"	0.004	0.037	0.0045
Dopamine	0.000	0.042	
"	0.004	0.054	0.0033

The constant $V_{max.}$ and K_i are calculated from the Michaelis-Menten equation according to Lineweaver and Burk (51):

$$1/V = 1/V_{max.} (K_s + K_s I/K_i) (1/S) + 1/V_{max.}$$

V = velocity

$V_{max.}$ = maximum velocity

K_s = dissociation constant of enzyme-substrate complex

K_i = dissociation constant of enzyme-inhibitor complex

S = concn. of substrate

I = concn. of inhibitor

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6 Modified from Alles and Heegaard (24).

Table V : Substrate Specificity of Amine Oxidases[a]

Substrate	Enzyme[b]			
Monoamines :	MO	DO	SO	PS
aliphatic amines (e.g., butylamine	+	-	+	+
tyramine	+	-	±	+
tryptamine	+	-	±	+
mescaline	+[c]	-	+	?
adrenaline	+	-	-	?
Diamines :				
putrescine	-	+	-	+
cadaverine	-	+	-	+
decamethylenediamine	+	-	+	?
histamine	-	+	-	+
agmatine	-	+	-	+
Polyamines :				
spermine	-	+	+	+
spermidine	-	+	+	?
Amino-Acid :				
D- or L-lysine	-	-	-	+

(a)
The signs employed have the following signification:

+ substrate is oxidized

± substrate is slowly oxidized

- substrate is not oxidized

? data not reported in the literature

(b)
MO = monoamine oxidase (30)

DO = diamine oxidase (30)

SO = "spermine oxidase" (36)

PS = amine oxidase from pea-seedling extracts (33)

(c)
in rabbit only (50)

4. Intracellular Distribution of Amine Oxidase

Monoamine oxidase is localized in the insoluble constituents of the cell (29). Howkins (52) studied the intracellular distribution of MO in rat liver suspensions and found that two-thirds of the enzymic activity was localized in the mitochondrial fraction, while the remaining third belonged to the microsomal fraction. These findings confirm previous work carried out by Cotzias et al (53). Attempts to purify MO have not met with much success so far (12, 21, 24). The enzyme in fact is tightly bound to the mitochondria, and until recently, has resisted solubilization. The solubilization of an active MO has been achieved by means of surface-active agents. Blaschko and coworkers (29), have found that treatment of tissue homogenates with bile salts or lysolecithin gave translucent solutions containing most of the MO originally present in the untreated tissue. Bile salts, per se, are, however, detrimental to MO activity (as measured manometrically), and these authors found it necessary to dialyze the salts out to obtain active preparations of MO (29). They suggest moreover, that the effects of the bile salts consist in the dispersion of the mitochondria into particles of smaller size, presumably the microsomes (29).

It may be mentioned here that other enzyme systems have been reported to be inhibited by bile salts. Cotzias et al (54) have succeeded in obtaining a soluble MO from rat and rabbit liver homogenates treated with detergents such as Alconox or Cutscum[6]. Since not only the mitochondria but also the microsomes are disintegrated when treated with these detergents, thereis in this case a more complete solubilization of the enzyme than obtained by means of bile salts. According to Cotzias et al (54), the detergents they employed for the solubilization do not affect MO activity as do the bile salts. Recently, Barsky et al (58), reported the preparation of non-sedimentable monoamine oxidase from hog-liver mitochondria by combined treatment with desoxycholic acid and sonic oscillation.

Now that MO has been solubilized, purification of the enzyme should meet with less difficulty than in the past. It must be emphasized that the so-called solubilized[7] MO preparations have only been assayed manometrically, and that

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6 These are two long-chain alcohols. Cutscum, or its active constituent, isooctylphenoxypolyethoxyethanol, was found to give the best results (54).

7 "Solutions" of MO obtained by means of bile salts are not true solutions in that the original particulate character of the solubilized enzyme is restored as soon as the bile salts are dialyzed out (55).

nothing is known as yet on the ability of such preparations to transfer electrons to acceptors other than molecular oxygen. It is possible, for instance, that the solubilized enzyme loses its ability to reduce other electron acceptors such as ferricyanide, which we have found to function as an electron acceptor during amine oxidation (see Appendix II). Work is in progress in our laboratory to obtain more information on the properties of solubilized MO preparations.

The mitochondrial localization of MO has some interesting implications. It means, first of all, that two toxic primary products of amine oxidation, namely, aldehyde and ammonia, can be removed *in situ* by enzymes present in the mitochondria concerned with aldehyde oxidation, the formation of urea, etc. Moreover, it has been suggested by Blaschko (29), that the cytochrome system, which is also localized in the mitochondria, may possibly act as "terminal oxidase" for amine oxidation in intact tissues. The participation of the cytochrome system in amine oxidation as studied *in vitro* has not been demonstrated. However, since amine oxidation *in vitro* is usually studied under high partial pressures of oxygen, such as do not obtain in intact tissues, it is suggested that amine oxidation may follow

pathways in intact tissues different from those followed in *in vitro* experiments. A number of flavine enzymes have been shown to behave in a similar way.

5. Inhibitors of Amine Oxidase

We shall briefly deal with some inhibitors of amine oxidase. In a preceding section, it was shown that MO differs from other amine oxidases in substrate specificity (cf. Table V). Similarly, there are marked differences in the inhibitor specificity of amine oxidases, depending on the source of the enzymes. This is illustrated in Table VI. It must be emphasized that the inhibitor studies cannot as yet yield some definite information concerning the homogeneity of the enzymes, since the particulate forms of amine oxidase have not been purified to any extent. At most, the behaviour of MO towards inhibitors can shed some light on what pathways are involved during amine oxidation.

A number of substances, not listed in Table VI are known to inhibit MO. These include some local anesthetics, antihistaminics, monoamidines, diamidines, monoguanidines, diguanidines, and diisothiourea derivatives (29, 30). Uric acid and adrenaline, after being oxidized by uricase (56) and cytochrome oxidase (57),

respectively, become inhibitors of MO.

Comments on Inhibitors Listed in Table VI

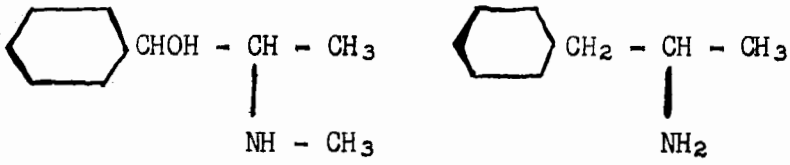
(a) Iproniazid and Isoniazid

Iproniazid and Isoniazid are amine oxidase inhibitors (cf. Table VI). Iproniazid, or 1, -iso-nicotinyl-2 - isopropylhydrazine (IIH), is a potent inhibitor of MO both *in vitro* and *in vivo* (58). IIH, also inhibits "spermine oxidase" (36). Zeller *et al* showed that the main requirements for inhibitors of MO could be represented by the formula = N - NH - R. It was found that the carbonyl reagent property of MO inhibitors is only incidental. Thus, it is unlikely that the inhibition of MO by phenylhydrazine (60) is indicative of an essential carbonyl group of MO phenylhydrazine; in fact, it is thought to inhibit MO in virtue of its oxidizing properties (60). In addition, MO is not inhibited by other carbonyl reagents such as semicarbazide (30) or hydroxylamine (30).

(b) Ephedrine and Benzedrine

Ephedrine and Bensedrine are two α-methylated amines which competitively inhibit MO *in vitro* (61, 62).

These two drugs have a strong affinity for MO, but are not oxidized by the enzyme since the amino group is not attached to the terminal C:



ephedrine

benzedrine

(Amphetamine

Benzedrine, which lacks an hydroxyl group on the side-chain of the molecule, has a greater affinity for MO than does ephedrine, and is therefore, the more potent inhibitor of the two drugs. Schayer et al (11) reported that ephedrine does not appear to inhibit MO in vivo. The inability of this drug to inhibit the enzyme in intact animals may be due to a number of reactions; for example (a) failure of the drug to reach the site of enzymic action because of too rapid destruction in the body, or for reasons of permeability; (b) differences between properties of MO when studied in vitro and in intact organisms. Mann and Quastel (62), who first discovered the inhibition of MO by benzedrine, in vitro, had suggested that the drug effects

of this compound could possibly be attributed, in part at least, to its effects on MO. Assuming that ephedrine and benzedrine act in a similar way, it would appear that Schayer's results (11), argue against the hypothesis suggested by Mann and Quastel (62). However, it is not unlikely that benzedrine (or ephedrine) affect the organism at some particularly sensitive site, producing the pharmacological effects; in that case, it is possible that such a small change cannot be discerned when one measures the total MO activity of the organism, as in Schayer's work (11). Hence, benzedrine may well owe some of its pharmacological property to its inhibitory effects on MO, though over-all changes in MO activity are not apparent. It may be added here that ephedrine, as well as some other MO inhibitors, also inhibit other enzyme systems, e.g. the pseudo-cholinesterases (63).

(c) Methylene Blue

In 1937, Philpot (64), reported the inhibitory effects of methylene blue on MO. These results have been confirmed for both animal and plant MO by Werle et al (27). Cotzias (65), reported that the inhibition is of the competitive type. West (66) found that

methylene blue was also inhibitory *in vivo*. In contrast to these findings, it has been found that methylene blue stimulates MO activity *in vitro* under special experimental conditions, presumably, by acting as a H- carrier (67).

(d) Narcotics

Werle *et al* (27) found that urethane is a potent inhibitor of MO. Phenobarbital inhibits "spermine oxidase", after pre-incubation with the enzyme preparation for 75 mins., while it is not affected by urethane (36). Quastel *et al* (68 to 70), have shown that many narcotics can be effective in either of two steps in the respiratory chain: they could prevent the transfer of hydrogens between reduced pyridine nucleotides and flavin carriers, or alternately, between the reduced flavin and the cytochrome system. Experiments by Greig (71) favour the latter possibility. The mode of action of narcotics on amine oxidation remains to be elucidated. It may be added that very little is known on the steps involved in amine oxidation (cf. "Mechanism of Action of Monoamine Oxidase", p. 32).

(e) Quinacrine

This anti-malarial drug has been shown by Haas (72) and others (73, 74) to inhibit flavine-linked enzymes in oxidative processes. The inhibitory effects of quinacrine can be reversed by riboflavinemonophosphate (FMN) (74). The inhibitory action of quinacrine is attributable to its combination with the protein part of the enzyme, which results in the displacement of the flavin component.

Quinacrine has recently been shown to inhibit the enzymic oxidation of monoamines by rat liver suspensions (75, 76). Previously, Allegretti and Vukadinovic (76) had demonstrated the inhibition of quinacrine of adrenaline oxidation both *in vitro* and *in vivo*. Theseauthors showed that this effect could be demonstrated more readily when studying MO activity of liver and kidney as compared to that of brain and heart. The enzyme activity could be restored by adding riboflavine. *In vivo* blood pressure measurements showed the inhibition by quinacrine of the oxidation of adrenaline in excess (1 mg.). Quinacrine (0.3 g.) was injected prior to adrenaline (1 mg.), while riboflavine (0.3 g.) was injected before or after the blood-pressure rise, its restoring potency being the same in both

cases. It was found by the same authors, that when brain MO was studies, contradictory results were obtained. They interpreted this as possibly due to the inability of quinacrine to pass through the hematoencephalic barrier ("blood-brain barrier"). Allegretti et al (76), conclude that the quinacrine inhibition "consists merely of replacing the flavin prosthetic group of the enzyme".

Hawkins (77), has shown that MO and D-amino acid oxidase activity of guinea-pigs kept on riboflavin-deficient diet is decreased by one-half. Addition of riboflavin to the diet dramatically restores the D-amino acid and oxidase activity, but reactivates MO only slowly. It was also found that inositol stimulated the restoring capacity of riboflavin (77). It was suggested that riboflavin may be involved in the synthesis of a prosthetic group or of a new enzyme protein.

Other evidence for the participation of flavin carriers during amine oxidation is presented elsewhere (cf. "Mechanism of Action of MO", p. 32).

(f) Alcohols

In 1947, Heim reported the inhibitory effects of

octyl alcohol on MO (78). This finding was subsequently confirmed by Werle and Roewer (27) in the case of plant and animal monoamine oxidase. Furthermore, Francis (79) found that the anaerobic reduction of tetrazolium salts by frozen tissue slices incubated with tyramine was abolished when thetissue slices were pre-incubated three hours with octyl alcohol. Tabor et al (36) showed that "spermine oxidase" (SO) is not affected by octyl alcohol. Octyl alcohol does not inhibit "diamine oxidase" (DO) (30). Recently, Cotzias and Serlin (80) have reported the effects of alcohols and hydrocarbons on the oxidation of monoamines by both particulate and "solubilized" enzyme preparations. These authors found that MO is subject to profound and irreversible modifications of behaviour when treated with alcohols, these changes being linked to the chain length of the compounds, and most pronounced with compounds having chains of five to eight carbon atoms. The inhibitory effects of these compounds on MO are inversely proportional to temperature between 4 and 60° C (80). These results are interpreted as demonstrating the crucial role of molecular geometry in the reactions catalyzed by MO (80).

(g) Sodium Azide

Sodium azide, NaN_3, which was introduced by

Keilin (81) as a metal reagent, is not generally considered as an inhibitor of MO (29). However, Werle et al (27) have reported the inhibition of both plant and animal MO by azide. Mann (33) found that his purified preparations of the pea-seedling amine oxidase are inhibited to a very small extent when ethanolamine or L-lysine are used as substrates. This author occasionally observed small activations by azide, which he attributed to the inhibition of catalase present in his enzyme preparations, resulting in an increase in the net oxygen uptake during amine oxidation (33).

(h) Cyanide

Cyanide is reported by several authors (12, 16, 82) not to affect amine oxidation by MO. Werle et al (27) found that cyanide, when added in high concentrations (0.3 M), reduced to one-half the oxidation of amines by plant and animal MO preparations. It is possible that this inhibition is due to the reducing of cyanide which could affect the state of the -SH groups essential for MO activity (83). "Spermine oxidase, which is the soluble plasma enzyme purified by Tabor et al (36) was found by these au hors to be inhibited by cyanide. When this enzyme assayed manometrically, it was found that the inhibition was progressive with time (36). Tabor et al (36) suggest

that cyanide inhibits "spermine oxidase" activity in virtue of its carbonyl reagent properties. Other carbonyl reagents are powerful inhibitors of the enzyme (36). Finally, it was reported by Mann (33) that cyanide inhibits amine oxidase activity of pea-seedlings. The author presents evidence which argues against the view that cyanide is acting as a carbonyl reagent, and suggests that it may be acting as a metal-reagent. He also found that other metal-reagents inhibit, and suggests that plant amine oxidase may be a metallo-protein. Examining plant amine oxidase preparations forheavy metals, Mann (33) found that they contained both coper and manganese in amounts of 0.03-0.06%, based on the protein content. However, he has not yet been able to demonstrate that the presence of these metals is essential for the activity of the enzyme.

Table VI : Effects of Inhibitors on Amine Oxidases

Substance[a]	MO[b]	SO	PS	DO
Ephedrine	+[63] o[9]	o[36]		
Benzedrine	+[64]	o[36]		
Quinacrine	+[77] (a-c)	+[36]		
Phenobarbital	+[27]	+[36]		
Urethane	+[27]	•[36]		
Octanol	+[27,79,81]	o[36]		o[30]
Methylene Blue	+[27,66-68]			+[30]
Isoniazid	o[59]	+[36]		+[59]
Iproniazid	+[59-61]	+[36]		•[59]
Semi-carbazide	o[30]	+[36]	+[33]	+[30]
Phenylhydrazine	+[62]			+[59]
Hydroxylamine	o[30]	+[36]		+[30]
Pyridoxamine		+[36]		
Pyridoxamine-phosphate		o[36]		
Azide	o[29] +[27]		+[33]	

Table VI : Effects of Inhibitors on Amine Oxidases (Cont'd.)

