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PREFACE

The mitochondrial enzyme, Monoamine Oxidase, (E C 1.4.3.4), is thought to be composed of a group of closely related enzymes with very narrow substrate specificities, capable of oxidatively deaminating such amines as tyramine, serotonin, adrenaline, noradrenaline, benzylamine, and monoalkylamines.

Cofactors implicated in the MAO reaction have included flavin, copper, and in this laboratory, iron as well. Although purification of the enzyme is less difficult at present, because many solubilization and purification procedures have been reported, only part of the present work was concerned with the purification of MAO.

The bulk of this work consisted in utilizing rats deficient in iron, copper, and both metals and noting the effect of such single and combined deficiencies on the kidney and liver MAO concentrations. It was with such nutritional studies that Joyce Hawkins, in 1952, demonstrated the importance of riboflavin in the rat liver mitochondrial enzyme, the only substantiated evidence to date concerning the cofactor requirements of the particulate MAO.

Kinetic studies, inhibitor studies, and the prolonged effect of deficiencies of iron and copper on MAO with regard to the inhibition by various metal chelators is also included in this report.

COFACTOR REQUIREMENTS OF RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE

By

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To Mrs. H. Glenn, I express my appreciation for typing this manuscript.

LIST OF ABBREVIATIONS

MAO	Monoamine Oxidase
DO	Diamine Oxidase
PPL	Pyridoxal Phosphate
PCMB	para-Chloromercuribenzoate
FMN	Flavin Mononucleotide
FAD	Flavin Adenine Dinucleotide
5-HT	5-Hydroxytryptamine (serotonin)
TCA	Trichloro Acetic Acid
%I	per cent Inhibition
NHI	Non-haem Iron

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I. INTRODUCTION

A. *Monoamine Oxidase, Diamine Oxidase, and Similar Enzymes from Serum and Other Sources*

1. *Historical*

In 1910, Ewins and Laidlaw (1) found that perfusion of tyramine or tryptamine (2) through the liver of the rabbit yielded *p*-hydroxyphenylacetic acid and indoleacetic acid, respectively. The transformation of amine to acid was thought to be of a hydrolytic nature by these workers.

$$\text{i.e.,}\quad RCH_2 - NH_2 + H_2O = RCH_2OH + NH_3 \qquad (1)$$

The possibility of an oxidative action arose from the discovery by Hare-Bernheim, in 1928, that rabbit liver homogenates consume oxygen in the presence of tyramine (3); this new enzyme, which was not inhibited by 10^{-3}M cyanide, was called tyramine oxidase. A few years later, two other enzymes were found capable of oxidizing aliphatic amines (4) and adrenaline (5, 6). It was soon concluded that the three enzymes were identical, having the ability to oxidize primary aliphatic amines as well as secondary amines. Zeller (7) proposed the name monoamine oxidase (MAO) to differentiate this enzyme from diamine oxidase (histaminase) (DO), first described by Best (8) in 1927, and which acts on short aliphatic diamines such as 1,5-diaminopentane (cadaverine) as well as on histamine. The reaction catalyzed by these two groups of enzymes is shown in equation two, and if catalase is present by equation three.

$$RCH_2NH_2 + O_2 + H_2O = RCHO + NH_3 + H_2O_2 \qquad (2)$$

$$RCH_2NH_2 + 1/2\ O_2 = RCHO + NH_3 \qquad (3)$$

The addition of 10^{-3}M cyanide or 10^{-2}M semicarbazide enables the measurement of MAO, as these compounds inhibit DO completely in the above concentrations, and decrease the oxygen uptake to the theoretical value of one atom/molecule of substrate (9), as would be expected if catalase were present.

2. *Properties of the Amine Oxidases*

These two groups of oxidases have been differentiated on the basis of substrate specificity and by the effects of various inhibitors. Overlapping of substrate specificities has been observed (10, 11) and might be expected owing to the close similarity of the catalysis afforded. The similarity in catalysis afforded suggests in turn a similarity in the mechanism of deamination (13). A novel finding in this direction has been Gorkin and Tatyanenko's recent work (14) on the transformation of the particulate MAO of rat liver mitochondria into a diamine oxidase-like enzyme *in vitro*, by the use of peroxides of higher unsaturated fatty acids (oleic). The true significance of this finding must await further work on the characterization of the cofactors of MAO. We will return to this topic of cofactors in some detail presently. The observation to be stressed, however, is that those monoamines which are oxidized by DO (both of animal and plant origin) are oxidized at a greatly reduced rate (11, 15) compared to diamines. A clearer distinction comes from examining the inhibitors of these two enzymes. MAO is not inhibited by carbonyl reagents such as cyanide, hydrazine, hydroxylamine, and semicarbazide, all of which in very small concentrations

inhibit DO, moreover, DO is found in the soluble fraction of tissue homogenates after centrifugation (16), whereas MAO is associated with the insoluble fraction (17); Hawkins (17) found about 2/3 of the enzymic activity in the mitochondria and 1/3 in the microsomal fraction. The finding that, in rabbit, DO is associated with the particulate fraction is the sole exception (18). The only finding of soluble MAO in tissues (kidney and liver of guinea pig) was that of Weissbach *et al.* (19), but this is only a small fraction of the total MAO. More recently, MAO activity in the thyroid gland has been shown to be distributed in a ratio of 3:2 between the mitochondria and microsomes (20). The microsomal enzyme, however, is not similar to MAO as it has the ability to oxidize spermine and is inhibited 50% by 10^{-3}M hydroxylamine. The soluble MAO of guinea pig kidney did not have the ability to oxidize benzylamine, but in all other respects it was imilar to MAO. Bernheim and Bernheim (21) discovered an enzyme in rabbit liver capable of oxidizing mescaline; this enzyme is distinct from the particulate MAO, and belongs to the DO group (22).

The next largest group of amine oxidases is found in the serum of many mammals. This enzyme is a soluble enzyme. It was first observed in beef serum by Hirsch (23). In the past few years, the enzyme from beef, pig, human and rabbit (24, 25, 26, 27) has been isolated, and in the former two species, characterized. These groups of enzymes, as can be seen from Table I, resemble more closely

the DO group of enzymes. Further evidence for this similarity comes from the most recent work of Mondovi (28) on hog kidney DO in which the metal associated with this enzyme has been shown to be copper. Whether PPL (pyridoxal phosphate) is a cofactor, remains to be proven, although Kappeler-Adler and McFarlane claim that it is (29). PPL is a known cofactor for the beef and pig serum amine oxidases (24, 25).

3. *Substrates and Inhibitors of Monoamine Oxidase*

(i) Primary amines (i.e., tyramine) are oxidized at a faster rate than the secondary amines (i.e., adrenaline), which in turn are oxidized faster than tertiary amines (i.e., hordenine).

(ii) Primary aliphatic amines are oxidized (excluding methylamine) more rapidly as the hydrocarbon chain is lengthened - maximal rates are obtained with amylamine and hexylamine. Branched chain members are oxidized more slowly, with the exception of isoamylamine.

(iii) The diamines of the series $H_2N\ (CH_2)_nNH_2$ are not oxidized by MAO unless the distance separating the two amine groups is large enough, i.e., $13 \leq n \leq 18$, possibly permitting the diamine to function as a monoamine at the vicinity of the active site.

(iv) Secondary carbinamines of the type RR_1CHNH_2 inhibit MAO.