Substance	MO	SO	PS	DO
Cyanide	o[12,16,82]	+[36]	+[33]	+[30]
Diethyldithio-carbamate	o[86]		+[33]	
Versene	o[86]	o[36]		
"-SH reagents"	+[87,88]			o[30]

(a)
A plus (+) sign indicates that the substance is inhibitory; a zero (o) indicates that the substance is not inhibitory.

(b)
MO = monoamine oxidase (30)
SO = "spermine oxidase" (36)
PS = pea-seedling amine oxidase (33)
DO = diamine oxidase (30)

6. Mechanism of Action of Monoamine Oxidase

Monoamine oxidase (MO) catalyses the oxidation of amines according to the following reactions (12, 16, 21):

$$R\text{-}CH_2NH_3^+ + O_2 \rightarrow R\text{-}CH{=}NH_2^+ + H_2O_2 \qquad (2)$$

$$R\text{-}CH{=}NH_2^+ + H_2O \rightarrow R\text{-}CHO + NH_4^+ \qquad (3)$$

The primary step, the dehydrogenation, yields an imino compound which on non-enzymatic hydrolysis gives ammonia and an aldehyde. This mechanism of action closely resembles the action of D-amino acid oxidase on α-amino acids (87) as well as that of diamine oxidase (DO) on diamines (30). Both of these enzymes are flavin-enzymes (30, 87), and by analogy, it is probable that MO also includes a flavin component[8]. The formation of H_2O_2 is characteristic of reactions catalysed by flavin enzymes (87).

MO reacts with molecular oxygen, probably by way of a flavin carrier. It does not require pyridine nucleotides and is able to by-pass the cytochrome system[9], although it is not known whether the cytochromes act as "terminal oxidase" during amine oxidation in living tissues.

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[8] Cf. p. 26.

[9] Cyanide, which is a potent inhibitor of cytochrome oxidase, was generally found to be without effect on amine oxidation by MO. Cf. p. 28.

In manometric experiments, MO activity is dependent on oxygen tension, and Kohn (21) has shown that the rate of amine oxidation under air is about one-third of that under pure oxygen. On the basis of these properties, MO can be classed as an "aerobic" or "flavin dehydrogenase" according to the criteria proposed by Green (89). These dehydrogenases are capable of by-passing the cytochrome system, although many of them are able to reduce cytochrome c (83). The fact that these enzymes, like MO, require high oxygen tensions to effectively transfer electrons to molecular oxygen militates against the view that such a pathway occurs in living tissues, where oxygen is present at much lower partial pressures. Moreover, MO is localized in the mitochondrial particles of the cell (29), in close proximity to the cytochrome system. It is conceivable that *in vitro*, MO can either react directly with molecular oxygen, or can alternately transfer electrons to oxygen *via* the cytochrome system, depending on the relative amounts of pure oxygen and of cytochrome c (or some other cytochrome pigment) present in the reaction mixtures. In the presence of sufficient amounts of cytochrome c, and at low oxygen tensions, MO would involve the particpation of the cytochrome system while, when the contrary situation obtains, electrons could be transferred directly to molecular oxygen with formation of H_2O_2.

MO requires free -SH groups for activity (57, 86). It is not known whether the essential -SH groups are cysteinyl residues of the enzyme-protein or whether they are present in the form of small molecules such as glutathione or α-lipoic acid; if the former compound is involved, it would have to be tightly bound to the enzyme, since prolonged dialysis does not eappear to affect amine oxidation by MO when this is assayed manometrically (16, 90).

Several dyes have been shown to act as suitable acceptors during the anaerobic oxidation of amines by MO. These include, O-bromoindophenol, O-cresolindophenol, 2,4-dichlorophenolindophenol and tetrazolium salts[10]. The reduction of tetrazolium salts is believed to necessitate the mediation of a flavin carrier (91): this may be interpreted as further evidence indicating the participation of a flavin in the action of MO. Results obtained studying the anaerobic reduction of dyes by MO has led to the suggestion that MO may transfer hydrogens <u>via</u> an hitherto unidentified H transporting system (30). Methylene blue and toluelene blue cannot replace oxygen as H-acceptors during amine oxidation, and in fact, these dyes inhibit MO (15)[11].

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10 Cf. Appendix I

11 Cf. also p. 24.

7. Physiological Role of Monoamine Oxidase

The presence of MO in the intestinal mucosa has led to the suggestion that it acts here to detoxify monoamines formed in the gut by bacterial decarboxylases (30). The presence of MO in the placenta has also been thought to imply a protective function of the enzyme (90).

Thereis much debate as to whether MO is related to adrenergic function. The fact that adrenaline and nor-adrenaline are substrates of the enzymes *in vitro* does not, *per se*, mean that they are physiological substrates for MO. These amines are in fact, relatively poor substrates for MO both *in vitro* and *in vivo* (92, 93). But Schayer *et al* (11) have reported that as much as one-half of adrenaline injected into rats is metabolized by MO. On the other hand, Griesemer *et al* (92) have found that the inactivation of the MO of liver and brain of cats by the administration of Marsilid[12] markedly potentiated the response of the nictitating membrane to injected tyramine, but not to adrenaline, suggesting that the latter is effectively metabolized by other enzyme systems. Koelle and Valk (44) have recently studied the cellular localization of MO in

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12 Cf. p. 21.

adrenergic neurones by a new histochemical technique, and concluded from their investigations that there does not appear to be any selective associations of MO with adrenergic neuro-effector systems in the cat. Although this would appear to militate against the view that MO is related to adrenergic function in a manner analogous to the action of cholinesterases of cholinergic nerves, Koelle and Volk (44) have proposed that, in view of the ubiquity of MO in the cat, MO may participate in a phase of amine metabolism which is common to all types of nerves.

In contrast to the findings obtained using adrenaline or nor-adrenaline, Udenfriend and his coworkers (93), have reported that serotonin (5-hydroxytryptamine) is rapidly oxidized by MO both *in vitro* and *in vivo*, and they suggested that serotonin may be the only physiological substrate of MO outside the intestinal mucosa. It is possible that MO is directly involved in limiting the physiologic action of serotonin just as cholinesterase controls the actions of acetylcholine (93).

Other pathways for the metabolism of sympathomimetic amines have been suggested (47). For example, Richter *et al* (94) found that when ingested orally, a number of sympathomimetic amines were excreted mainly as their sulfate conjugates. In contrast with the views

of Burn and his coworkers (63), who support the concept that MO is the main effective inactivating enzyme for catechol amines, Von Euler et al (95) have recently suggested that while amines which are released by physiological processes may be metabolized chiefly by MO, circulating catechol amines are inactivated only to a minor degree by this enxyme. These authors (95) suggested that peroxidases, which are capable of transforming catechol amines into inactive compounds (96), may play an important role in the inactivation of circulating amines.

A relation between MO and the development of hypertension has been investigated by several workers (97 to 100). It is interesting to note that the ability of monoamines to raise blood pressure is related to their affinity, in vitro, to MO (99). It is believed by some authors that experimental renal hypertension is due to the liberation into the blood stream of amines formed in the ischemic kidney (97).

C. METAL IONS AND ENZYME SYSTEMS

Part of the work reported in this thesis deals in particular with the susceptibility of MO towards a number of metal ions. It had previously been shown that the enzyme is inhibited by mercurials and arsenicals (57, 58), but a search of theliterature has not revealed any systematic study of the effects of ions on the oxidation of amines by MO. There is at present no evidence indicating that MO activity involves the participation of metal ions. Recently, however, Mann (33) has purified an amine oxidase from pea-seedling extracts which he found to be inhibited by a number of metal-reagents, suggesting that a metal may be contained in the prosthetic group of the enzyme. It is not unlikely that if this is the case, amine oxidases obtained from other sources may also contain a metal as part of their prosthetic groups. In view of this possibility, and also because this is pertinent to the experimental results obtained in our studies on MO, we should like here to review briefly what is known on the role of metal ions in enzyme systems[13]. In what concerns the susceptibility of enzymes towards metals, in particular, towards heavy metals, the reader is referred to section IV of this thesis, where comments along these lines

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[13] Excellent reviews dealing with the role of metals in enzyme systems have appeared in the literature (101, 102). Earlier work on the subject is presented in Oppenheimer and Stern's "Biological Oxidations" (103), and in Warburg's classical treatise, "Heavy Metal Prosthetic Groups" (104).

accompany the experimental results obtained during our investigation.

Metals are generally known to participate in enzymic reactions in two ways:

A : The metal is an indispensable part of the enzyme system, in which case, it is highly specific for the enzymatic reactions it catalyses, and can only be dissociated from the enzyme by drastic chemical means. In such systems, removal of the metal component from the enzyme results in a marked lowering of enzymic activity. Metallo-enzymes of this type include the copper-enzyme tyronsinase and iron-containing metallo-enzymes such as the catalases and the cytochromes.

B : The metal is only loosely bound to both enzyme and substrate, in which case its removal (which can be accomplished by ordinary chemical means) does not necessarily result in a decrease in enzymic activity. Moreover, in such systems, the metal specificity for the enzyme is low. Included among this type of metallo-enzymes are peptidases, carboxylases and phosphatases.

The metals which most commonly particpate in enzymic reactions are found in groups Ia, Ib, IIa and VII of the periodic table of elements. Many of them belong to the transition series of elements, sharingthe common property of having unfilled electron shells other than the outer shell. This means that electron transfers mediated by such metals can involve electronic re-arrangements other than those related to changes in valency.

Lately, it has been found that a number of flavin-containing enzymes also contain metals tightly bound to both the flavin and protein moieties of the enzyme molecule

(moieties of the enzyme molecule (metallo-flavoproteins) (105 to 107). The concept of metallo-flavoproteins, which is due primarily to the work of Green and his school, has now been well estabilished, and provides additional conformation of the pivotal role of metals in electron transport. Green and Mahler (105) have summarized the characteristic nature of these enzymes in the following way:

> "Metallo-flavoproteins are a group of enzymes containing both metal and flavin in firm linkage with the protein and in definite proportions.... The metal plays no role in the inter-action of reduced enzyme with organic oxido-reduction dyes, quinones and molecular oxygen.... The metal is absolutely required for the inter-action of reduced enzymes with one-electron acceptors such as cytochrome c and ferricyanide".

Metallo-flavoproteins hitherto described include DPNH-cytochrome reductase (Fe^{+++}) (106), xanthine and aldehyde oxidase (Mo^{+VI}) (107) and butyryl-CoA dehydrogenase (Cu^{++}) (107). Recent findings by Singer and Kearney (109) suggest that succinic dehydrogenase may also be a metallo-flavoprotein containing iron.

It is not unreasonable to speculate that MO may be a metallo-flavoprotein enzyme, if indeed, the claim that it is a flavin-enzyme, is a valid one. The lack of inhibition of MO by metal-reagents such as cyanide or Versene[14] does not constitute sufficient evidence against the participation of a metal in amine oxidation. In fact, when these reagents were tested for their effects on MO, the enzyme was assayed manometrically, under which conditions, reduced MO reacts directly with molecular oxygen (forming hydrogen peroxide), thus mediating a two-electron transfer which, according to Green et al (105), can proceed independently of the metals in the case of metallo-flavoprotein enzymes. It would be useful to study the effects of metal-reagents on the inter-action of MO with one-electron acceptors, however, no studies in which MO was assayed in a system involving the transfer of electrons to such acceptors have been reported in the literature[15].

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14 Cf. Table VI.

15 We have found that MO can be assayed spectrophotometrically measuring the reduction of ferricyanide, which, it is thought, acts as a one-electron acceptor under the conditions of our experiments. (Cf. Appendix II).

ABBREVIATIONS

BAL	=	British Anti-Lewisite or 2,3-dimercaptol propanol
DPN	=	diphosphopyridinenucleotide
DPNH	=	reduced DPN
FAD	=	flavin adenine dinucleotide
FMN	=	riboflavin-5'-phosphate
GSH	=	glutathione
GSSG	=	oxidized glutathione
ISO	=	iso-amylamine
MO	=	monoamine oxidase enzyme system
PCMB	=	p-chloromercuribenzoate
TGA	=	thioglycollic acid
TRIS	=	tris (hydroxymethyl) aminomethane
TRYP	=	tryptamine - HCl
TYR	=	tyramine - HCl
Versene	=	ethylenediaminetetracetic acid (di-sodium salt)

MATERIALS

BAL was kindly supplied to us by Dr. O.F. Denstedt.

The amine substrates employed during the course of this work were obtained from the Eastman Kodak Company and from Merck and Company.

γ-Amino-n-butyric acid was obtained from the California Foundation for Biochemical Research.

Quinine and atebrin were obtained through the courtesy of Dr. L.E. Hokin (Department of Pharmacology, McGill University).

All other materials employed during this investigation were obtained either from Merck and Company or Eastman Kodak Company.

Metal salts tested were of Reagent Grade.

IV EXPERIMENTAL

A. Effects of Ions on Monoamine Oxidase Activity of Rat Liver Suspensions

1. Methods

(a) Preparation of Tissue

Adult male rats obtained from a commercial dealer and weighing between 100 and 200 gms. were employed. The rats were guillotined and bled, and the liver was immediately excised, rinsed in cold tap water, weighed and homogenized in an all-glass Potter-Elvehjem or a Teflon homogenizer to give a 20% tissue suspension made up in distilled water. This suspension was then transferred to a Cellophane dialysis bag and dialysed against distilled water at 3° C for about twenty hours. The dialysis medium was changed three times in the first three hours and then once more three hours prior to use. The purpose of this dialysis was to remove endogenous substrates and so reduce the oxygen uptake of the tissue blanks. Dialysis did not decrease the rate of oxygen consumption in presence of added substrate (cf. Table VII). In some cases, dialysis was carried out against cold running tap water, and here too, no decrease in the rate of amine oxidation was observed when such preparations were compared to non-dialysed portions of the same liver suspension.

(b) Reaction Mixtures

Reaction mixtures were prepared following a standard pattern, (cf. Table VI). Tyramine and tryptamine, used as their hydrochlorides, as well as iso-amylamine, were chosen as representative substrates for rat liver MO, and were employed as their aqueous solutions, after being brought to a neutral pH. The substrate solutions were made so as to give a final concentration in the reaction mixtures of 0.02 M. It has been shown by Kohn (21) and Cotzias (65) that at this concentration, the enzyme is saturated with substrate, and that further increments in substrate concentration do not increase MO activity.

TRIS buffer (final concentration: 0.037 M; pH: 7.4), was employed instead of phosphate buffer, since the latter is known to form insoluble complexes with a number of metals. In some cases, however, it was found preferable to use phosphate buffer (final concentration: 0.02 M; pH: 7.4), and this will be indicated with the experimental results.

(c) Procedure

For measuring oxygen uptake, the manometric technique was used (109). The experiment was carried out as follows. The liver suspension was pipetted into the main compartment of the Warburg vessels (containing all other ingedients) which had been chilled on cracked ice for 15 mins. Flasks were then hooked to the manometers and gassed with pure oxygen for about 5 mins. After equilibration of the flasks in the water bath

at 38° C for 10 to 15 mins., the manometer taps were closed, and one or two readings were taken in rapid succession to check if equilibration had been reached. The substrate was then tipped into the main compartment of the reaction vessel ("zero time"). Readings were taken every 10 mins. during a period ranging between 1 and 1.5 hours. The results are then generally expressed as µl. O_2 consumed in the first 30 mins. Any deviations from the procedures described above are mentioned with the results.