(v) Serotonin and tyramine are excellent substrates of MAO.

(vi) Substitution in the phenolic ring alters the activity of MAO, e.g. dopamine is oxidized more slowly than tyramine.

(vii) Substitution of the sidechain of tyramine alters the activity of MAO as well, e.g. noradrenaline is oxidized at a slower rate than epinine. If two methyl groups are introduced in the β carbon, no activity is noted. This latter finding is complicated by the oxidation of benzylamine, a compound containing no beta hydrogens in the sidechain. (For i-vii cf. (30, 31)). Table II lists some of the biologically important compounds that MAO acts upon.

In general, "the amines that are substrates of amine oxidase are chiefly present as ions at the pH at which the enzyme is active" (31). This statement does not apply in the case of the amine oxidases of rabbit serum acting on short chain aliphatic diamines, nor for β-phenylethanolamine (26), nor for the deamination of substrates by the human serum enzyme (27).

Inhibitors of MAO fall under two headings - reversible inhibitors and irreversible inhibitors. A reversible inhibitor may be removed from the enzyme preparation by dialysis and full enzymic activity noted thereafter. An irreversible inhibitor cannot be removed either by repeated washing or dialysis. Some reversible inhibitors are α-methylated amines.

i.e. (a) amphetamine (β-phenylisopropylamine) (32)

(b) ephedrine (33)

(c) 2-amino-4-methyl-*n*-butane (31)

As was noted earlier, the introduction of a β hydroxy group in the tyramine side chain decreases the activity of MAO for this molecule. Similarly, we would expect the same to hold for an inhibitor. This was found to be the case, and amphetamine is a better inhibitor than ephedrine (31). Harmaline and harmine, as well, are reversible inhibitors, and are more potent than the amphetamines. Recently (34) harmaline was found to be a competitive inhibitor of the enzyme isolated from the insect *Tribolium confusum Duval*. The remaining reversible inhibitors (metal chelators) will be discussed under cofactor requirements of the enzyme.

(a) *Irreversible Inhibitors*

As already mentioned, MAO and DO can be clearly separated by the effect of carbonyl reagents upon them, for DO is inhibited by carbonyl reagents such as hydrazine, hydroxylamine, bisulfite and cyanide. Zeller (35) has shown that MAO is inhibited by compounds possessing the structure =N-NHR, whereas DO is inhibited by compounds having the hydrazine backbone, $-N-NH_2$, which imparts the carbonyl binding property. Phenylhydrazine inhibits both enzymes. An important observation noted by Davison (36) was that there is a decrease in the extent of inhibition when the enzyme and hydrazine analogues are incubated in the presence of nitrogen instead of oxygen. Some of the hydrazine compounds studied displayed more specificity for this effect than others, e.g. iproniazid as opposed to isopropylhydrazine or isocarboxazide (37); iproniazid requires O_2 (36) for inhibition.

In concluding that an inhibitor of MAO is reversible or irreversible, the substrates used should be mentioned; for, although the inhibitor parnate sulfate was shown to be irreversible with respect to tyramine, it is reversible in action with respect to phenylbutylamine and tryptamine.

Furthermore, MAO activity is known to be enhanced in the presence of an atmosphere of pure oxygen (3). Novick (38)recently has focused attention on this overlooked property of MAO. He confirmed earlier findings that high oxygen tension increases the activity of MAO; he used tissues of rats, mice and guinea pigs as the enzyme source. Tyramine and serotonin were deaminated with equal speed by rat liver preparations in 20% oxygen. However, the rate of tyramine oxidation was 3.1 times that of serotonin in an atmosphere of pure oxygen. Rat brain MAO showed almost no increase in oxidizing serotonin, tryptamine or β-phenylethylamine in 100% oxygen as compared with air; however, rat liver MAO showed 1.4, 2.1 and 5.1 times the activity, respectively in 100% O_2 as compared with air. The percent inhibition of MAO by iproniazid, d,l-amphetamine and trans-2-phenylcyclopropylamine (parnate) was not found to change by altering the oxygen tension.

TABLE I

SIMILARITIES AND DIFFERENCES OF THE VARIOUS AMINE OXIDASES

Enzyme	Source	Maximally Active Substrate(s)	Semicarbazide	Octyl Alcohol PCMB*	Histamine	Colour of Purified Enzyme	Absorption Maxima mμ	Molecular Weight	Cofactors
Monoamine Oxidase E.C. 1.4-3.4	Rat Liver	Tyramine	-	+	-	Yellow	410	280,000	FAD
	Beef Liver	Serotonin				Yellow	410,450,480		Fe ?
	Beef Kidney	(Tyramine (Benzylamine					410,450,485		FAD
(Histamine) Diamine Oxidase E.C. 1.4-3.6	Pig Kidney	Cadaverine	+	-	+	Yellow-Pink		87,000	Cu Ppl
	Pea Seedling	Spermine Lysine	+	-	+	Rose-Pink	500	96,000	Cu Ppl
Plasma Amine Oxidases	Beef	Benzylamine	+	-	±	Pink	480	260,000	Cu, Ppl
	Pig	Benzylamine		-	±	Pink	470	195,000	Cu, Ppl
	Human	Benzylamine	+	-	-				
	Rabbit	Benzylamine Tyramine	+	-	±				
	Aspergillus niger	Benzylamine Tyramine Mescaline	+	-	+	Pink	500	252,000	Cu Ppl

± Acted upon weakly

\+ Actively oxidized or inhibitor

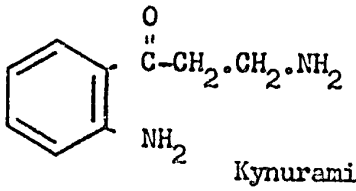
\- Not oxidized or not inhibited

? Partial evidence (suggested)

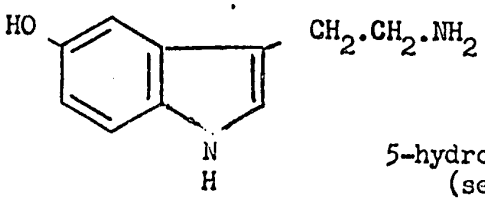
* *p*-chloromercuribenzoate

TABLE II

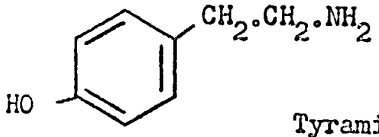
SUBSTRATES OF MONOAMINE OXIDASE



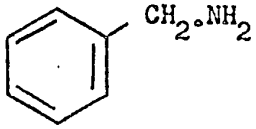
Kynuramine



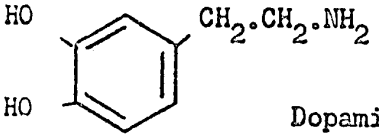
5-hydroxytryptamine (serotonin)



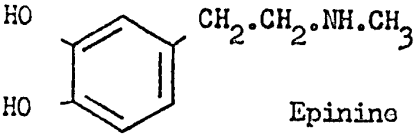
Tyramine (4-hydroxyphenethylamine)



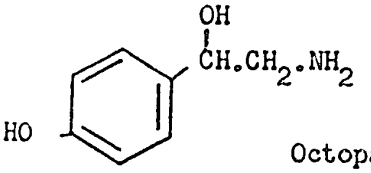
Benzylamine



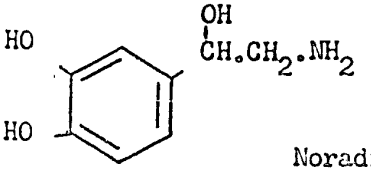
Dopamine (3,4-dihydroxyphenethylamine)