Table VII : Effects of Dialysis on Amine Oxidation

Liver Preparation	Expt. 1		Expt. 2		Expt. 3	
	Substrate					
	-	+	-	+	-	+
Fresh Suspension	40	274	28	230	40	95
Dialysed vs. distilled H_2O	10	255			10	65
Dialysed vs. tap H_2O			13	131		

Values shown represent µl. O_2/hr./100 mg. wet wt. of tissue (before dialysis)
Substrate: expts. 1 and 2: tyramine; expt. 3: tryptamine.
Gas phase: oxygen.
Temperature: 38° C.

Table VIII : Model Reaction Mixture

Warburg Vessel	Constituent	Concn.	Vol. added	Final concn.
Main well	metal salt tissue		0.5 ml.	
	water		0.2 ml.	
	TRIS	0.37 M	0.2 ml.	0.037 M
Side-bulb	substrate	0.10 M	0.4 ml.	0.020 M
Center well	KOH	30%	2 drops	

Gas phase: oxygen.

(d) Ions Tested

The ions tested for their effects on MO activity are listed in Table IX.

The results foreach ion are presented in order of its appearance in the periodic table. A general summary of these results will be presented at the end of this section.

A number of anions were tested to check if these could affect MO activity. It was found that high concentrations of salts containing these anions had little if any effects on amine oxidation, (cf. Table X). This makes it unlikely that the anionic moieties of the metal salts tested contributed to the effects obserbed.

We have found that if the liver suspension was made up in isotonic KCl and employed without dialysis, the endogenous respiration was very high as compared to that of non-dialysed preparations made up in distilled H_2O. Moreover, using homogenates made up in isotonic KCl, it was found that the total oxygen uptake in the presence of added substrate never exceeded that of the tissue blank. This may be due to the protecting effects of isotonic KCl on the mitochondria, thence on the activity of mitochondrial oxidizing enzymes which could possibly compete for molecular oxygen (or some other H acceptor) with the MO system (111). That the amine substrate was being oxidized, to some extent at least, is evidenced by

the appearance in all cases of melanin-like pigments when tyramine or tryptamine were used as substrates, since such pigments are characteristic of the oxidation products of these substrates (16).

Table IX : Ions Tested

Group I	Group II	Group III	Group IV	Group VI
Sodium	Magnesium	Aluminium	Titanium	Chromium
Potassium	Calcium		Tin	Selenium
Copper	Zinc		Cerium	Molybdenum
Silver	Cadmium		Lead	Uranium
	Barium			
	Mercury			
	Group VII		Group VIII	
	Manganese		Iron	
			Cobalt	
			Nickel	

Table X : Effects of Anions

Expt.	Addition	Final Concn	µl. O_2/30 mins.	
			Endogenous	Substrate TYR
1	none		-6	80
	KCl	0.01 M	-2	76
	K_2SO_4	"	-2	71
	NaCl	"	-5	80
	Na_2SO_4	"	4	99
2	none		11	84
	Na_2SO_4	0.01 M	4	79
3	none		2	78
	NH_4Cl	0.01 M	5	85

2. Results

(a) Effects of Copper

Aqueous solutions of $CuSO_4 \cdot 4H_2O$ (Cu^{II}) and of$(CuCl)_2$ (Cu^{I}) were tested. Cuprous chloride was added as a suspension, since it is only sparingly soluble in water (15 gm./l at 25° C).

At final concentrations of the order of 0.0001 M, both cuprous and cupric ions reduced amine oxidation. This confirms previous findings reported by Bernheim (60), who suggested that this inhibition is due to the effects of copper salts on essential -SH groups of amine oxidase (60). We have found, however, that within a narrow range of concentrations, copper ions apparently activated amine oxidation. This activating effect is as yet without explanation. The results obtained are shown in Table XI, and illustrated in Figure 1.

Table XI : Effects of Copper

Expt.	Addition	Final Concn.	µl. O_2/30 mins.			
			Endogenous	TYR	TRYP	ISO
1	none		0	136		
	$CuSO_4$	1×10^{-7}M	0	143		
	"	1×10^{-6}M	0	135		
	"	1×10^{-5}M	0	125		
	"	1×10^{-4}M	0	147		
	"	3×10^{-4}M	0	146		
	"	1×10^{-3}M	0	88		
	"	1×10^{-2}M	0	14		
2 (a)	none		42	69		
	$CuSO_4$	2.5×10^{-5}M	17	96		
3 (b)	none		37		67	114
	$CuSO_4$	2.5×10^{-5}M	37		88	106
	"	2.5×10^{-2}M	5		12	14
4 (c)	none		13	105	64	
	$(CuCl)_2$	5×10^{-6}M	17	128	72	
	"	5×10^{-2}M	17	26	36	

Table: XI: (Continued).

Expt.	Addition	Final Concn.	µl. O_2/30 mins.			
			Endogenous	TYR	TRYP	ISO
5	none		0	128		
	$(CuCl)_2$	1×10^{-6}M	0	154		
	"	1×10^{-5}M	0	132		
	"	1×10^{-4}M	0	91		
	"	1×10^{-3}	0	105		
	"	1×10^{-2}	0	27		

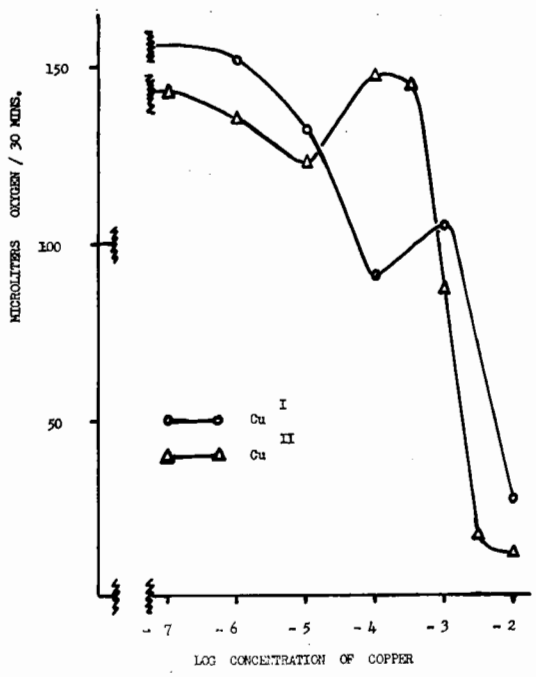
a
Liver suspension dialysed at 3° C two hrs. vs. 0.001M versene, pH 8.1, then one hr. vs. TRIS buffer 0.1 M, pH 8.1.

b
Liver suspension not dialysed.

c
Liver suspension dialysed 18 hrs. vs. TRIS 0.1M, pH 8.1 at 3° C.

Figure 1 : Effects of Copper Ions on Amine Oxidation by Rat Liver Homogenates.

(Cf. Experimental results, Table XI.)



(b) Effects of Silver

Silver ions were tested in the form of silver nitrate, $AgNO_3$. It was found that tyramine oxidation was inhibited 50% when $AgNO_3$ was added at a final concentration of the order of 10^{-3}M. In some cases, silver ions activated the endogenous respiration of the tissue after the first 30 mins. of incubation (cf. Table XII expts. 1 and 5). Exceptionally, silver ions, added in final concentrations of the order of 10^{-4}M, produced a small apparent activation of amine oxidation (cf. Table XII, expt. 4). During the course of amine oxidation, silver ions were reduced and metallic silver deposited on the walls of the Warburg flasks.

Silver ions are known to inhibit a number of enxymes by combining with free -SH groups essential for activity (112). It was found that BAL could prevent the inhibition of MO by silver ions (cf. Table XII, expt. 4).

Table XII[(a)] : Effects of Silver

Expt.	Addition	Final Concn.	µl. O_2/30 mins.		Extra O_2	% of control
			Endogenous	TYR		
1	none		9	143	134	100
	$AgNO_3$	1×10^{-3} M	20	0		0
	"	5×10^{-3} M	23	0		0
2	none		7	124	117	100
	$AgNO_3$	2×10^{-3}M	0	0		0
3	none		8	98	90	100
	$AgNO_3$	2×10^{-4} M	3	84	81	90
	"	6.5×10^{-4} M	9	35	26	29
4	none		8	125	117	100
	BAL[(b)]		7	125	118	100
	$AgNO_3$	1×10^{-4} M	6	137	131	112
	"	4×10^{-4} M	0	107	107	91
	"	8×10^{-4} M	0	71	71	61
	"	4×10^{-4} M[(b)]	11	115	104	89
	"	8×10^{-4} M[(b)]	5	113	108	93
5	none		0	87	87	100
	$AgNO_3$	1×10^{-4} M	0	80	80	90
	"	5×10^{-3} M	12	62	50	57

Table: XII (Continued)

Expt.	Addition	Final Concn.	µl. O_2/30 mins.		Extra O_2	% of control
			Endogenous	TYR		
6	none		11	88	77	100
	$AgNO_3$	1×10^{-6} M	19	92	73	95
	"	1×10^{-5} M	13	87	74	96
	"	1×10^{-4} M	10	74	64	83
	"	1×10^{-3} M	5	2	-3	0

(a) TRIS buffer was employed in expt. 5 and 6; in all other expts., 0.02 M phosphate buffer, pH 7.4, was used.

(b) Here 1.6 µmoles of BAL were tipped in main well from second side-arm of Warburg flask at "zero time". (see also p. 64).

(c) Effects of Magnesium

Magnesium was tested as its sulfate, $MgSO_4 \cdot 7\ H_2O$. When added at a final concentration of 0.02 M, magnesium sulfate was found to decrease amine oxidation to a small extent (cf. Table XIII, expt. 1). In one experiment, it was found that magnesium sulfate apparently reactivated MO activity of a tissue preparation dialysed against versene (cf. expt.2). Since, at other times, versene did not seem to affect MO activity, this experiment could not be repeated. Moreover, it is possible that this apparent reactivation is only due to the effects of $MgSO_4$ on the endogenous respiration, which it reduced by half. Sulfate anions do not appear to affect amine oxidation (cf. Table X), but it is possible that they are the cause of the effects observed on the endogenous respiration of the tissue preparation dialysed against versene (cf. Table XIII, expt. 2).

Table XIII : Effects of Magnesium.

Expt.	Addition	Final Concn.	$\mu l.O_2$/30 mins.		"Extra O_2"
			Endogenous	TYR	
1	none		0	85	85
	$MgSO_4$	1×10^{-3} M	0	87	87
	"	1×10^{-2} M	0	69	69
2(a)	none		42(33)(b)	69(97)	27(64)
	$MgSO_4$	5×10^{-6} M	20	68	48
	"	5×10^{-3} M	22	75	53

Table: XIII (continued)

(a) Liver suspension dialysed 2 hrs. vs. 0.001 M versene pH 8.0, then 1 hr. vs. distilled H_2O (to remove the versene) at 3°C.

(b) Figures in brackets give values for non-dialysed sample of same liver preparation.

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d) Effects of Calcium

Calcium chloride, $Ca(Cl)_2$, appears to inhibit amine oxidation to a small extent at some of the concentrations tested (cf. Table XIV).

Table: XIV Effects of Calcium

Expt.	Addition	Final Concn.	µl. O_2/30 mins.		Extra O_2
			Endogenous	TYR	
1	none		28	141	113
	$Ca(Cl)_2$	5×10^{-4} M	18	128	110
	"	5×10^{-3} M	25	125	100
2	none		2	70	68
	$Ca(Cl)_2$	1×10^{-3} M	12	70	58
	"	1×10^{-2} M	1	65	64

(e) Effects of Zinc

Zinc sulfate, $ZnSO_4$, is a powerful inhibitor of amine oxidation, and it was found to suppress the oxidation of tyramine and of isoamylamine when added at a final concentration of 0.001 M (cf. Table XV).

Table: XV Effects of Zinc

Expt.	Addition	Final Concn.	µl. O_2/30 mins.		
			Endogenous	TYR	ISO
1	none		0	44	33
	$ZnSO_4$	0.001 M	2	2	0

(f) Effects of Cadmium

Cadmium was tested as the chloride, $Cd(Cl)_2$. The results obtained are shown in Table XVI and illustrated in Figure 2. At relatively low concentrations, cadmium may enhance amine oxidation (cf. Table XVI, expts. 3 and 4). At final concentrations above 10^{-4}M, cadmium is definitely inhibitory. Flasks containing the metal at a final concentration of 10^{-2}M showed flocculation of protein.

Although no reactivation experiments were carried out, it seems likely that the toxicity of cadmium towards MO is in part at least due to its effects on the essential -SH groups of the enxyme. Indeed, cadmium is a well-nown inhibitor of other -SH enzymes, and is believed to owe its toxic effects *in vivo* to the fact it combines with essential -SH groups of these enzymes (113). Moreover, it has been shown by a number of workers that BAL can reverse the toxic effects of cadmium both *in vivo* and *in vitro* (113).

Table XVI : Effects of Cadmium

Expt.	Addition	Final concn.	µl. O_2/30 mins Endogenous	TYR	"Extra O_2"	% of control
1	none		5	107	102	100
	$Cd(Cl)_2$	1×10^{-6}M	19	101	82	80
	"	1×10^{-2}M	0	0	0	0
2	none		11	134	123	100
	$Cd(Cl)_2$	1×10^{-5}M	14	130	116	94
	"	1×10^{-4}M	9	122	113	92
	"	1×10^{-3}M	8	109	101	82
				TRYP		
3	none		16	37	21	100
	$Cd(Cl)_2$	1×10^{-6}M	5	41	36	172
	"	1×10^{-5}M	2	32	30	162
	"	1×10^{-4}M	17	46	29	138
	"	1×10^{-3}M	14	17	3	14
				ISO		
4	none		14	57	43	100
	$Cd(Cl)_2$	1×10^{-6}M	3	46	43	100
	"	1×10^{-5}M	2	56	54	126
	"	1×10^{-4}M	2	67	65	151
	"	1×10^{-3}M	20	30	10	23

Table XVI : Effects of Cadmium (Continued)

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	ISO	"Extra O_2"	% of control
5	none		16	62	46	100
	Cd)Cl)$_2$	1 x 10^{-6}M	22	60	38	83
	"	1 x 10^{-5}M	18	61	43	94
	"	1 x 10^{-4}M	19	68	49	107
	"	1 x 10^{-3}M	24	29	5	11

(g) Effects of Barium

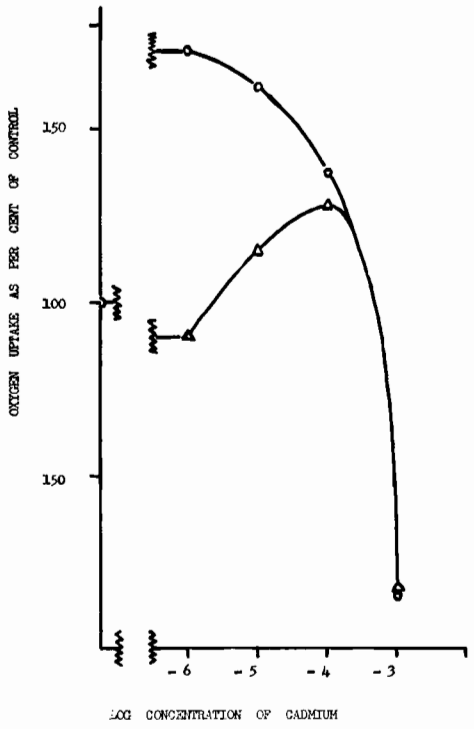
At the concentrations tested, Ba(Cl)$_2$ inhibited oxygen uptake in the presence or absence of added tyramine (cf. Table XVII).