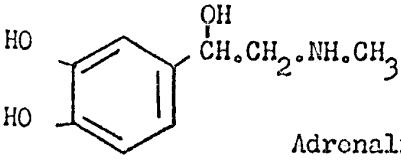
Epinine (3,4-dihydroxyphenethylamine)



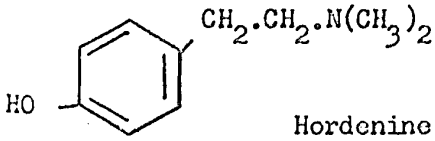
Octopamine (4-hydroxyphenethanolamine)



Noradrenaline (3,4-dihydroxyphenethanolamine)



Adrenaline (3,4-dihydroxyphenethanolmethylamine)



Hordenine (4-hydroxyphenethyldimethylamine)

4. *Homogeneity of MAO*

The classical studies of Alles and Heegard (39) on the oxidation of a wide variety of amines by liver preparations from different species (rabbit, guinea pig, cat, cattle) showed great differences in the substrate specificity of these MAO enzymes. Some highlights of their observations are as follows: Whereas the rabbit and guinea pig enzymes were not found capable of oxidizing ethylamine, the enzymes from cat and cattle were found to be capable of doing so. The very low activity of rabbit and guinea pig enzymes toward benzylamine was in sharp contrast to that of the cat and cattle enzymes which oxidized benzylamine readily. All four of the liver MAO enzymes oxidized tyramine optimally. The assumption that the amines attacked by MAO were acted on by a single enzyme was thus questioned for the first time. Sarkar and Zeller (40, 41) later extended these studies, comparing MAO of rabbit and cattle, and came to the conclusion that the "active sites" of the two enzymes differed sharply. They speculated that MAO must comprise a whole family of closely related homologous enzymes. Barbato and Abood (42) supported this contention. Working with a twenty-fold purified beef liver enzyme, they were able to obtain a pronounced difference in inhibition, using *o*-phenanthroline, at pH 6.5 - 7.0, compared with pH 8 - 8.5. At the former pH, inhibition was 95%, at the latter, inhibition was 30% (using 2 x 10^{-3}M *o*-phenanthroline). Youdim and Sourkes (43) have confirmed

these results with rat liver homogenates. These workers also observed that heat-inactivated preparations of the enzyme yield two peaks for activity vs. pH and were able to inhibit selectively each of these two peaks with various metal chelators (8-hydroxyquinoline, *o*-phenanthroline, D-penicillamine) and various other inhibitors such as parnate, iproniazid and pargyline. Furthermore, the activity at pH 6.5 was more susceptible to heating than was the activity at pH 7.4. Previously, Severina and Gorkin (44) had shown that the oxidation of tyramine was considerably more sensitive to controlled heating than was the oxidation of benzylamine. Nagatsu and Yagi (45), more recently, have shown that the heat stability of the enzyme deaminating serotonin is exceptional, 100% of the enzymic activity being retained after 10 minutes' heating at 60°C. This was true for rat liver but not for rat kidney, brain, or heart MAO. From the work of Youdim and Sourkes (43), one can calculate that only 20% of the activity of rat liver remains to oxidize kynuramine under these conditions. Furthermore, Nagatsu and Yagi (45) found that about 50% of the total activity of the heated homogenate is retained in the supernatant fraction after centrifugation at 19,000 x g for 20 minutes whereas in unheated homogenates, 100% of the activity is sedimented under the above conditions. The activity in the heated homogenate (or its supernatant after centrifugation) was not inhibited by harmaline, ephedrine, or octyl alcohol. Yet, PCMB (*p*-chloromercuribenzoate) still inhibited this activity by 100% (1×10^{-3}M), as did *o*-phenanthroline (4×10^{-3}M).

Semicarbazide did not affect the activity. Nagatsu and Yagi (45) are currently investigating the nature of this enzyme to see whether it is MAO. One possibility they mentioned was that the heat treatment might have solubilized the enzyme, a crucial finding if this is actually the case. The actual physical separation of two distinct types of MAO was accomplished by Gorkin (46), one attacking *m*-nitro-*p*-hydroxybenzylamine and the other *p*-nitrophenylethylamine. The MAO were separated on a 'Brushite' column with increasing molarity of phosphate buffer. Finally, Severina and Gorkin have (47) shown selective inhibition with various MAO inhibitors such as proflavine, chloracizine, chlorpromazine, and various metal chelators.

The only real evidence supporting the homogeneity of MAO comes mainly from the work of Blaschko *et al.* (48) on controlled simultaneous oxidation of two substrates in which the rate of oxidation was found to be intermediate between the rates of the two amines tested separately.

5. *Cofactor Requirement*

The lack of inhibition of MAO by cyanide and the early findings by Friedenwald and Herrman (49) that rabbit mucosa preparations of MAO were inhibited by sulfhydryl reagents was all that was known concerning the chemistry of MAO for almost three decades. Lagnado and Sourkes (50) tested a great number of sulfhydryl compounds and found them to be inhibitory. These studies present evidence that a sulfhydryl group might function in the catalytic

action of enzyme.

With purification, the enzyme becomes much more susceptible to inhibition by sulfhydryl agents. Friedenwald and Herrman (49) found about 85% inhibition with 1.5 x 10^{-2}M PCMB. Barbato and Abood (42) obtained complete inhibition with a twenty-fold purified enzyme preparation using 10^{-3}M PCMB. Youdim (51) found 90% inhibition with 10^{-4}M PCMB on a purified preparation of the enzyme. This gives greater strength to the importance of the sulfhydryl group as functioning in the active enzymatic process. As mentioned earlier, Gorkin and Tatyanenko's latest findings (14) show that peroxides of higher unsaturated fatty acids completely inhibit MAO during the course of preincubation with the enzyme. The content of SH groups simultaneously decreases. If one incubates serotonin with the peroxide of the higher unsaturated fatty acids simultaneously, the activity of the enzyme for serotonin is retained, but for another substrate (tryptamine) it is absent. It is not unlikely that the SH groups impart a certain specificity to the enzyme as concerns the substrates it will act on.

(a) Metals Implicated in MAO

As most oxidases contain iron, copper or molybdenum, workers studying the properties of MAO became interested in knowing whether any of these metals was associated with MAO. Lagnado and Sourkes (50), the first workers to study the effect of metals on the enzyme, noted

that in very low concentrations (10^{-5} - 10^{-6}M), copper sulfate and iron sulfate activated the enzyme. In higher concentrations these metals inhibited MAO. The anaerobic amine/tetrazolium system employed by these workers (50A) as opposed to the aerobic amine system was sensitive to cyanide. However, since reversal of cyanide inhibition by metal salts was not observed, it is not likely that the inhibition stemmed from metal chelation. *o*-Phenanthroline, however, was found to inhibit the aerobic system by these workers. Gorkin and Romanova (52) extended this work with the use of many other specific metal chelators (8-hydroxyquinoline, plumbone, diethyldithiocarbamate, *o*-phenanthroline) and found that 8-hydroxyquinoline was the most potent inhibitor of the enzyme. Reversal of the inhibition caused by 8-hydroxyquinoline and cyclohexanediamine tetraacetate was achieved by the addition of certain divalent ions (Zn, Cu). Inhibition of MAO by some of these chelators can be reversed by dialysis as well.