Table XVII : Effects of Barium

Expt.	Addition	Final concn.	µl. O_2/30 mins Endogenous	TYR	"Extra O_2"
1	none		17	137	120
	Ba(Cl)$_2$	2.5 x 10^{-5}M	4	108	104
	"	2.5 x 10^{-3}M	0	94	94

Figure 2 : Effects of Cadmium Ions on Amine Oxidation by Rat Liver Homogenates

(Cf. page 60 and Table XVI.)



o—o Tryptamine used as substrate

Δ—Δ Iso-amylamine used as substrate

(h) Effects of Mercury

Mercuric acetate, $Hg(AcO)_2$ was found to reduce MO activity by one-half when added at a final concentration of the order of 10^{-4} M. Low concentrations of $Hg(AcO)_2$ apparently activate tyramine oxidation (cf. Table XVIII).

In an attempt to reverse the mercury inhibition with BAL, it was found that at the concentrations used, BAL inhibited MO in the absence of added mercury, and this makes it difficult to draw any conclusions from the re-activation experiments.

Our results on the inhibitory effects of Hg on amine oxidation corroborate previous findings reported in the literature (85, 86).

The activating effects of mercury added in low concentrations were more apparent when non-dialysed liver suspensions were employed as source of MO (cf. Table XVIII, expt. 2). The significance of this finding has not been explained as yet.

Table XVIII :- Effects of Mercury

Expt	Addition	Final concn.	µl. O_2/30 mins.		"Extra O_2"	% of control
			Endogenous	TYR		
1	none		7	121	114	100
	$Hg(AcO)_2$	5×10^{-5}M	10	118	108	95
	"	2×10^{-4}M	23	112	89	78
	"	3×10^{-4}M	15	95	80	70
	"	4×10^{-4}M	8	37	29	25
	"	5×10^{-4}M	4	0	-4	0
2(a)	none		30	143	113	100
	$Hg(AcO)_2$	5×10^{-6}M	25	186	161	142
3	none		8	87	79	100
	$Hg(AcO)_2$	5×10^{-7}M	6	97	91	115

(a) Liver preparation not dialysed for this expt..

Table XVIII Effects of Mercury (Continued)

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2"	% of control
4	none		11	107	96	100
	$Hg(AcO)_2$	4×10^{-4}M	9	31	22	23
	BAL(b)	4×10^{-4}M	15	75	60	63
	BAL(b) + $Hg(AcO)_2$	4×10^{-4}M	18	63	45	46
	BAL(b)	8×10^{-4}M	24	52	28	29
	BAL(b) + $Hg(AcO)_2$	4×10^{-4}M	18	52	34	35

(b) BAL tipped from second side-arm at "zero time".

(i) Effects of Aluminium

Aluminium sulfate, $Al_2(SO_4)_3 \cdot 18\ H_2O$, when added at a final concentration of 10^{-3}M, inhibited MO activity more than 50% (cf. Table XIX).

Table XIX : Effects of Aluminium[a]

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2"
1	none		2	119	117
	$Al_2(SO_4)_3$	1×10^{-4}M	13	131	118
	"	1×10^{-3}M	9	56	47

(j) Effects of Titanium

Titanium sulfate, $TiOSO_4$, was inert towards amine oxidation at the concentrations tested (cf. Table XX).

Table XX : Effects of Titanium

Expt.	Addition	Final concn.	µl. O_2/65 mins. Endogenous	TYR
1	none		22	152
	$TiOSO_4$	4×10^{-4}M	29	168
	"	4×10^{-3}M	29	164

(a) In these experiments, phosphate buffer (final concn. : 0.02 M, pH : 7.4) was used instead of TRIS buffer.

(k) Effects of Tin

Tin, added as stannous chloride, $Sn(Cl)_2$, activates to a small extent at the higher concentration tested. (Table XXI).

Table:XXI Effects of Tin

Expt.	Addition	Final Concn.	µl. O_2/60 mins.		
			Endogenous	TYR	"Extra O_2"
1	none		26	151	125
	$Sn(Cl)_2$	5×10^{-6} M	28	138	110
	"	5×10^{-3} M	41	181	140

(l) Effects of Cerium

Ceric sulfate, $Ce(SO_4)_2$, apparently activated amine oxidation when added in low concentrations (cf. Table XXII). In testing this ion, guinea-pig liver was used as source of MO instead of rat liver.

Ceric salts are known to stabilize H_2O, forming cerium peroxide. Wieland (114) has made use of this property for quantitatively determining H_2O_2 formed during the oxidation of xanthine by xanthine oxidase. In this method, cerium peroxide formed during aldehyde oxidation by xanthine oxidase is decomposed by acid to give H_2O_2 and ceric ions which are determined by titration. The H_2O_2 is then decomposed by catalase. When catalase is present in a biological system, however, it reacts more rapidly with H_2O_2 than does the cerium salt, thus preventing the formation of cerium peroxide.

Since catalase is present in our tissue preparations, it is unlikely that added cerium salts can form peroxides with H_2O_2 produced during amine oxidation.

In our experiments, all flasks containing the ceric sulfate showed flocculation of protein.

Table XXII : Effects of Cerium

Expt.	Addition	Final concn.	µl. O_2/20 mins. Endogenous	TYR	TRYP	ISO
1(a)	none		10	81	70	
	$Ce(SO_4)_2$	5×10^{-7}M	0	85	76	
	"	5×10^{-6}M	4	84	72	
	"	5×10^{-4}M	4	75	84	
2(b)	none		13			54
	$Ce(SO_4)_2$	5×10^{-6}M	2			55
	"	5×10^{-3}M	11			54

(a) Non-dialysed liver suspension employed.

(b) Liver suspension dialysed 18 hrs. vs .03 M TRIS, pH 8.0.

(m) Effects of Lead

Lead acetate, $Pb(AcO)_2$, inhibited amine oxidation at the concentrations tested (cf. Table XXIII).

Table XXIII Effects of Lead

Expt.	Addition	Final concn.	µl. O_2/20 mins. Endogenous	TYR
1	none		0	107
	$Pb(AcO)_2$	4×10^{-5}M	0	96
	"	4×10^{-3}M	0	77

(n) Effects of Chromium

Chromium was tested as potassium chromate and as sodium dichromate. Both these compounds are known oxidizing agents by virtue of their anions, $CrO_4^{"}$ and $Cr_2O_7^{"}$, respectively. Potassium chromate showed a small inhibitory effect, whilst sodium bichromate had both activating and inhibitory effects in different experiments (cf. Table XXIV).

Table XXIV: The effects of Chromate and Dichromate

Expt.	Addition	Final concn.	"Extra O_2"	% of control
1	none		115	100
	K_2CrO_4	2.5×10^{-5}M	111	97
	"	2.5×10^{-3}M	105	91

Table XXIV The effects of Chromate and Dichromate (Continued)

Expt.	Addition	Final concn.	"Extra O_2"	% of control
2(a)	none		83	100
	$Na_2Cr_2O_7$	1×10^{-3}M	81	98
	"	4×10^{-3}M	60	72
	"	8×10^{-3}M	48	58
3(a)	none		114	100
	$Na_2Cr_2O_7$	1×10^{-3}M	128	112
	"	4×10^{-3}M	226	198
	"	8×10^{-3}M	42	37
4(a)	none		90	100
	$Na_2Cr_2O_7$	4×10^{-4}M	81	90
	"	4×10^{-3}M	57	63

(e) Effects of Selenium.

Selenium salts can readily combine with -SH groups (115), and thereby inactivate a number of enzymes requiring free -SH groups for activity. Succinoxidase is inhibited

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(a) In these experiments, 0.02 M phosphate buffer, pH 7.4, was employed instead of TRIS buffer.

by selenite (116), although the inhibition does not occur immediately upon addition of the salt. Bernheim et al (117) found that a similar latent period occurs before added selenite inhibits MO or choline oxidase, both "-SH enzymes. These authors interpret this retarded inhibition as an indication that rather than combining with the -SH groups of the enzyme, in which case, the inhibition would occur immediately upon addition of the salt, selenite catalyzes the oxidation of the essential -SH groups (117). Bernheim et al have, moreover, observed that selenite can produce a small activation of MO activity during the first hour of incubation, which is then followed by an inhibition. No explanation has been suggested for this activating effect during the latent period (117).

In our experiments, incubations were carried out for only one hour when testing the effects of selenium, and it was found that relatively high concentrations of Na selenite produced no effects on tyramine oxidation during this time. Testing selenium dioxide, it was found that, whilst at the lower concentration tested there was a small activation, relatively high concentrations of SeO_2 inhibited MO activity during the first hour of incubation. (Cf. results shown in Table XXV).

Table XXV : Effects of Selenium

Expt.	Addition	Final concn.	µl. O_2/60 mins. Endogenous	TYR	"Extra O_2"	% of control
1	none		30	258	228	100
	SeO_2	5×10^{-6}M	20	266	246	108
	"	5×10^{-3}M	52	250	198	89
2	none		-4	135	139	100
	Na selenite	1×10^{-3}M	13	156	143	103
	"	1×10^{-2}M	15	154	139	100

(p) Effects of Molybdenum

Molybdenum was tested both as the trioxide, MoO_3, and as the ammonium salt, $(NH_4)_6\ Mo_7O_{24} \cdot 4\ H_2O$. Molybdic trioxide which is only sparingly soluble in water (1.06 gm./l. at 18°C), was added as a suspension.

Since the results obtained with either MoO_3 or the ammonium salt were very similar, only the effects of ammonium molybdate are reported (Table XXVI).

Ammonium molybdate reduced amine oxidation to one-half when added in concentrations of the order of 10^{-3}M. Figure 3 illustrates the fact that the effects of Mo were very similar, regardless of what substrate was employed.

Table XXVI : Effects of Molybdenum

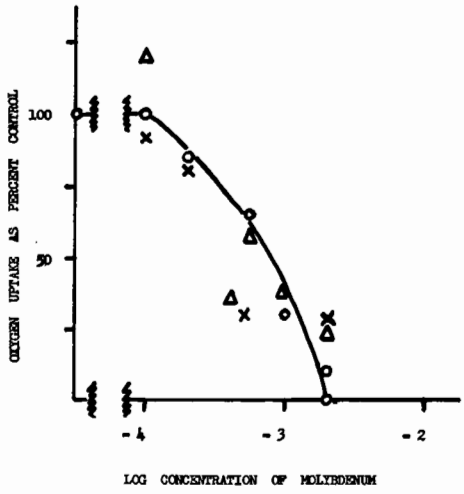
Expt.	Addition	Final concn.	µl. O_2/30 mins.			
			Endogenous	TYR	TRYP	ISO
1	none		0		44	39
	Mo^{VI} (a)	1×10^{-4}M	0		53	42
	"	2×10^{-4}M	0		52	30
	"	4×10^{-4}M	0		25	13
2	none		5	108	54	56
	Mo^{VI}	5×10^{-4}M	4	70	30	20
	"	1×10^{-3}M	0	25	21	12
	"	2×10^{-3}M	0	0	0	0
3(b)	none		34	142	62	87
	Mo^{VI}	1×10^{-4}M	30			78
	"	2×10^{-4}M	32	123	52	74
	"	4×10^{-4}M	35		45	53
4(b)	none		31	151	71	86
	Mo^{VI}	5×10^{-4}M	33	153	59	
	"	1×10^{-3}M	2	38	17	18
	"	2×10^{-3}M	5	18	14	5

(a) Mo^{VI} stands for ammonium molybdate.

(b) Liver preparations not dialysed in these experiments.

Figure 3 : Effects of Ammonium Molybdate on Amine Oxidation by Rat Liver Homogenates

(Cf. ps. 73 - 74)



Substrate used: Tyramine —o—o—

Tryptamine Δ

Iso-amylamine ×

(q) Effects of Uranium

Uranium acetate, $UO_2 (C_2H_3)_2)_2 \cdot 2H_2O$, at neutral pH, exists in solution predominantly as UO^{++} ions, other forms being unstable. Dounce et al have reported some work on the effects of uranium on a variety of enzyme systems in (Pharmacology and Toxicology of Uranium Compounds" (118). They found that uranium acetate does not affect MO activity, using adrenaline as substrate. (In their experiments, the uranium salt was employed at the relatively low concentration of 5×10^{-4}M). The same authors also found that UO_2^{++} ions have no affinity for -SH groups, while they readily form complexes with ionized -COOH groups.

In our experiments, it was found that UO_2^{++} ions inhibit only when added at concentrations of the order of 10^{-2}M (cf. Table XXVII). At low concentrations, uranium acetate sometimes activated amine oxidation; the cause of this activating effect is not known. When added in concentrations above 10^{-3}M, uranium acetate produced a flocculation of proteins in the flasks. It is possible that the inhibitory effects observed when the metal is added in high concentrations is due to the precipitation of proteins, and not to some specific effects on MO.

Table XXVII : Effects of Uranium

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	TRYP
1	none		20	92	59
	uranium acetate	5×10^{-7}M	15		59
	" "	5×10^{-6}M	14	137	73
	" "	5×10^{-5}M	12		52
	" "	5×10^{-4}M	16		56
	" "	5×10^{-3}M	29		53
	" "	2.5×10^{-2}M	0	18	16
2(a)	none		27		103
	uranium acetate	5×10^{-6}M	39		166
	" "	2.5×10^{-2}M	0		15
3	none		18	101	55
	uranium acetate	5×10^{-7}M	19	106	54
	" "	5×10^{-6}M	16	100	55
	" "	5×10^{-5}M	13	98	50

(r) Effects of Manganese

Manganese was tested as its sulfate, $MnSO_4$ and as its permanganate, $KMnO_4$. $MnSO_4$ was inhibitory at all concentrations tested except in one experiment, where Mn^{II} produced an apparent activation when added in high concentrations (cf. Table XXVIII, expt. 1). In this last case, the results cited are the means of duplicate determinations. It is possible that the activations observed are due to the sulfate anion, since it was previously shown that SO_4" ions can apparently activate amine oxidation when added in high concentrations (cf. Table IV).

Potassium permanganate, at relatively high concentrations, reduces MO activity (cf. Table XXVIII). When TRIS buffer was employed, it was found that $KMnO_4$ was immediately decolorized and reduced when added to the reaction mixture prior to the addition of the enzyme preparation. In this case, no inhibition of MO activity was observed until relatively high concentrations of permanganate were added. On the other hand, using phosphate buffer, permanganate produced a marked inhibition at low concentrations. The inhibitions observed with permanganate may be due to oxidation of -SH groups essential for MO activity. It is known that permanganate readily oxidizes free -SH groups at neutral pH (112).

Table XXVIII : Effects of Mn^{II} and Potassium Permanganate

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2"
1	none		0	86	86
	$MnSO_4$	1×10^{-4}M	2	85	83
	"	1×10^{-3}M	13	82	69
	"	1×10^{-2}M	7	118	111
		"			
		(duplic.)	9	121	111
2	none		3	77	74
	$MnSO_4$	1×10^{-2}M	2	55	53
3	none		18	141	123
	$MnSO_4$	2.5×10^{-4}M	9	118	109
	"	1×10^{-2}M	30	123	93
4(a)	none		17	137	120
	$KMnO_4$	1.5×10^{-3}M	21	140	119
	"	3×10^{-3}M	3	85	82
5	none		0	132	132
	$KMnO_4$	1×10^{-3}M	-5	69	74
	"	4×10^{-4}M	-6	27	33

(a)
Here, TRIS buffer was used (0.037 M, pH 7.4). In all other experiments, phosphate buffer was employed (0.02 M, ph 7.4).

(s) Effects of Iron

Fe^{II} and Fe^{III} were tested under various experimental conditions; the results obtained are summarized in Table XXIX.