Recently, workers (34) studying MAO in *Coleoptera (Tribolium confusum Duval)* found that homogenates of this insect when dialyzed, or homogenates to which 0.5M EDTA were added, lost 70% of their original activity. Addition of Zn^{++} or Cu^{++} (10^{-3}M) to the original homogenate in the presence of EDTA not only restored the lost activity but also activated the enzyme considerably. Other workers studying the effect of copper deficiency (53, 54) have found decreased levels of amine oxidase in pig and ovine sera.

In the sheep, the regression equation relating enzyme activity to plasma copper suggested that a small component of the MAO activity of ovine plasma may not be associated with copper. Thus, the possibility of other metals substituting for copper or traces of amine oxidase without a metal cofactor might exist in ovine plasma. The purification of the various amine oxidases of serum has shown copper to be associated with the enzyme from beef (24) and pig (29). The amine oxidase of pea seedling (55) as well as that in *Aspergillus niger* (56) and *Sarcina lutea* (57) have been crystallized and shown to be cuproproteins. Not much has been presented to date regarding the particulate MAO of vertebrates. Youdim (51) found no decrease in activity of the liver MAO of copper deficient animals compared with that of control animals.

Barbato and Abood (42) and Gorkin (46) have succeeded in purifying MAO approximately twenty-fold. With these preparations both authors have relied mainly on chelation experiments to prove the metallo-protein properties of this enzyme. With the exception of Guha and Krishna Murti (58), whose purification could not be reproduced (59), Youdim and Sourkes (60) working with the rat liver, and Nara working with beef liver (61), have succeeded in obtaining the purest soluble preparations to date. Both of these workers have presented evidence for the presence of small amounts of copper in their purest preparations; the amount of iron found in Youdim and Sourkes' (60) preparation was four times that of copper.

(b) Role of Flavin

Richter (12) postulated in 1937 that amine oxidase might be a flavoprotein, owing to the similarity of the reaction, oxidative deamination, observed for both MAO and D-amino acid oxidase. Hawkins (62) was the first to show a direct relationship between MAO activity and riboflavin. By placing rats on a riboflavin-free diet she obtained a 50% decrease in hepatic MAO activity compared with that of the control animals. Sourkes (70) has confirmed these findings. In Hawkins' experiments the D-amino acid oxidase activity was restored to normal by adding flavin adenine dinucleotide (FAD) to 'deficient' liver preparations *in vitro*, but this was not true for MAO activity. Moreover, upon administering riboflavin to rats already deficient in this vitamin, MAO activity was not restored for a period of several days, whereas D-amino acid oxidase activity was restored rapidly.

Whether riboflavin is involved in the synthesis of the enzyme or whether it is the nature of its attachment (63) to MAO that causes this delay remains to be elucidated. Mepacrine (atebrine), an inhibitor of yellow enzymes, block the metabolism of adrenaline *in vivo* and *in vitro* (64); Belleau and Moran (65) explained the inhibitory effects of harmine on MAO as possibly due to its interaction with a flavin component of MAO. Gorkin (66), studying the effects of proflavine on MAO activity, has a similar explanation for this inhibitor as well. However, he cautions

against this mechanism as being the only possible explanation. For, as he demonstrated, atebrine, a known antagonist of yellow enzymes, inhibits spermine oxidase as well, an enzyme containing no flavin. The separation of a flavin component from mitochondrial MAO has recently been accomplished by Nara *et al.* (67), but whether FMN (flavin mononucleotide) or FAD (flavin adenine dinucleotide) is the cofactor was not decided. Youdim (51) has also presented evidence of a flavin prosthetic group in rat liver MAO. More recently, "tyramine oxidase" from *Sarcina lutea* (57) and beef kidney MAO (68) have been shown to contain FAD. Interestingly, the amount of copper detected in the purified beef kidney MAO (68) was not considered significant.

Davision (69) suggests that MAO might consist of a dehydrogenase system as part of a more complete system including flavins and cytochromes. Lagnado and Sourkes (50A) have succeeded in linking the amine dehydrogenase system directly with tetrazolium salts. Adenylic acid or ribonucleotide derivatives containing an adenyl group were found obligatory for the deamination of amines with mitochondrial material from rat liver serving as the source of enzyme.

(c) Role of Pyridoxal Phosphate

Pyridoxal phosphate is the prosthetic group of spermine and benzylamine oxidase (25). The possibility that this cofactor serves mitochondrial MAO was explored by Sourkes (70), who found no decrease in the activity of hepatic MAO in rats maintained on a

diet deficient in this vitamin. This finding, in conjunction with this enzyme's resistance to carbonyl reagents, detracts from the role of pyridoxal phosphate as a cofactor for MAO in rat liver. However, in pyridoxine deficiency, DO activity is decreased in the intestine and lung of rats (71). Goryachenkova (72) and Abdel-Aziz and Boullin (73) have confirmed these results and found that riboflavin deficiency decreases the enzyme level as well.

6. *Determination of MAO Activity*

Although Creasey (9) has improved the long standing manometric assay by the use of semicarbazide and cyanide, which inhibit secondary oxidative processes, the lack of sensitivity of the method is its main drawback. As mentioned earlier, oxygen has a profound effect on MAO activity and therefore "gas" conditions must be carefully controlled.

Udenfriend *et al.* (74) have developed three specific methods for the estimation of 5-HT (serotonin) in biological material. All three methods depend on the initial extraction of 5-HT into alkaline solutions and re-extraction into an acidic solution. The ultraviolet absorption of this solution, or the colorimetric estimation of this solution produced by a reaction with 1-nitroso-2-naphthol, or the fluorescence of this solution can then be obtained. The advantage of this assay lies in its degree of sensitivity and its specificity. Tyramine, as well, can be assayed after removal of the protein, by a reaction with

1-nitroso-2-naphthol which can be followed colorimetrically (75). The need of multiple extractions, precipitations, and handling of large volumes, detracts from this assay's usefulness in situations where rapid assaying is necessary.

Sourkes *et al.* (76) have also used a spectrophotometric method for following the disappearance of 5-HT and tryptamine in crude tissue homogenates by the action of MAO.

A recent spectrophotometric method possessing good sensitivity and simple procedures was developed by Weissbach *et al.* (77). The enzymatic reaction may be carried out in Beckmann cuvettes to facilitate kinetic studies. Kynuramine, the substrate, is oxidized to an aldehyde, which then undergoes a nonenzymatic intramolecular condensation to form 4-hydroxyquinoline. Furthermore, either the disappearance of kynuramine at 360 mμ and/or the appearance of the product at 315 mμ may be measured. Kraml (78) has further adapted the kynuramine assay for spectrophotofluorimetric determination enabling greater sensitivity and specificity.

Several workers have determined MAO activity by measuring the aldehydes or their breakdown products which accumulate owing to the action of MAO. Green and Houghton (79) conducted their assay in the presence of semicarbazide, thus allowing the aldehyde formed (*p*-hydroxyphenylacetaldehyde) during the course of the incubation to be converted to aldehyde semicarbazone, followed by conversion to the 2,4-dinitrophenylhydrazone in alkaline medium,

which is extracted into benzene. This procedure has since been modified and two extractions into benzene have been used, recovering about 80% of the 2,4-dinitrophenylhydrazone (80). Gorkin (80) has recently described a method for the determination of MAO activity based on measuring the aldehyde formed from benzylamine. Benzaldehyde can be measured during the course of incubation (with a solubilized solution of MAO) directly in the cuvette or after precipitation of the protein at 250 mμ.