(i) Effects of $FeSO_4$

Ferrous sulfate, at final concentrations of the order of 10^{-2}M, reduced amine oxidation to one-half. It was found, moreover, that low concentrations of the salt produced an apparent activation of amine oxidation, (cf. Table XXIX, expts. 5, 6 and 7). This finding confirms data reported in the literature (84).

(ii) Effects of Ferric Salts

In the absence of added substrate, ferric salts produced a marked activation of oxygen uptake when added in final concentrations of the order of 10^{-4}M or higher, However, in the presence of added substrate, the oxygen uptake never exceeded that of the tissue blanks when in the presence of added ferric ions[(a)]. It would appear from such experiments that amine oxidation is suppressed. However, there is formation of pigment when tyramine or tryptamine are used as substrates, and this may be taken as an indication that amine oxidation did occur to some extent at least (16). The activating effects of ferric salts on the endogenous respiration was observed both when incubations were carried out under air and under pure oxygen. It is possible that the increase oxygen uptakes are due to a peroxidatic reaction for which ferric salts are known to act as catalysts (103).

(a) Cf. Figure 4.

Table XXIX : Effects of Iron

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	TRYP	ISO
1(a)	none		33	114	100	
	$Fe(Cl)_3$	5×10^{-5}M	42	114		
	"	2.5×10^{-2}M	178	61	112	
	$Fe_2(SO_4)_3$	2.5×10^{-2}M	105	105		
2	none		0	198		
	$Fe(Cl)_3$	1×10^{-4}M	184	170		
3(b)	none		0	30	39	
	$Fe(Cl)_3$	1×10^{-4}M	158	123	123	
3(b)	none		0	35		
	$Fe(Cl)_3$	1×10^{-4}M	180	178		
4(a)	none		35	115		65
	$FeSO_4$	5×10^{-4}M	42	118		70
	"	2.5×10^{-2}M	25	60		35

(a)
Liver suspension not dialysed in this experiment.

(b)
Here reactions were carried out in an atmosphere of air (i.e., no gassing with pure oxygen).

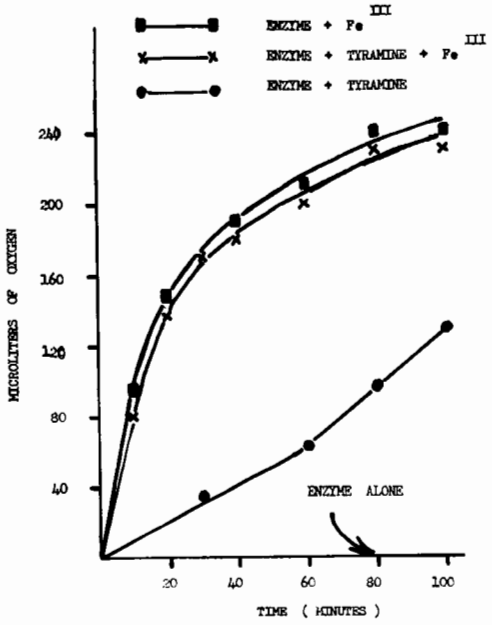
Table XXIX Effects of Iron (Continued)

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	TRYP	ISO
5	none		0	86		
	$FeSO_4$	1×10^{-6}M	0	100		
	"	1×10^{-5}M	0	106		
	"	1×10^{-4}M	0	108		
6(b)	none		0	46		
	$FeSO_4$	1×10^{-3}M	0	66		
	"	1×10^{-3}M	0	38		
7(b)	none		0	35		
	$FeSO_4$	1×10^{-3}M	0	48		

(b)
Here reactions were carried out in an atmosphere of air (i.e., no gassing with pure oxygen).

Figure 4 : Effects of Ferric Ions on Tyramine Oxidation by Rat Liver Homogenates

(Cf. page 80 and Table XXIX.)



Effects of Cobalt

Cobaltous nitrate, $Co(NO_3)_2$, enhanced MO activity to a small extent at the concentrations tested (cf. Table XXX).

The activating effect of cobaltous salts found in our experiments confirms some results reported by Perry et al who were using guinea-pig liver brei as source of MO (84).

Table XXX : Effects of Cobalt

Expt.	Addition Final concn.	µl. O_2/30 mins.		
		Endogenous	TYR	"Extra O_2"
1	none	30	143	113
	$Co(NO_3)_2$ 5×10^{-6}M	20	140	120
	" 5×10^{-3}M	23	168	145

Effects of Nickel

Nickelous ions, added as $Ni(Cl)_2$, inhibited MO activity at the concentrations tested. Some flocculation was observed in the flasks containing added nickel. The results are shown in Table XXXI .

Table XXXI : Effects of Nickel

Expt.	Addition	Final concn.	µl. O_2/20 mins. Endogenous	TYR	TRYP	"Extra O_2"
1	none		12	75		63
	$Ni(Cl)_2$	4×10^{-4}M	0	69		69
	"	8×10^{-4}M	7	49		42
2	none		2		50	48
	$Ni(Cl)_2$	1×10^{-3}M	0		32	32
3(a)	none		0	40		40
	$Ni(Cl)_2$	1×10^{-3}M	0	28		28

(a) In this experiment the gas phase was air instead of pure O_2.

SUMMARY

In Table XXXII are listed a number of ions which were found to produce an apparent activation of amine oxidation.

A number of metal ions inhibited amine oxidation. The Table XXXIII are shown the concentrations at which specific % inhibitions were observed.

The following ions were either inert towards amine oxidation or slightly inhibitory (less than 15%): Ti^{IV}, Ce^{IV}, Ba^{II} and Ca^{II}.

Table XXXII : Activation of MO by Metal Ions

Ion	Table Ref.	Final concn.	% of control
Cu^{I}	XI	$1 \times 10^{-6}M$	120
		$5 \times 10^{-6}M$	122
Cu^{II}	XI	$2.5 \times 10^{-5}M$	133
		$1 \times 10^{-4}M$	108
Ag^{I}	XII	$1 \times 10^{-4}M$	112
Mg^{II}	XIII	$5 \times 10^{-6}M$	177
		$5 \times 10^{-3}M$	197
Cd^{II}	XVI	$1 \times 10^{-6}M$	172
		$1 \times 10^{-5}M$	144
		$1 \times 10^{-4}M$	132
Hg^{II}	XVIII	$5 \times 10^{-7}M$	115
		$5 \times 10^{-6}M$	142
Sn^{II}	XXI	$5 \times 10^{-3}M$	112
Cr^{VI}	XXIV	$1 \times 10^{-3}M$	112
Se^{IV}	XXV	$5 \times 10^{-6}M$	108

Table XXXII :Activation of MO by Metal Ions (Continued)

Ion	Table Ref.	Final concn.	% of control
U^{VI}	XXVII	$5 \times 10^{-6}M$	162
Mn^{II}	XXVIII	$1 \times 10^{-2}M$	130
Co^{II}	XXX	$5 \times 10^{-3}M$	141

Table XXXIII Inhibition of MO by Metal Ions.
Concentrations at which specified % inhibitions are observed

Ion	Table Ref.	% Inhibition		
		25 - 49%	50 - 74%	75 - 100%
Cu^{I}	XI	$5 \times 10^{-2}M$(a)		$1 \times 10^{-2}M$(b)
Cu^{II}	XI	$1 \times 10^{-3}M$		$1 \times 10^{-2}M$
Ag^{I}	XII		$6.5 \times 10^{-4}M$	$1 \times 10^{-3}M$
Zn^{II}	XV			$1 \times 10^{-3}M$
Cd^{II}	XVI			$1 \times 10^{-3}M$
Hg^{II}	XVIII	$3 \times 10^{-4}M$		$4 \times 10^{-4}M$
Al^{II}	XIX		$1 \times 10^{-3}M$	
Pb^{II}	XXIII	$4 \times 10^{-3}M$		

Table XXXIII : Inhibition of MO by Metal Ions. (Continued)
Concentrations at which specified
% inhibitions are observed

Ion	Table Ref.	% Inhibition		
		25 - 49%	50 - 74%	75 - 100%
Cr^{VI}	XXIV	4×10^{-3}M	8×10^{-3}M	
Mo^{VI}	XXVI	4×10^{-4}M	1×10^{-3}M	2×10^{-3}M
U^{VI}	XXVII			5×10^{-2}M
Mn^{II}	XXVIII	1×10^{-2}M		
Fe^{II}	XXIX	2.5×10^{-2}M		
Fe^{III}	XXIX			1×10^{-4}M
Ni^{II}	XXXI	1×10^{-3}M		

(a)
Using tryptamine as substrate

(b)
Using tyramine as substrate

B. Effects of Sulfhydryl Compounds

Starting with the observation that MO activity could be inhibited by BAL (cf. p. 64), a number of preliminary experiments were carried out in order to assess the effects of BAL as well as some other -SH compounds. Testing the effects of -SH compounds manometrically, it was found that in general, added BAL, thioglycollic acid, cysteine and oxidized glutathione (GSSG) inhibited MO activity, while reduced glutathione (GSH) produced an activation when experiments were carried out in an atmosphere of air (this is in contrast to results obtained when the gas phase was oxygen). Tables XXXIV to XXXIX, summarize the results obtained. Someof the manometrically obtained results were further confirmed assessing MO activity spectrophotometrically (cf. Table XXXVIII), by themethod of Sourkes et al (119). Finally, it was found that dialysis of the tissue preparation agains M/50 cysteine, pH 8.1, consistently inhibited MO activity (cf. Table XXXVII).

1. Procedure

Rat liver suspensions were prepared as indicated previously (cf. p. 55). Reaction mixtures for the manometric assay follow the same model system previously described (cf. p. 45). The sulfhydryl compounds and substrates were added in separate side-bulbs of the flasks containing the reaction mixtures, and both were tipped at "zero time".

Spectrophotometric assay of MO activity was carried out according to the method described by Sourkes et al (119). Briefly, theprocedure consists in the following steps. After

incubation for a given time in the Warburg bath, during which time O_2 consumption is measured, 0.1 ml. of 60% $HClO_4$ is tipped from the side-bulb or added to the flasks immediately after removal of these from the bath, to precipitate the protein contents of the reaction mixtures. The flask contents are then transferred into test tubes for centrifugation and the supernatants are diluted appropriately. Tryptamine absorbs maximally at 279 mμ in the Beckmann DU spectrophotometer. The aldehyde formed during enzymic oxid ation is precipitated with proteins, and does not interfere with the optical density readings. Therefore, by examining the optical density of the deproteinized flask contents at different time intervals, one can measure the amount of tryptamine remaining (i.e., not deaminated by MO). Subtracting the O.D. values at a given time from those found for the "zero time" flasks, which contain the original amount of amine added, one determines the amount of tryptamine which has disappeared, and this in turn is a measure of MO activity. This method is particularly useful in cases where O_2 consumption measurements alone cannot tell us whether any amine is being oxidized.

2. Results

(a) Effects of BAL

At the concentrations tested, BAL is inhibitory in all cases except one (Table XXXIV). At concentrations of the order of 10^{-3}M, BAL itself is being rapidly oxidized and thus produces high O_2 uptake in the blanks (i.e., no added substrate). In the absence of tissue, BAL is less readily oxidized. The tissue contributes therefore, to the oxidation of added BAL, and this could be done in at least two

ways: in virtue of the metals present in the tissue; or in virtue of enzymes present in the tissue capable of catalyzing the oxidation of BAL. It is known that BAL can be rapidly oxidized in the absence of enzymatic catalysts by traces of copper, iron or manganese, heavy metals which form unstable complexes with thiol groups, in contrast to Cd, Hg, Ag, which form mercaptides with small dissociation constants (112).

(b) Effects of Thioglycollic Acid

Thioglycollic acid (TGA) inhibited MO activity consistently (cf. Table XXXV), and this was confirmed spectrophotometrically (cf. Table XXXVIII). Like BAL, it also enhanced the respiration of the tissue blanks at the higher concentrations tested.

The appearance of a yellow-green precipitate was observed in flasks containing TGA. This is possibly due to complex formation of the mercaptan with metal ions such as Fe^{II}, Mo^{VI} or Sn^{II}, which are known to form yellow complexes with TGA and neutral pH.

(c) Effects of Cysteine and Cystine.

Cysteine, like BAL, was found to inhibit most readily when it was itself being oxidized to some degree, thus raising the endogenous respiration, (cf. p. 64, Table XXXVI). For this reason, it was thought useful to find what effects dialysis of the tissue against cysteine would have on MO activity. Most of the cysteine was then removed by further dialysis against distilled water. Table XXXVII indicates that dialysis against cysteine M/50 consistently decreased MO activity, and had little, if any effect, on the endogenous respiration.

Cystine, on the other hand, only decreases amine oxidation when it is ground with the tissue. The low solubility of cystine in water, hence its unavilability, may account for the effects observed.

(d) Effects of Glutathione

Added glutathione was found to enhance MO activity, when the gas phase was air (Table XXXIX), without affecting respiration of the blanks to any extent. In addition, it was found that DPN sometimes increased the activating effects of GSH. Under oxygen, GSH either activated or inhibited MO activity to a small extent. On the other hand, oxidized glutathione (GSSG), inhibited under oxygen, but not under air. These preliminary results warrant further investigation on the effects of glutathione.

Table XXXIV : Effects of BAL

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2	% of control
1	none		9	113	104	100
	BAL	4×10^{-4}M	15	111	96	92
	"	8×10^{-4}M	25	84	59	57
2	none		11	107	96	100
	BAL	4×10^{-4}M	15	75	60	62
	"	8×10^{-4}M	24	52	28	29
3(a)	none		8	125	117	100
	Bal	8×10^{-4}M	7	125	118	100

(a) Phosphate buffer (final concn. 0.02M; pH 7.4) is employed instead of TRIS buffer.

Table XXXIV: Effects of BAL (Continued)

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2"	% of control
4(a)	none		0	127	127	100
	BAL	8×10^{-4}M	10	108	98	77
5	none		0	113	113	100
	BAL	8×10^{-4}M	11	92	81	72
6	none		0	90	90	100
	BAL	8×10^{-3}M	152	202	50	56
	"	1.6×10^{-3}M	232	278	46	51
	"	" " (b)	126	116	-10	0
7	none		5	100	95	100
	BAL	4×10^{-4}M	42	114	72	78
	"	" " (b)	21	13	-8	0

(b) No tissue added to flasks.

Table XXXV Effects of Thioglycollic Acid

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2"	% of control
1[a]	none		0	100	100	100
	TGA	2×10^{-3}M	48	22	-26	0
2	none		0	84	84	100
	TGA	2.5×10^{-3}M	9	25	16	17
3	none		5	100	95	100
	TGA	5×10^{-3}M	46	47	1	1
	"	" "	56	55	-1	0
	"	" "[b]	6	-3	-9	0

(a) Phosphate buffer (final concn. 0.02M; pH 7.4) is employed instead of TRIS buffer.

(b) Boiled enzyme preparation employed.

Table XXXVI ; Effects of Cysteine and Cystine

Expt.	Addition	Final concn.	µl. O_2/30 mins. Endogenous	TYR	TRYP	"Extra O_2"	% of control
1.	none		15	99		84	100
	cysteine	3.16×10^{-2}M	5	2		-3	0
	cystine	3.16×10^{-2}M	14	95		81	97
	"	6.32×10^{-2}M	7	91		84	100
2.(a)(b)	none		56		99	43	100
	cysteine	4×10^{-3}M	85		104	19	44
3.(a)(b)	none		20		140	120	100
	cysteine	2×10^{-4}M	42	152		110	92
	"	2×10^{-3}M	75	140		65	54

(a)
Phosphate buffer (final concn. : 0.02 M ; pH 7.4) employed instead of TRIS buffer.