Methods based on ammonia production (82, 81) have long been claimed to be the most reliable and accurate assay procedure for measuring MAO activity. However, the laboriousness of the ammonia assay, its relatively small sensitivity, and its unsuitability for kinetic studies have limited the ammonia procedure extensively. The direct reaction of ammonia and hypochlorite (83, 84) may help ease the labor of these methods; as this system has been tested and found suitable for MAO determination (83).

Hydrogen peroxide formation can be used as a measure of the activity of MAO in special circumstances (20).

7. *Iron and Monoamine Oxidase*

As was mentioned earlier, Youdim and Sourkes (60) found four times as much iron as copper in their purest rat liver MAO preparations. Moreover, they also found that there was a direct linear relation between the specific activity of their MAO preparations and the amount of iron these contained; the increase in specific activity of MAO was directly proportional to the increase

in the amount of iron these preparations contained. Earlier, Youdim and Sourkes (43), had observed that the specific copper chelator, diethyldithiocarbamate, was much less effective than iron chelators such as thenoyltrifluoroacetone, *o*-phenanthroline, and α,α-dipyridyl in inhibiting rat liver MAO. Copper deficiency resulted in no decrease in activity of hepatic MAO in the rat. For these reasons we undertook to study the effect of iron deficiency in rats. Animals were to be divided into four groups: Iron withheld, copper withheld, iron and copper withheld, and animals fed both iron and copper.

The inclusion of animals deficient in both iron and copper is necessary, for, if lowered levels of MAO activity could not be demonstrated in animals deficient in either iron or copper, the possibility of lowered levels of MAO in the case of a deficiency of both metals cannot be ruled out on theoretical grounds. Not only is the metabolism of iron and copper very intimately interrelated, as will be shown presently, but the possibility that one metal might be necessary, in trace amounts, for the direct or indirect incorporation of the other metal into the enzyme MAO must be considered.

B. *Deficiency Experiments - A Study of Past Approaches and Their Uses in Enzymology and in the Metabolism of Trace Metals*

1. *Historical*

In the light of the results to be presented, it is appropriate to give a brief review of some of the earlier work with such studies. Let us first examine the design of the deficiency experiments studied by early workers, in particular, Elvehjem (85). Elvehjem and Sherman (86) first rendered rats anemic by allowing the young to partake solely of their mothers' milk until 21 days of age. These animals were then fed a diet of cow's whole milk. When anemia became severe (approximately 14 days later), the animals were fed an iron and copper supplement. The haemopoietic response and tissue levels of iron were measured. This type of experimental approach may be called therapeutic: treatment is not initiated until anemia had developed; the control animals are allowed to continue on an anemic diet and are compared to animals given copper or iron. Alternatively, the experiment may be designed as a prophylactic one: supplementation with one metal commences at the same time as the rats are placed on a milk diet; the control animals are fed a milk diet, supplemented with copper and iron, and anemia is thus prevented. The therapeutic approach has been viewed skeptically by later workers (87) who have used only prophylactic experiments, claiming these to be more fully controlled and their deductions more readily drawn. Although

the prophylactic approach does have these advantages, one cannot rule out the fact that conclusions of equal validity can be drawn from either approach. Understandably, if anemia is allowed to progress for too long a period of time, irreversible metabolic changes may occur; however, the early work of Elvehjem does not suggest such a possibility (85).

2. *Experimental Methods for Producing Iron Deficiency*

Iron is found in most tissues in three forms; ferritin (water-soluble) and hemosiderin (water-insoluble), both of which are non-haem iron proteins, and in the form of haem proteins. Kaldor (88) has shown that the sum of the water-soluble and water-insoluble non-haem iron fractions equals the total tissue non-haem iron. Thus, one may equate non-haem iron with storage iron. These proteins act as a reserve in case of an inadequate dietary intake of iron, at which time they become available for the synthesis of iron containing enzymes, hemoglobin, and myoglobin. Body iron requirements are increased as well during a period of rapid growth.

Experimental iron deficiency has been produced by feeding animals semi-synthetic diets very low in iron. If iron requirements are high, either because the animals are growing (86), or because of induced chronic bleeding (89), the deficiency develops more quickly. McCall *et al.* (90) have given the most thorough requirements for an iron deficiency experiment; they showed that when supplemented with iron, their diet would support normal growth, blood formation, and

reproduction in rats for at least three generations. With this diet, the minimum iron content for normal growth was found to be 40 mg/Kg of diet. As mentioned in these workers' papers, if one in addition bleeds the animals (or produces partial hepatectomy) to reduce their iron stores further, other essential constituents will be removed and an 'uncomplicated' iron deficiency will not be obtained. The semi-synthetic diets, in general, are made up with or without the addition of added vitamins.

The diet described in this thesis was developed by my colleague (91) and has proven successful for inducing copper deficiency. For iron deficiency, iron was omitted and replaced with copper. The details of the diet will be presented under Methods.

It is not necessary to follow changes in the storage iron of a variety of organs, as it has been shown (92) that changes in storage iron of one organ (liver and spleen) correlate well with changes observed in other organs. As the main objective of my deficiency experiments was to determine whether there is a decrease in the concentration of an enzyme (MAO) owing to a deficiency of iron or copper or both, I shall focus attention on earlier evidence that this type of deficiency can in fact lead to decreases in tissue metallo-enzymes.

3. *Effect of Iron Deficiency on the Iron Enzyme Content*

In 1936, Hahn and Whipple (93) perfused the tissues of a single anemic dog free of blood and found that the iron content of

the perfused liver after washing was the same as the enzymic iron content calculated theoretically, indicating that the iron-containing cytochromes were not decreased in iron deficiency. However, since it is not actually possible to remove all traces of blood, and since 1 ml of blood has 6,000 times as much iron as 1 gm of liver's iron cytochrome content, there are good grounds to believe that some of the iron demonstrated in this dog's liver might have originated from hemoglobin. Cohn and Elvehjem, in 1934, had shown (94) a decrease in the cytochrome content of iron deficient tissues from rats and cows. Beutler's (95) experiments have further disproven the theory proposed by Hahn and Whipple (93) that iron enzymes are held at all costs even in severe iron deficiency. Beutler's (95, 99, 100) series of experiments on the relation of iron depletion on the activity of iron enzymes in various tissues of the rat has shown that not all iron enzymes are affected in the same way by iron deficiency, and that the same enzyme might behave differently in different tissues. Beutler (95) has observed a decrease in the concentration of cytochrome c of the kidneys of iron-deficient rats. Gubler *et al.* (96) have also found a decrease in the concentration of cytochrome c in the kidney and heart of iron-deficient pigs. McCall *et al.* (97) pointed out that there is no decrease of this enzyme in the course of iron deficiency in the kidney and liver of rats, rather, the amount of cytochrome c had simply failed to increase with respect to the increase in the size of these tissues.

The catalase activity was found to remain normal in the red cells and liver of iron-deficient rats (98). However, Gubler *et al.* (96) found a decrease in the catalase activity in liver of iron-deficient swine. Beutler and Blaisdell (99) showed that succinic dehydrogenase, a non-haem iron-containing enzyme, showed a slight decrease in heart and kidney, but not in the liver of iron-deficient rats. Aconitase, an enzyme requiring iron as a cofactor, was found to decrease steadily in the kidneys of iron-deficient rats by Beutler (100). When Beutler tried to "activate" the homogenates of iron-deficient kidneys with iron and ascorbic acid, no increase in the aconitase activity over the deficient homogenate levels was noted. He concluded that "in the absence of the cofactor iron, there was inadequate synthesis of the apoenzyme". However, when he fed iron to iron-deficient rats, the kidney aconitase returned to normal. Furthermore, the decrease in aconitase activity was shown to be due specifically to a lack of iron by the following experiment. Beutler (100) fed rats with phenylhydrazine to produce anemia, and bled rats to produce anemia; as controls, he bled rats and supplied them with iron, and included normal rats as well. Only the rats that were bled without receiving the iron supplement became depleted of aconitase activity.

Beutler (101) noted a decrease in the concentration of cytochrome oxidase in the kidneys of iron-deficient rats. These results are at variance with those of Cohn and Elvehjem (94), and Gubler *et al.* (96). Cohn and Elvehjem (94) used indophenol as the substrate for cytochrome oxidase, whereas Gubler *et al.* (96) used

cytochrome c. Beutler (101) questions the validity of drawing negative results with the use of only three pigs by Gubler *et al.* (96), and furthermore, points out that Gubler *et al.* froze the animals tissues, which Beutler claims causes a rapid decline in cytochrome oxidase activity. Hill and Matrone (102) found that copper deficiency resulted in a definite lowering of the cytochrome oxidase activity in the heart of the chicken, whereas iron deficiency did not, even though the copper-deficient chickens were not anemic. Gubler *et al.* (96) have found that the myoglobin concentration in the leg muscle and heart muscle of iron-deficient swine was markedly reduced. Cusack and Brown (103), studying the effect of iron deficiency in the rat, confirmed the observations of Beutler and Blaisdell (98) that there is no change in catalase activity in liver or red blood cells of iron-deficient animals. These workers (103) did not note a decrease in the myoglobin concentration in the hind leg even after 12 weeks of dietary iron deprivation.

From a consensus of these findings, it will be noted that an impairment in the synthesis of many of the known heminproteins has been obtained by the above workers in iron deficiency. Thus, it seems that after the loss of storage iron, competition for the available iron leads to a decrease not only of hemoglobin but also of various hemin enzymes, non-haem enzymes which require iron, and myoglobin.

Several workers (104, 105, 90) have shown that the iron stores cannot be lowered below a certain minimum level. The term "parenchymal iron" has been used to describe the non-haem storage iron which is not mobilized even in severe iron depletion.

This parenchymal iron fraction constitutes a very small fraction of the total non-haem iron of liver and spleen. Hallgren (92) considers that this residual iron should not be regarded as storage iron.

4. *Copper Deficiency*

(a) Effect upon Cytochrome Oxidase

Cohn and Elvehjem (94) and Schultze (106) found a reduction in cytochrome oxidase in the tissues of copper-deficient animals. Gubler *et al.* (96) have more recently confirmed these results. They found (96) an 88% reduction in the cytochrome oxidase activity in the heart and a 67% reduction in the liver of the copper-deficient swine.

(b) Effect upon the Amine Oxidase in the Aorta

Recently, the role of amine oxidase in the formation of elastin in the aorta of chicks has been studied extensively (107). A decrease in the elastin of the aorta occurs in chicks on a copper deficiency diet. Hill *et al.* (107) speculate that the desmosine and isodesmosine components of elastin are formed from the condensation of four lysine groups pre-existing in straight chain elastin precusors. They postulate that for this reaction to take place, the ε-amino groups of the lysine residue would have to be oxidized, possibly to an aldehyde. This type of a reaction is catalyzed by amine oxidases (diamine oxidase, in particular) which contain copper. They (107) went on to say, "If such an enzyme

existed in the aorta of chicks, and if the activity were sensitive to copper deficiency, an argument could be advanced for this as the biochemical lesion behind the physiological lesion of reduced elastin content in copper deficiency." These workers set out with a prophylactic type of deficiency study and found no detectable amine oxidase activity using benzylamine as the substrate in the aorta and decreased levels of activity in the liver of copper-deficient chicken. Hill and Kim (108) have further strengthened their stand by conducting experiments with a diet deficient in pyridoxal phosphate, as this vitamin has also been shown to be associated with amine oxidases. They found a decrease, as well, in the activity of aortic amine oxidase from chicks deficient in this vitamin. The specificity of this effect was demonstrated by placing other chicks on a potassium-deficient diet, in which case the amine oxidase activity of the aorta was well above the levels found in pyridoxal deficiency.

5. *Copper Intake in Relation to Iron Storage*

(a) Increased Iron Stores in Copper Deficient Animals - Early Findings, Later Diagreements

Elvehjem and Sherman (86) found that when iron was fed to rats deficient in both iron and copper, the liver and spleen of these animals accumulated this metal in proportion to the amount fed. Feeding of 0.1 - 0.33 mg Fe per day (without copper) resulted in an accumulation of 0.25 - 0.45 mg Fe/gm dry liver respectively; any further increase in the amount of iron fed led to a gradual

levelling off of the total amount of iron capable of being stored. Maximum storage was 0.5 mg Fe/gm dry liver when 0.5 mg Fe per day was fed, and it was not possible to increase the iron content above 1 mg Fe/gm dry liver even after prolonged iron feeding. No increase in hemoglobin was noted even with the higher amounts of iron fed. In the presence of copper, hemoglobin formation was proportional to the amount of iron fed, and the liver showed no iron storage until 0.3 mg or more of iron was fed per day. Above 0.3 mg iron per day in the presence of copper only half the amount of iron stored without copper was found in the liver. The iron content of the livers of the rats which were depleted of iron and copper was0.1 mg Fe/gm dry liver. These workers (86) concluded that "in the absence of copper, inorganic iron is readily assimilated and stored in the liver and spleen. The iron so stored cannot be used for hemoglobin formation unless copper is supplied, when the greater part of the iron in the liver is removed and built into hemoglobin." Schultze (109) was of the same opinion in his review of this subject in 1940.

Houk *et al.* (110), fed animals a diet deficient in iron and copper and compared these animals with others receiving a diet supplemented with iron or copper or both. The results of this experiment (110) clearly indicated that addition of copper to a copper-deficient diet resulted in a marked increase in the retention of iron. This work seemingly contradicts the previous work of Elvehjem and Sherman (85) and has since been quoted in support of the work of Gubler *et al.* (87) to be presented shortly.

However, the amount of iron fed animals is of extreme importance with regard to the storage of this element, as has been discussed. The amount of iron Elvehjem and Sherman (89), and McCall *et al.* (90) used in feeding animals as a basal minimum for good health was about forty times the amount used by Houk *et al.* (110). Therefore, the conclusion that copper-supplemented rats retain and store more iron than copper-deficient rats is, in my opinion, true only for the experimental conditions used by these workers, i.e., subnormal feeding of iron.