(b)
Tissue suspension not dialysed.

Table XXXVI : Effects of Cysteine and Cystine (Continued).

Expt.	Addition	Final Concn.	µl. O_2/30 mins. Endogenous	TYR	TRYP	"Extra O_2"	% of control
4(a)(c)	none		0	81		81	100
	cysteine	3.2×10^{-6}M	6	85		79	
	"	8×10^{-5}M	2	84		82	
	"	4×10^{-4}M	6	91		85	
	"	2×10^{-3}M	9	88		79	
	"	1×10^{-2}M	55	87		32	40
5(d)	none		8	141		133	100
	cystine	6.16×10^{-2}M	8	117		109	82

(a)
Phosphate buffer (final concn. : 0.02 M ; pH 7.4) employed instead of TRIS buffer.

(b)
Values represent µl. O_2/20 mins.

(d)
In this experiment, the dialysed liver preparations were re-homogenized in a Teflon homogenizer in the presence of 20 mg. cystine/ml. of tissue suspension. A control was run by re-homogenizing a sample of the suspension in the absence of added cystine. This last preparation gave exactly the same value as the untreated liver tissue.

Table XXXVII : Effects of Dialysis against M/50 Cysteine[a]

Expt.	Preparation	µl. O_2/30 mins. Endogenous	TYR	"Extra O_2"	% of control
1	control	10	125	115	100
	experimental	20	85	65	57
2	control	25	165	140	100
	experimental	20	120	100	71
3	control	7	122	115	100
	experimental	24	107	83	72

Table XXXVIII : Effects of Cysteine and Thioglycollic Acid

Expt.	Addition	Final concn.	µM O_2/30 mins.	µM TRYP lost in 30 mins.
1	none		1.34	3.75
	TGA	4×10^{-3}M	0.02	0
2	none		2.2	6.1
	Cysteine	3.16×10^{-2}M	0	4.3

(a)
Tissue suspensions were dialysed 6 hrs. against M/50 cysteine, pH 8.1, then 12 hrs. against distilled H_2O to remove the cysteine. Controls were dialysed against distilled H_2O for 18 hrs. All dialysis were carried out at 3° C.

Table XXXIX Effects of Glutathione

Expt.	Gas phase	Addition per flask	µl. O_2/hr. Endogenous	TYR	ISO	"Extra O_2"
1	Air	none	6	53		47
		3 mg. GSH	3	116		113
		3 mg. GSSG	0	49		49
	Oxygen	none	0	183		183
		3 mg. GSH	2	166		164
		3 mg. GSSG	14	134		120
2	Air	none	12		48	36
		3 mg. GSH	14		62	48
		0.5 mg. DPN	28		63	35
		GSH + DPN	16		76	60
3(a)	Air	none	3	68		65
		2 mg. GSH	5	112		107
	Oxygen	none	12	194		182
		2 mg. GSH	16	211		195

(a) In this experiment , 140 mg. wet wt. of tissue suspension was used per flask.

DISCUSSION AND CONCLUSIONS

In a previous section, the results of a study of the effects of ions on the monoamine oxidase enzyme complex[16] of rat liver suspensions have been presented. The purpose of this study was to scan various metal ions in order to obtain an over-all picture of their effects on the oxidation of monoamines. Since routine assay of monoamine oxidase (MO) consisted in measuring total oxygen uptake, it was not possible to determine whether the ions acted specifically on the MO oxidizing system or on secondary reactions which occur concomitantly with amine oxidation. An increase in the net oxygen uptake, for instance, can result from an inhibition of catalase, (cf. Footnote 16, reaction (3) as well as from an activation of MO or of aldehyde oxidase (see Footnote 16 reactions (1) and (2).

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Monoamine oxidase enzyme complex (MO), i.e., the enzymes involved in the following reactions:

(1) $R\text{-}CH_2NH_3^+ + O_2 + H_2O \xrightarrow{\text{"amine oxidase"}} R\text{-}CHO + NH_4^+ + H_2O_2$

(2) $R\text{-}CHO \xrightarrow{\text{"aldehyde oxidase"}} R\text{-}COOH$

(3) $H_2O_2 \xrightarrow{\text{Catalase}} H_2O + 1/2\ O_2$

(4) net reaction: $R\text{-}CH_2NH_3 + O_2 \xrightarrow{\text{"MO"}} R\text{-}COOH + NH_4^+$

The ions which produced an apparent activation of MO did so only when added in concentrations of the order of 10^{-4}M or less. It is possible to attribute these effects to an inhibition of catalase. A survey of the literature has not revealed many previous studies on the effects of metal ions on catalase, but Cu^{II}, and to a lesser extent, Pb^{II}, have been claimed to inactivate catalase of wheat sprout. Alternately, the activations observed may be caused by the ions, activation of reactions (1) or (2). Among the metals which activate at these concentrations are some heavy metals which, when added in larger amounts, are toxic towards MO. These include Hg,Cd,Cu^{I}, Cu^{II} and Fe^{II} [17]. It may be mentioned here that other instances have been reported in the literature in which substances acted biphasically towards enzymes, i.e., substances which activate at low concentrations and inhibit when added in higher concentrations (121).

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Since copper, in concentrations of the order of 10^{-4}M, activated amine oxidation when tyramine or tryptamine were used as substrates, but not in the case of isoamylamine, it is possible that the activating effects are due to ring oxidation of the substrate, or to some other effect on the ring of the substrates.

The activating effects of Mg^{II}, U^{VI}, Ti^{II}, Sn^{II} and Co^{II} have not been explained. When used in higher concentrations, these ions where less inhibitory towards amine oxidation than those previously mentioned.

The most toxic metals tested were heavy metals, whose common property of forming stable mercaptides with thiols must be considered in greater detail. MO is considered to require free -SH groups for activity (29). It is a generally accepted fact that metals which form mercaptans with thiols can also react in a similar way with the -SH groups of proteins (120). Furthermore, there is evidence indicating that the inhibition of "SH-enzymes" by heavy metals is directly related to the ability of these metals to form insoluble mercaptides with thiols (120). It is suggested that the heavt metals tested inhibit, in part at least, by combining with -SH groups essential for MO activity. In support of this suggestion is the observation (Table XII.), that the inhibition of MO by silver salts can be prevented by the addition of BAL to the reaction mixtures. The results with respect to the inhibition of MO by Hg, confirm the findings of Friedenwald et al (57) and others (85). In comparison with other "SH-enzymes", MO appears to be relatively insensitive towards Hg.

In spite of the above considerations, one cannot exclude the possibility that heavy metals may combine with other functional groups of the enzyme protein. For example, Haarmann (122) reported that mercury salts were able to combine with carboxyl and amino groups in proteins as well as with -SH groups. Benesch, in a recent symposium (123), has warned against the too-commonly-accepted idea that mercury salts and other "-SH reagents" combine exclusively with thiol groups in proteins.

Several other metal ions tests were only moderately inhibitory towards amine oxidation. In contrast with the more toxic metals, these ions did not inhibit maximally at the beginning of the reaction. The course of the inhibition produced by these ions suggests that they catalyse a change in some active group of the MO system, and that they do not combine with some group on the enzyme (as do for example Hg, Ag, Cd, etc). A progressive inhibition (with time) can also mean that a product of the enzymic reaction is cause of the inhibition. For example, in the case of amine oxidation, it is possible that certain metals inhibit aldehyde oxidase, leading to an accumulation of the aldehyde formed during the reaction, which would then act itself as an inhibitor of MO. Liver aldehyde oxidase is an "SH-enzyme", and the comments concerning MO inhibition by metals apply to this enzyme also. Moreover, it has been shown by Oster (97) that addition of aldehydes to the reaction mixtures can depress MO activity.

In view of the interest in the -SH groups of MO elicited from the experiments on metal effects, it was decided to test the effects of various -SH compounds. Thiol groups generally can affect enzyme systems in two ways: (i) by combining with metals which have an effect on the enzyme; (ii) by acting as reducing agents. It is known that certain thiol compounds can activate enzymes by the removal of traces of toxic metals. On the other hand, thiol compounds are capable of inhibiting a number of enzymes by combining with metal ions of metallo-protein systems. For example, Webb et al (124) have shown that BAL, in concentrations of the order of 10^{-3}M, markedly inhibits phenol oxidase (Cu^{II}), carbonic anhydrase (Zn^{II}), catalase (Fe^{III}) and peroxidase (Fe^{III}).

We have found that thioglycollic acid (TGA), cysteine and BAL all inhibited amine oxidation when added in concentrations of the order of 10^{-3}M. At these concentrations, moreover, these thiol compounds are themselves oxidized to some extent, and it was necessary to check their inhibitiory effects with techniques independent of oxygen uptake measurements. Employing the MO assay of Sourkes et al (119), measuring substrate disappearance, the inhibitory effects of BAL and TGA were confirmed. Moreover, it was found that dialysis of the tissue against weakly alkaline cysteine (M/50) caused a decrease in MO activity. Addition of cobalt, iron, manganese and copper did not restore the MO activity of the dialysed preparations.

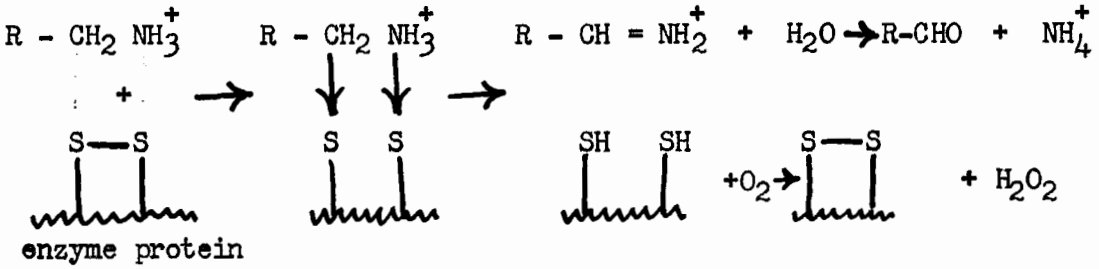
Two conclusions emerge from

these experiments: firstly, the thiol compounds mentioned inhibit amine oxidation, and this inhibition is not simply due to elevated blank values because of the oxidation (or autoxidation) of these substances; secondly, the effects of dialysis against cysteine are probably not due to the removal of iron, copper, cobalt or manganese from the MO system, since these ions did not reactivate the system. Whether other ions can reactivate it remains to be ascertained.

Barron et al (125) have reported the inhibition of succinoxidase, cytochrome oxidase and, to a lesser extent, cholinesterase by BAL. These authors attributed the inhibition to the reducing effects of BAL. A similar explanation may be applied to the case of MO. Possibly, SH compounds maintain the sulfhydryl troups of MO in the reduced state; alternately, the inhibition may be due to the oxidized form of the thiol compounds tested (the disulfides). This may explain why inhibitions by BAL, cysteine and TGA occur most markedly when there is oxidation (or autoxidation) of the thiol compounds tested. Additional evidence in support of this possibility comes from the observation that cystine and oxidized glutathione inhibited MO. We have also found that other oxidizing substances are

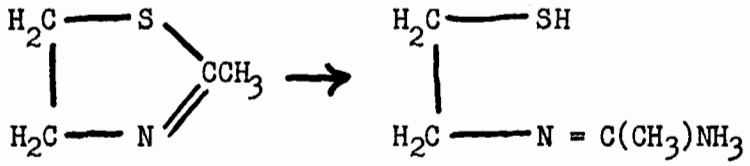
inhibitory towards MO: $KMnO_4$ and Na_2O_7 depressed amine oxidation markedly when added in concentrations of the order of 10^{-3}M. Others have reported the inhibition of MO by iodine, a very reactive oxidizing agent, which is known to inactivate "SH enzymes" (85). On the other hand, we have found that ascorbic acid can, in some cases, inhibit MO, presumably by virtue of its reducing power.

To explain the effects of thiol compounds on MO, the following suggestions are made. It is conceivable that the functional -SH groups of MO act in a shuttle-like mechanism, being alternately reduced and oxidized in the course of amine oxidation. The addition of compounds which maintain them in either the oxidized or reduced state would then prevent the normal dynamic function of these groups, hence inhibiting the enzyme. Furthermore, a possible mode of action of the functional SH groups of MO is proposed. This is illustrated below:



In such a reaction, all the primary products of amine oxidation are accounted for.

Linderstrøm-Lang (126) has formulated an attractive hypothesis to explain the functioning of thiol groups of proteins. He postulated that the -SH groups (those of the sluggish and masked types) do not exist as such, but are combined in the form of cyclic reversible structures such as the thiazolidines. The -SH groups of such compounds become freely reacting in the presence of ammonium salts which readily open the thiazolidine ring. This is illustrated below:



Finally, we may mention a number of preliminary experiments reported in this thesis concerning the effects of glutathione, when amine oxidation is assayed under air instead of under high partial pressures of oxygen. It is recalled that under air, amine oxidation proceeds at only one-third of the rate under oxygen. Reduced glutathione (GSH) was generally found to stimulate amine oxidation under these conditions. On the other hand, oxidized glutathione (GSSG) had no effects. Under oxygen, GSH inhibited or activated amine oxidation to a small extent, while GSSG usually inhibited. No explanation has been found to account for the stimulatory effects of GSH. From considerations previously

stated in this thesis (cf. "Introduction"), the possibility exists that amine oxidation proceeds through different routes under air than under high partial pressures of oxygen. Further studies on the mechanism of action of MO under air are needed before any attempts are made to explain the effects of GSH. The possibility that GSH activates by the removal of metals to which MO is more sensitive under air than under oxygen remains to be explored.

S U M M A R Y

1. The effects of ions and monoamine oxidase activity of rat liver suspension was studied manometrically measuring oxygen consumption (cf. summary of results obtained given on pages 86 to 89).

2. The effects of sulfhydryl compound on MO activity was studied. It was found that BAL, thioglycollic acid, cysteine, and to a lesser extent, cystine and oxidized glutathione, depressed amine oxidation when this was studied manometrically, measuring oxygen consumption. Confirmation of these results was obtained assaying MO by the spectrophotometric method of Sourkes et al (119). Glutathione, on the other hand, appears to activate MO activity when this is assayed manometrically and the gas phase is air instead of pure oxygen.

3. The anaerobic reduction of tetrazolium salts during amine oxidation was studied (cf. Appendix I). It was found that cyanide, cysteine and atebrine, as well as the iron-binding agent, o-phenanthroline, markedly inhibited this reaction.

4. Preliminary experiments indicate that ferricyanide can act as an electron acceptor during the enzymic oxidation of amines by rat liver suspensions. MO activity could be assayed measuring the reduction of ferricyanide spectrophotometrically.

5. Preliminary studies were carried out testing the effects of various metal-binding agents on amine oxidation by MO. These experiments did not yield consistent results, (cf. Appendix III).

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APPENDIX I

The Reduction of Tetrazolium Salts by Amine Dehydrogenase

A. INTRODUCTION

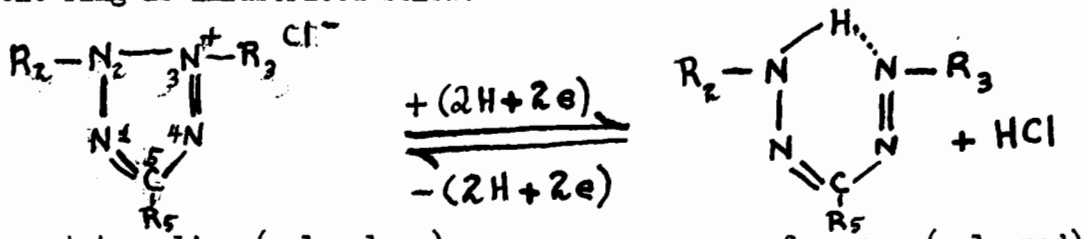
1. General Properties of Tetrazolium Salts

Tetrazolium salts (TTZ) are a class of compounds whose properties make them a useful tool in the study of cellular metabolism. They were first described by Pechmann *et al* (127) in the last decade of the nineteenth century, although it was only in 1941 that attention was drawn to their possible utility in biochemical research (128).