(b) Iron Absorption in the Swine

Gubler and co-workers have conducted many prophylactic copper and iron deficiency studies in swine (87, 111), and it is clear that they do not support Elvehjem's conclusions (85) regarding increased iron stores in copper-deficient animals. Gubler and co-workers have found decreased levels of iron in the livers and spleens of copper-deficient swine. It is interesting to note that these workers have also observed a few cases of increased copper levels in the livers of iron-deficient swine (87, 111). Furthermore, these workers obtained a significant increase in the levels of copper in the kidneys of iron-deficient swine (96). The swine receiving iron were given 30 mg/Kg body weight. "No supplements other than iron and copper were added to the diet of any of the pigs." (96). Only if these workers injected 1,000 mg Fe into copper-deficient animals was the amount of iron in the liver and spleen of copper-deficient swine greater than the control level (96).

In a recent paper by Nacht *et al.* (112) the lack of accumulation of iron in copper-deficient swine was thought to be due to the lack of a copper containing carrier which serves to transport iron across the gastro-intestinal tract. The amount of iron accumulating in the mucosa of copper-deficient animals was much greater than that in control animals. This "mucosal block theory" awaits further studies. On the other hand, sheep grazing on copper-deficient pastures were found to have an increased retention of iron (113). Whether Gubler's differences are due to a species difference or the omission of added vitamin supplements in his studies, or to some other experimental variation is not clear.

(c) Iron Absorption in Normal and Iron-Deficient Rats and in Rats fed a Corn Grit Diet

Under normal conditions, the absorption of dietary iron is regulated by the need of the body for iron. Hahn *et al.* (114) and Copp and Greenberg (115) have demonstrated that in iron deficiency, absorption of iron is increased several fold. Hahn *et al.* (114) postulated that there exists a receptor substance in the intestinal mucosa capable of reversibly combining with iron and passing it into the blood stream. Granick (116) believes that ferritin plays this role. Kinney *et al.* (117) maintain that normally there is some block to iron absorption, for animals consuming a large dietary intake of iron on a Purina dog chow ration fail to absorb excessive iron. On the other hand, these workers (117) have found that this

gastrointestinal block may be overcome by feeding rats a corn grit diet supplemented with iron. These animals store 4 - 6 times as much iron over the control animals, and progessively lose more weight than the controls.

(d) Recent Work Backing Elvehjem's Early Findings

Undritz (118) has confirmed Elvehjem's (85) work in rats, using the same technique; young rats were rendered anemic by a pure milk diet, and iron or copper or both were fed to these deficient animals. Hemoglobin levels in the anemic rats fell to 1/3 of the normal levels and the feeding of iron or copper raised the hemoglobins to half of the normal level. The rats fed iron were found to accumulate iron in the liver. The rats fed copper became depleted of iron in the liver. "Apparently copper mobilizes the entire iron reserve for hemoglobin formation."

Undritz (118) further reports an observation of heightened importance, remembering the findings of Hill *et al.* (107, 108). Rats rendered anemic on a diet of cow's milk all eventually die (175 days after onset). Rats fed iron in addition all eventually die as well (205 days after onset). However, rats fed only copper in addition to cow's pure milk survive almost as well as the rats fed both iron and copper (experiment terminated 235 days after onset). Undritz (118) used 68 µg Fe and 6.8 µg Cu per 100 g/day in feeding the various groups of rats. This level of iron might be the lower limit of iron intake necessary to observe increased iron storage in copper

deficiency. The observation that copper-deficient animals all eventually die is understandable in light of the findings reported by Hill *et al.* (107, 108), that the immediate cause of death in such animals is lesions of the aorta owing to the deficiency of a copper containing diamine oxidase enzyme which leads to impaired elastin formation in the aorta.

Recently, an interesting complication due to iron deficiency was observed in the rat (119). Iron-deficient rats on a diet containing adequate folic acid, appeared to have signs of folic acid deficiency, such as increased excretion of formimino-glutamic acid. It was later found that iron-deficient rats had a decreased level of activity of the enzyme(s), (liver) glutamate formimino-transferase, whose optimum activity is dependent on iron and thus caused the observable defects in folate metabolism.

(e) Interaction of Trace Metals in Deficiency Studies and Further Backing of Elvehjem's Early Findings

In concluding this section, a brief and pertinent example of trace metal interactions in metabolism will be mentioned. Bunn and Matrone (120), after depleting rats of copper, fed some of these rats cadmium and withheld cadmium from the rest. If the animals fed cadmium were fed zinc or copper as well, their hemoglobin and body weights were still much lower than those animals which were not fed cadmium and received zinc or copper. Only if both zinc and copper were fed to the cadmium-supplemented rats did their hemoglobin and body weights increase. In another experiment, these workers (120)

used rats which were fed a normal diet in the same manner as above, i.e., some they supplemented with cadmium and some they did not. Neither zinc nor copper nor both could reverse the poor progress of these cadmium supplemented animals.

The points of most interest in this discussion are the following:

(1) the overall effect of cadmium is to lower liver iron.

(2) zinc has the effect of increasing liver iron.

(3) animals fed dietary copper have a lower level of liver iron.

(4) when copper is omitted from the diet and when zinc without copper is added to the diet, iron accumulates in the liver, because the copper concentration is inadequate for the normal mobilization of liver iron.

This last point proves that the therapeutic experiments of Elvehjem and co-workers are indeed as valid as the prophylactic experiments of Gubler and co-workers, at least from an empirical viewpoint; for had not Elvehjem come to the same conclusion three decades past?

II. EXPERIMENTAL

A. *Chemicals*

1. *Substrates of Monoamine Oxidase*

Kynuramine dihydrobromide (lot P 17-113-3) was purchased from the Regis Chemical Co., Chicago, Illinois. Benzylamine was purchased from the Matheson Coleman and Bell Co. Inc., Norwood, Ohio.

2. *Metal Chelators*

o-Phenanthroline, 8-hydroxyquinoline, sodium diethyldithiocarbamate and thenoyltrifluoroacetone were purchased from the Fisher Scientific Co., Montreal. Cuprizone and cupferron were purchased from the G. Frederick Smith Chemical Co., Columbus, Ohio. An initial sample of cuprizone was generously donated by Dr. H. Bolker of the Pulp and Paper Institute of McGill University. Riboflavin was purchased from Chas. Pfizer and Co., Inc., New York, New York.

3. *Diets for the Deficiency Experiments*

Benson's Original Corn Starch was purchased from Steinberg's Ltd., Montreal. Crino powdered milk was purchased from the Coopérative Agricole de Granby. Nicotinic acid, biotin, calcium pantothenate, vitamin B_{12}, folic acid, *p*-aminobenzoic acid, and riboflavin-5'-PO_4 were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Pyridoxine hydrochloride was purchased

from Chas. Pfizer and Co., Inc., New York, New York. Cupric sulfate, thiamine hydrochloride and manganese sulfate were purchased from Merck and Co. Ltd., Montreal. i-Inositol was purchased from the Anachemia Chemicals Ltd., Montreal. Ferrous sulfate was purchased from the J. T. Baker Co., Phillipsburg, New Jersey. Choline chloride was purchased from Fisher Scientific Co., Montreal.

4. *Materials for Column Chromatography*

Sephadex G-200, lot no. 9405 (particle size 40 - 120 μ) and DEAE sephadex A-50, lot no. 9012 (particle size 40 - 120 μ, capacity 3.5 ± 0.5 meq/gm) were purchased from Pharmacia, Montreal Ltd.