Nineham (129) has recently provided an extensive general survey of TTZ and their reduced derivatives, the formazans. Shorter reviews, more specifically concerned with the use of TTZ salts in biochemical research, are also available (128).

Tetrazolium salts are easily soluble quaternized tetrazoles containing a ring of 1 C and 4 N atoms, one of which is quaternary. The formazans belong to the groups of "formazyl compounds" (132) which contain the characteristic side-chain:

The most characteristic property of TTZ salts is the ability of these comparatively colourless compounds to accept hydrogen from suitable donors with formation of coloured water-insoluble formazans. This presents a marked advantage over a number of other dye-stuffs (e.g., indophenols, azines, oxazines, flavins, etc.) which, upon reduction yield colourless (or leuco-) compounds. The reduction of TTZ salts occurs with the rupture of the tetrazole ring as illustrated below:



tetrazolium (colourless) formazan (coloured)

TTZ salts, as well as their formazans, are light-sensitive (133). Exposure of these salts to ordinary light transform them to pale yellow compounds, while exposure to ultra-violet light of short wave-length brings about their reduction to the formazan. Formazans exposed to visible light fade, and this is usually attributed to a reversible cis-trans isomerization (134).

2. Biochemical Applications

TTZ salts have been successfully employed for the localization of several respiratory enzymes (129). Kuhn and Jerchel (128) found

that plant tissues reduce a number of TTZ salts to their formazans according to equation (1):

$$\text{TTZ} \xrightarrow{2H + 2e,\ \text{red.}} \text{formazan} \qquad (1)$$

This reaction occurs at pH 7.2, and could not therefore be attributable to substances such as glutathione, cysteine or ascorbic acid, normally present in cells, since they only reduce TTZ above pH 9 (129). Moreover, sugars are only effective at highly alkaline pH values (ca.,pH,11). In 1947, Lakon (135,136) showed that plant tissues heated to 80°C do not reduce TTC (triphenyltetrazolium chloride), and suggested therefore that the reduction is an enzymatic one. This suggestion is borne out by subsequent investigations.

The demonstration of particular dehydrogenases by means of TTZ salts has been reported by several authors (137-140). In 1951, Seligman and Rutenberg (139) detected the presence of several enzymes, including succinic oxidase, cysteine desulphurase, lactic and malic dehydrogenases, by incubating thick slices of animal tissues with Blue Tetrazolium (BT). In 1951, Kún demonstrated that several flavin-linked enzymes could reduce TTC (140). He further observed that the reduction of TTC by glycolytic enzymes was demonstrable only when mitochondrial fractions were added to the system (140).

Brodie and Gots (141,142) presented evidence that the reduction of TTZ salts necessitated the mediation of a flavin carrier. This was shown in particular in the case of a bacterial flavoprotein which, when dialysed against dilute HCl and potassium monophosphate to remove the flavin moiety (143), lost its ability to reduce TTC or NTC (Neo-tetrazolium chloride). Activity could be restored by addition of FAD (flavin-adenine-dinucleotide) to the dialysed enzyme preparation.

Farber et al (144) detected the presence of several DPN- and TPN-linked dehydrogenases after incubation of frozen rat kidney slices with TTZ salts. These authors were able to show that dye reduction necessitated the participation of a flavin carrier between the reduced DPN and TPN and the dye. ("DPN diaphorase" and TPN diaphorase"), (144). This confirmed the findings of Brodie and Gots (141,142).

In conclusion, it may be said that flavin carriers seem to be essential for the enzymic reduction of TTZ salts. Whether or not the reduced flavin directly transfers electrons to the dye is not certain. If, for example, the cytochrome system were involved in the reduction of the dye, it should follow that cyanide would inhibit the reduction of TTZ salts by dehydrogenases. Seligman and Rutenberg (139) have reported that the reduction of BT by tissue slices is not affected by KCN (at least anaerobically),

while Rutenberg et al (138) found that KCN completely inhibits the reduction of BT by tissue homogenates.

3. Reduction of Tetrazolium Salts by "Amine Dehydrogenase"

In 1953, Dianzani (145) detected amine oxidase activity in isolated mitochondria by means of di-tetrazolium salts. Comparing the activity of this enzyme with that of D-amino-acid oxidase, lactic and malic dehydrogenases, Dianzani (145) found that "amine oxidase activity" was quite weak. Later, Francis (79) obtained more successful results using NTC (Neo-tetrazolium) salts as H-acceptors. He incubated frozen sections of guinea-pig and rabbit liver with tyramine, and found that NTC reduction was abolished when the sections were pre-incubated for three hours without substrate, in the presence of octyl alcohol (79), which is a powerful inhibitor of monoamine oxidase (78). KCN, at a concentration of 3×10^{-3} M, did not affect dye reduction in these experiments (79).

Earlier, Blaschko (29) reported some unpublished observations on the reduction of TTC by amine oxidase. He stated that ... "this substance (TTC) forms a red reduction product in the presence of a fresh amine oxidase preparation and tyramine"(29). We have been able to confirm this observation using dialysed rat liver and brain suspensions as source of MO (cf. Tables I and Figs.I and 2). We have also tested the effects of a few inhibitors on TTC reduction

by amine dehydrogenase (cf. Table II). Preliminary work reported in this thesis has warranted further investigations on the ability of tissue homogenates to reduce TTC and NTC in the presence of amines, and a systematic study of amine dehydrogenase is presently under way in our laboratory.

B. EXPERIMENTAL

1. Procedure

Rat liver and brain suspensions were incubated in the presence of TTC and various amine substrates at 38°C in small test-tubes. More or less anaerobic conditions were maintained, since only a small surface of the reaction mixtures was exposed to air. The red water-insoluble reduction product of TTC, formazan, was extracted with acetone after the reactions were stopped by the addition of a 100% trichloracetic acid solution (0.2 ml./tube), and the amounts of formazan formed were measured spectrophotometrically in the Beckmann spectrophotometer (DU model) by reading at 485 mμ, the wave-length at which the formazen shows an absorption peak (129). The O.D. values obtained are then converted to μg of formazan, on the basis that a solution of 20.6μg formazan/ml. give a reading of 1.000 O.D at 485 mμ.

2. Preparation of Tissues

Rat liver suspensions were prepared as previously described (cf. p. 44). Rat brain suspensions were prepared as follows: rat brains were excised from the guillotined and bled animal, weighed, then homogenized in phosphate buffer (0.01 M,pH 7.4) to give a 20% suspension. This suspension was then spun at 20,000 g. (International Refrigerated Centrifuge) in the cold, and the residue, which contained most of the MO activity was re-suspended in 0.01 M phosphate buffer, pH 7.4

C. RESULTS

1. Time Curve

Fig.I shows that reduction by rat liver suspensions in presence of tyramine is linear with time, after the first 10 mins., during which there is a lag in activity.

2. Effects of Enzyme Concentration

Reduction of TTC increases with increasing concentration of enzyme (cf. Fig. 2), although there is no straight line relationship between enzyme concentration and activity.

3. Effects of Substrate Concentration

Using tyramine, it was found that in some cases, high

concentrations of substrate wereinhibitory. This was always the case when iso-amylamine was tested (cf. Fig. 3).

4. Effects of Various Amines on TTC Reduction

The aim of these experiments was to study the substrate specificity of amine oxidase in brain and liver. Preliminary results are shown in Table I. It can be seen that both mono- and diamines reduce TTC, although there are some marked differences in the reduction rates when one compares liver and brain tissues.

Table I : Enzymic Reduction of TTC by amines

Substrate	Formazan / 35 mins. / 120 mg. wet wt. tissue	
	Brain	Liver
Tyramine	0	25.80
Iso-amylamine	1.34	27.50
Adrenaline	20.25	21.30
1,2-Ethylenediamine		1.15
1,4-Diaminobutane (cadaverine)	5.68	5.95
1,6-Diaminohexane	0	13.82

Reaction mixtures contain : 200 mg. (wet wt.) of rat brain suspension or 100 mg. (wet wt.) rat liver suspension ; 40 µM substrate (neutralized ; 20 µM Phosphate buffer, pH 7.4 ; 0.2 ml. Tri-phenyl-

tetrazolium chloride solution, containing 5 mg. of dye/ml. In control tubes, substrate is replaced by equivalent volume of distilled H_2O. Volume made to 2.0 ml. with distilled H_2O. Reactions are stopped by the addition of 0.2 ml. 100 % trichloracetic acid and 5 ml. of acetone are added to extract the formazan.

5. Effects of Inhibitors

Cyanide, cysteine and atebrine inhibited the enzymic reduction of TTC by amines (cf. Table II), when added to the reaction mixtures. Liver suspensions dialyzed against 0.02 M ortho-phenanthroline, then against distilled water, did not reduce TTC in the presence of amines to any extent. This confirms results obtained manometrically in which we found that liver preparations dialyzed against o-phenanthroline lost their ability to oxidaize amine substrates. Interestingly enough, we also found that with such tissue preparations, succinic dehydrogenase activity as measured by TTC reduction, was also abolished. Normally, this enzyme shows very strong activity with the TTC reduction method. (For manometric results on the effects of o-phenanthroline, see Appendix III).

Table II : Effects of Inhibitors

Inhibitor	Final Concn.	Substrate	% of control*
NaCN	5×10^{-4}M	TYR	81
		ISO	89
NaCN	5×10^{-3}M	TYR	14
		ISO	23
NaCN	2×10^{-2}M	TYR	0
		ISO	10
		adrenaline	25
		1,2-ethylenediamine	0
		1,4-diaminobutane	0
		1,6-diaminohexane	3

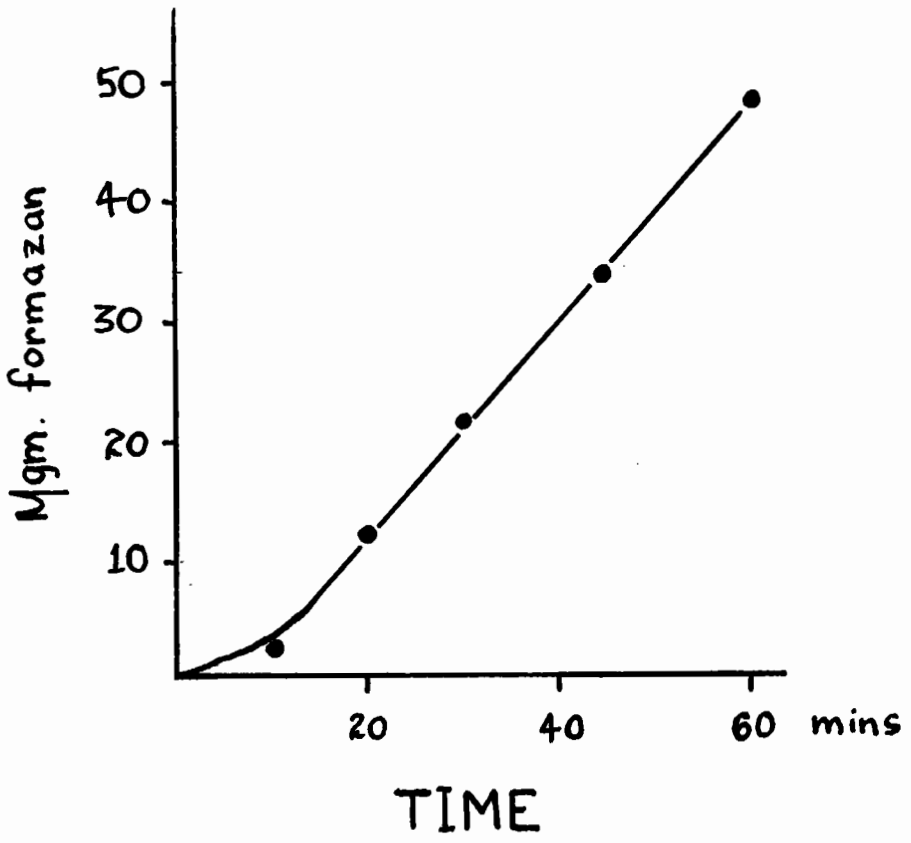
*
% of control (no inhibitor) as measured in µg. formazan produced in excess of blank values.

Table II : Effects of Inhibitors (Continued)

Inhibitor	Final Concn.	Substrate	% of control
Cysteine	1×10^{-3}M	TYR ISO	0 0
Atebrine	1×10^{-3}M	TYR ISO	29 17
o-phenan-throline	1×10^{-2}M	TYR ISO	0 0

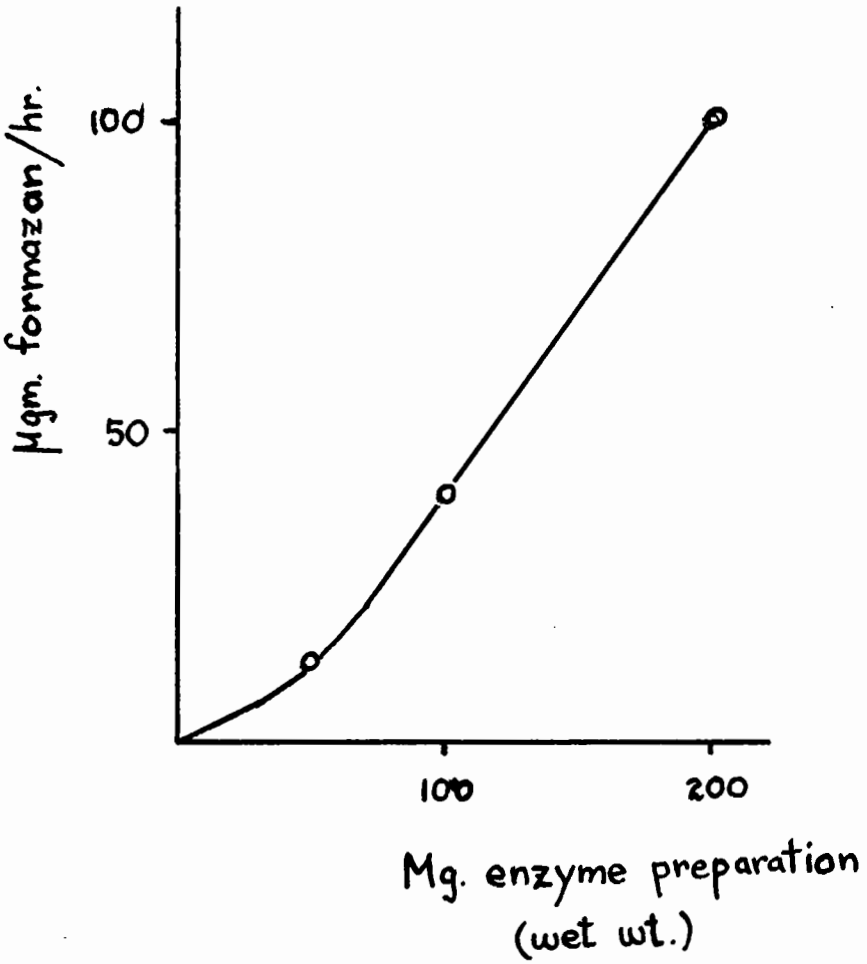
Reaction mixtures contain : 100 mg. (wet wt.) rat liver suspension ; 40 µM substrate (neutralized) ; 20 µM Phosphate buffer, pH 7.4 ; 2 mg. triphenyltetrazolium chloride (contained in 0.2 ml. distilled H_2O ; inhibitor, as indicated. Volume made to 2.0 ml. with distilled H_2O_3

Figure 1 : Reduction of TTC during Amine Oxidation: Time Curve



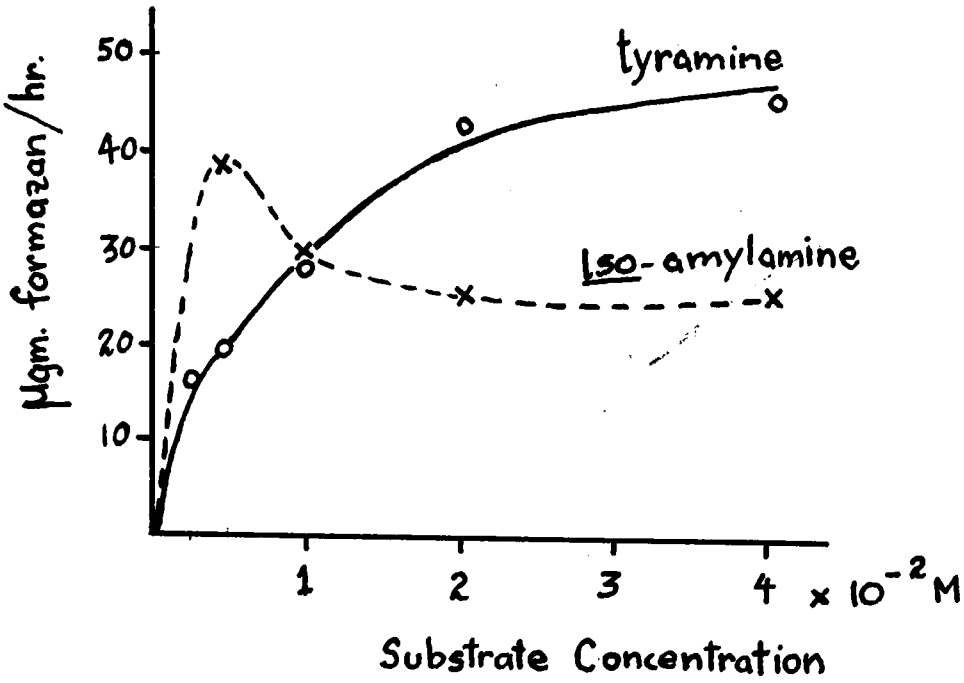
Reaction mixtures contain : 100 mg. (wet wt.) of dialysed rat liver suspension (prepared as indicated on p. 44) ; 20 μM phosphate buffer, pH 7.4 ; 40 μM tyramine-HCl (neutralized) ; 3 mg. TTC (contained in 0.2 ml. H_2O) ; dist. H_2O to make final vol. : 2.0 ml. Control tubes contain no substrate (replaced by same vol. of dist. H_2O). Reaction stopped with 0.2 ml. tri-chloracetic acid and formazan extracted with 5.0 ml. acetone. The formazan was then measured by reading solutionsin the Beckmann spectrophotometer at 485 mμ. O.D. values converted to μgm. formazan on the basis that a solution containing 20.6 μgm. formazan / ml. gives an O.D. reading of 1.000. In Fig. 1, μgm. formazan formed in excess of that produced in control tubes is plotted against time (= time when reaction is stopped).

Figure 2. : Effects of Enzyme Concentration



Reaction Mixtures : as in Fig. 1, except for amounts of enzyme (i.e., amounts of rat liver suspension).

Figure 3 : Effects of Substrate Concentration



Reaction mixtures: as in Fig. 1, except for substrate concentration.

APPENDIX II

Reduction of Ferricyanide as a Method of Assay for Amine Oxidase

A. Introduction:

In 1937 Haas (147) showed that ferricyanide could accept electrons from reduced coenzyme I (DPN). Later, Quastel and Wheatley (148) developed a manometric assay for dehydrogenases using ferricyanide. The reactions involved are:

(1) Substrate (2H) + DPN → substrate (oxid.) + DPNH + H^+

(2) DPNH + 2 Fe $(CN)_6^{'''}$ → DPN + H^+ + 2 Fe $(CN)_6^{''''}$

Addition of (1) and (2) gives the net reaction:

(3) Substrate (2H) + 2 Fe $(CN)_6^{'''}$ → substrate + Fe $(CN)_6^{''''}$

The H^+ ions liberated during these reactions then react with the bicarbonate in the medium and 2 molecules of CO_2 are evolved per molecule of substrate dehydrogenated.

This simple method has been used extensively in the study of many dehydrogenase enzymes, including succinic dehydrogenase, which, although not couples to pyridine nucleotides, reduces ferricyanide via cytochrome b .

Potassium ferricyanide absorbs maximally at 420 mμ., while the reduced ferrocyanide does not. It should then be

possible to follow an enzymic reduction of ferricyanide spectrophotometrically measuring the decrease in absorption during the reduction. On the suggestion of Mr. P. Ottolenghi (Department of Biochemistry, McGill University), this was tried as an assay for amine oxidase activity.

B. Experimental

Although only preliminary experiments have been carried out, the results shown below indicate that ferricyanide can act as an electron acceptor during the enzymic oxidation of amines by rat liver suspensions. It may be added, however, that in assaying amine oxidation manometrically by the method of Quastel et al (148), we obtained negative results.

C. Results

I. In a first experiment, it was found that dialysed rat liver suspension, in the presence of tyramine, reduced ferricyanide as measured in a Beckman spectrophotometer (DU model). The decrease in absorption of ferricyanide at 420 mμ. was followed during 20 minutes. From the results illustrated in Fig. 1, it is seen that in the absence of enzyme or of substrate, negligible decreases in the absorption take place.

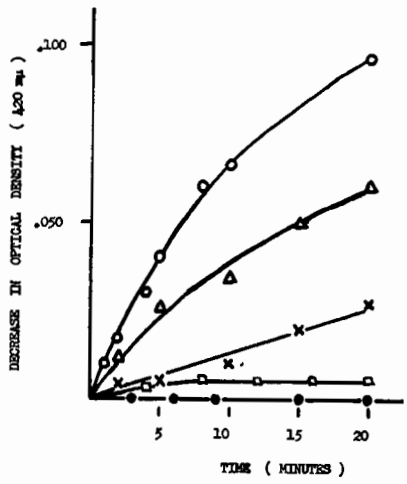
II. Effects of Enzyme Concentration

Figure 2, shows that ferricyanide reduction is linear with enzyme concentration. In this and subsequent experiments using ferricyanide, 10% rat liver suspensions were homogenized with a 0.5 or 1.0% oxbile solution prior to dialysis against water. This treatment disperses the mitochondria and "solubilizes" the amine oxidase bound to these particles (29). After dialysis, the liver suspension was spun at low speed (600 g.) and the translucent supernatant containing the amine oxidase activity was employed. This makes for more convenient readings in the Beckman, although the amine oxidase activity appears somewhat reduced. Blaschko (29), has reported that bile salts are detrimental to amine oxidase activity when this is tested manometrically, and that removal of the bile salts by dialysis yields enzyme preparations with original amounts of activity.

III. Enzymic Activity using Tryptamine and Iso-amylamine as Substrates.

The results which had been obtained using tyramine (cf. Fig. 1, curve 1) were qualitatively confirmed with both tryptamine and iso-amylamine (cf. Fig 1, curves 4 and 5 respectively).

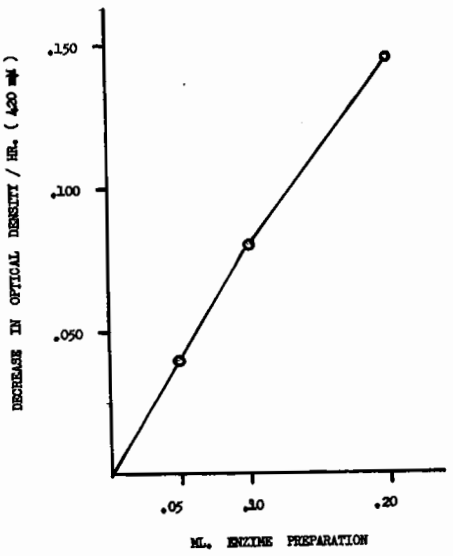
Figure 1 : Enzymic Reduction of Ferricyanide by Amine Oxidase.



Beckmann cuvettes (light path : 1 cm.) contain the following ingredients: 0.03 ml. of a 20% rat liver suspension(a); TRIS buffer, 1.4 x 10^{-2}M (pH 7.4); amine, 1.5 x 10^{-2}M (neutralized); $K_3Fe(CN)_6$, 5 x 10^{-4}M. Final volume of 2.6 ml. made up with dist. H_2O. (1) o——o , represents curve obtained using tyramine as substrate, and read vs. control containing only buffer and H_2O; (2) □——□ , same as (1) replacing substrate by equivalent volume of H_2O; (3) ●——● same as (1) replacing liver suspension by equivalent vol. H_2O; (4) Δ——Δ , same as (1), but using tryptamine as substrate; (5) ×——× , same as (1) using iso-amylamine as substrate.

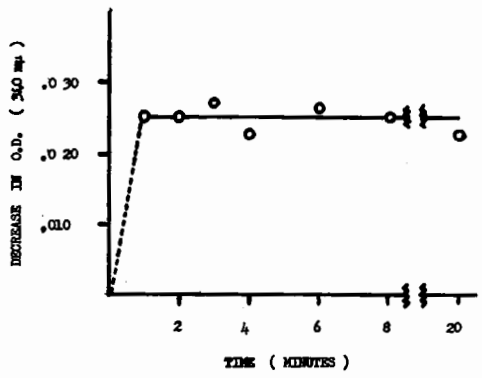
(a) prepared as indicated on p. 44.

Figure 2 : Effects of Enzyme Concentration



Cuvette contents: As in Figure 1 (curve 1), except for amount of enzyme added. Read vs. control containing only buffer and H_2O. Final vol. : 2.5 ml.

Figure 3 : Reduction of DPN by Amine Oxidase



Cuvette contents: TRIS buffer, 0.2M, pH 7.4; tyramine, 1.6×10^{-2}M (neutralized); 0.03 ml. of a 20% rat liver suspension(as for Fig. 1); DPN, 0.5 mg. Final vol. made up to 2.5 ml. with dist H_2O.

ERRATA: Ordinate in Fig. 3 should read INCREASE instead of DECREASE in O.D.

D. Comments

In a recent paper, Pressman (149) studied several enzymes concerned with the processes of oxidative phosphorylation, and on the basis of his findings, he suggested that ferricyanide functions as an electron acceptor acting in the proximity of cytochrome c.

Under the conditions of Quastel and Wheatley's assay, ferricyanide accepts electrons from DPNH, TPNH, or cytochrome b. In that method, high concentrations of ferricyanide are employed (0.2 M, final), as opposed to the low concentrations (5×10^{-4} M, final) used in our experiments, the latter being comparable to those employed by Pressman (149).

Assaying amine oxidation by the ferricyanide method of Quastel et al, we obtained negative results both in the presence and absence of added DPN. Presumably, then, DPN is not involved in amine oxidation by our crude enzyme preparations. We have found, moreover, that DPN was not reduced by these enzyme preparations, measuring the reduction of DPN spectrophotometrically at 340 mμ. (150) (cf. Fig. 3).

APPENDIX III

Effects of Metal-Binding Agents on MO

Preliminary experiments were begun to test the effects of some known metal-binding agents on amine oxidation, assaying MO activity by the manometric technique. It was found, in particular, that cysteine[1] and ortho-phenathroline inhibited amine oxidation when added in high concentrations, while oxine (8-hydroxyquinoline) inhibits 25% when it is added at final concentration of about 10^{-4}M. Pyrophosphate produced a small activating effect when added in high concentrations, while Versene and α,α'-dipyridyl were without effects at the concentrations tested. These results are presented in Table I.

Dialysis of the enzyme preparations (rat or guinea-pig liver homogenates) against ortho-phenanthroline (0.01 M), cysteine[1] (0.02 M, pH 8) and sodium cyanide (0.01 M, pH 8), resulted in a decrease in activity towards monoamines. Dialysis against Versene (0.001 M) did not usually affect MO activity. The results of the dialysis experiments are summarized in Table II.

Whether or not theinhibitions of MO activity produced by some of the reagents tested is due to the removal of metals involved in amine oxidation is difficult to assess. According to

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[1] The effects of cysteine on amine oxidation are given in Tables XXXVL to XXXVIII. (Cf. "Effects of Sulfhydryl Compounds", p. 89 of this thesis).

Mahler and Elowe (106), enzymic reactions involving 2-electron transfers, for example, reactions in which molecular oxygen is the H-acceptor, are independent of the metal components of metallo-enzymes[2]. In our experiments, MO was assayed manometrically, in which case, molecular oxygen is theprimary H-acceptor. Then, if Mahler and Elowe are correct, the inhibition of MO by some of the metal-binding reagents would not be due to the removal of metals from the enzyme molecule[3]. It may be that cysteine, ortho-phenanthroline and cyanide owe their inhibitory effects of their action on essential -SH groups of MO. These three substances were also found to inhibit markedly the reduction of tetrazolium salts by "amine dehydrogenase" (cf. Appendix I, Table II).

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2 Cf. "Metal Ions and Enzyme Systems", p. 38 of this thesis.

3 We have also found in the course of our experiments, that the inhibition of MO produced by cysteine could not be relieved by addition to the reaction mixtures of Fe^{++}, Cu^{++}, Mo^{+VI}, Ni^{++} or Co^{++}.

Table I : Effects of Metal Reagents

Expt.	Addition	Final concn.	Substrate	% Control
1	o-phenanthroline	1×10^{-5}M	TYR	114
		1×10^{-4}M	"	114
		1×10^{-3}M	"	100
		1×10^{-2}M	"	50
2	oxine	2.5×10^{-4}M	ISO	75
3	pyrophosphate	1×10^{-2}M	ISO	124
	α,α'-dipyridyl	1×10^{-2}M	ISO	100
4	Versene	2.5×10^{-4}M	ISO	100

Reaction mixtures in Warburg vessels contain 0.5 ml. of enzyme preparation (rat liver homogenate, except in Expt. 3, where guinea-pig liver is employed as source of MO); 2×10^{-2}M potassium phosphate buffer, pH 7.4, 2×10^{-2}M substrate(neutralized), and metal reagent as indicated, in a total volume of 2.0 ml. The metal reagent was in contact with the enzyme preparation 10-15 mins. prior to the tipping of the substrate from the side-arm ("zero time"). Gas phase was pure O_2. Values (% Control) are based on the µl. O_2 consumed during the first 30 mins. of incubation in excess of blank values. In the control flasks, metal reagents are replaced by equivalent volumes of distilled H_2O.

Table II: Effects of Dialysis

Expt.	Dialysis	Substrate	% Control
1	Vs. NaCN (0.01M, pH 8) 15 hrs., then 10 hrs. vs. dist. H_2O	TYR	56
2	Vs. O-phenanthroline (0.01M, pH 7) then 5 hrs. vs. dist. H_2O	TYR	80
3	Vs. Versene (0.001M, pH 7.4) 12 hrs., then 3 hrs., vs. 0.01M TRIS buffer (pH 8)	TYR	93
4	Vs. oxine (0.001M, pH 7.4) 2 hrs., then 2 hrs. vs. 0.01M TRIS buffer (pH 8)	TYR	129

Contents of Warburg vessels: as indicated for controls in experiments in Table . For dialysis controls, enzyme preparation was dialysed against distilled H_2O instead of against reagent. Gas phase: pure O_2. Otherwise, conditions same as indicated for experiments in Table I .