5. *Animals Employed for the Deficiency Experiments*

The animals used in the deficiency experiments were male albino rats of the Sprague-Dawley strain and were purchased from the Canadian Breeding Laboratories, Laprairie, Quebec. Their initial weights were about 40 grams.

B. *Methods*

1. *Preparation of Copper, Iron, and Copper and Iron-Deficient Diets*

The bulk of the diet for all the animals was 50% (w/w) powdered milk and 50% corn starch. This diet contained very low amounts of copper and iron. Minerals and vitamins were added directly to the glass drinking bottles of the various animal groups. The stock vitamin solution contained the following amounts of vitamins and minerals.

Nicotinic acid	200 µg/ml
Biotin	1 µg/ml
Calcium pantothenate	800 µg/ml
Vitamin B_{12}	1 µg/ml
Folic Acid	40 µg/ml
Choline chloride	40 mg/ml
p-aminobenzoic acid	200 µg/ml
Riboflavin-5'-PO_4	100 µg/ml
Pyridoxine hydrochloride	20 µg/ml
Thiamine hydrochloride	100 µg/ml
i-Inositol	10 mg/ml
Manganese sulfate (as $MnSO_4.7H_2O$)	0.1 mg/ml

The stock solution of ferrous sulfate ($FeSO_4.7H_2O$) contained 0.8 mg/ml of iron and the stock solution of cupric ($CuSO_4.5H_2O$) sulfate contained 0.1 mg/ml of copper.

The control animals were fed 10 ml of the stock vitamin solution, 10 ml of the stock iron and 10 ml of the stock copper solution in 300 ml of glass distilled water. The deficient animals received the same concentration of vitamins as the control animals. Similarly, equal concentrations of copper were fed the iron-deficient animals. All animals were allowed to drink and eat *ad libitum*.

2. *Dry Ashing Procedure*

The procedure is essentially that described by Thiers (121) and modified to suit our purposes. Tissues for iron and copper determinations were weighed and placed in a Vycor 30 ml crucible. These crucibles were then placed on a Lindberg heater and covered with an inverted Petri dish, fumes being allowed to escape, yet dust being prevented from entering the crucibles. A General Electric Reflection 250 watt infra-red lamp was placed above the Vycor crucibles to initiate the pre-ashing procedure. At intervals of a half-hour the lamp was lowered until it barely touched the top of the Petri dish, at which time the Lindberg heater was switched on "low". The heating capacity was increased until all the tissue samples were blackened and no more fumes were observed. The crucibles were then removed, their lids replaced, and put into a Blue M Lab Heat Muffle Furnace (initial temperature 200° C). The heat was increased at a moderate rate to 450° C, at which temperature the tissue was ashed completely in about twenty hours.

Tissues being analyzed for copper have to be ashed at 600° C for twenty hours, because of the much greater amount of tissue used for detecting copper. Also, iron is more volatile in the presence of chloride than is copper and a lower temperature must, therefore, be maintained.

3. *Determination of Presence of Copper*

All copper determinations were carried out by Mr. K. Lloyd of this laboratory. Three ml of 6N HCl was added to the ashed tissue samples in the Vycor crucible and heated for 15 minutes, but not allowed to boil. This was then transferred, along with three 1 ml glass-distilled water rinses, to a test tube. Five ml of saturated sodium pyrophosphate was added to eliminate any interference from colour owing to iron. Then, 5 ml of 0.03% dibenzyldithiocarbamate ("Arazate", Naugatuck Chemicals, Elmira, Ontario) in CCl_4 was added and shaken for 30 seconds. The organic phase was transferred to a clean tube and a small amount of anhydrous sodium sulphate was added. The clear liquid was read in a Coleman Junior Spectrophotometer at 440 mμ.

4. *Determination of Presence of Iron*

Three ml of 3N HCl was added to the ashed tissue in the Vycor crucible and with the aid of a clean glass rod the grey ash was finely dispersed. The crucibles were left for at least four hours. 1.5 Ml was then removed from the crucible and transferred to a clean test tube. 3.0 Ml of 3N sodium acetate (Fisher) was added; the final pH was about 6.0. 0.1 Ml of a freshly prepared

0.5M sodium sulfite (Fisher) solution was then added, followed by the addition of 0.4 ml of a 0.1% *o*-phenanthroline solution. The mixture was then shaken for 15 seconds and allowed to stand for maximum colour development. After 40 minutes, the colour was fully developed and was read in a Coleman Junior Spectrophotometer at 510 mμ. This method is a modification of that described by Gubler *et al.* (87).

All determinations of copper and iron reported in this thesis were carried out on perfused liver samples. All glassware used in conjunction with the deficiency experiments and iron determinations was first washed with hot detergent. The glassware was then transferred to a plastic trough filled with 3N HCl and allowed to soak overnight. Repeated rinsing of this glassware with glass distilled water proved sufficient in providing glassware free of iron or copper.

5. *Determination of Hemoglobin*

The alkaline haematin method (122) was used without any changes. 0.05 Ml of fresh blood was transferred to a tube containing 4.95 ml of 0.1N NaOH and allowed to boil for five minutes. A standard solution of pure haemin was prepared as follows: 37.5 mg haemin (recrystallized, Eastman Organic Chemicals), was dissolved in 1 liter 0.1N NaOH. This gives a colour similar to 7.4 gm% of haemoglobin. Readings were taken at 540 mμ in the Coleman Junior Spectrophotometer.

6. *Perfusion of the Liver*

A disposable, plastic, 25 ml syringe with a fine needle (28 gauge) was filled with ice-cold 0.30 M sucrose. Immediately following decapitation, the rat's abdomen was opened and the heart removed. The needle was inserted in the vena cava and half of the sucrose content was allowed to perfuse slowly through the liver. The remaining half of the sucrose was injected into the portal vein and perfusion was continued until the liver had turned a homogeneous fawn colour.

7. *Preparation of Liver MAO*

The following procedure was employed in studies pertaining to the deficiency experiments. The animals employed were Sprague-Dawley male rats. Their livers were all perfused before proceeding to measure MAO activity in this organ.

A randomly chosen lobe of the liver was severed, weighed and cut into smaller portions. These slices were then placed in an all-glass homogenizer with a teflon pestle. A small volume of freshly prepared ice cold sucrose, 0.3M, was then added and homogenization was carried out with an electrically driven pestle (Arthur H. Thomas Co., Philadelphia, Pa.), for three minutes. The homogenizing vessel was immersed in a vessel containing ice and water during the three minute period. Enough 0.3 M sucrose was added to obtain a 5% (w/v) suspension.

8. *Preparation of Kidney Monoamine Oxidase*

The kidneys were cleaned of fat, decapsulated, and cut into small pieces. The homogenization was the same as that described for the liver. The final kidney suspension, however, was made up to 20% (w/v) with 0.3M sucrose.

9. *Determination of Protein*

The method of Lowry *et al.* (123) was employed and no changes were introduced. Human serum albumin (Cutter Laboratories, Berkley, Calif.), was used for preparing a standard curve. The directions prescribed by Lowry *et al.* were for a total volume of 1.3 ml, or any multiple of this value. I employed a total volume of 6.5 ml and thus used protein solutions contained in 1 ml of water. Readings were taken at 500 mμ or 750 mμ in the Zeiss PMQ II Spectrophotometer with the appropriate filter.

10. *Preparation of Sephadex Gel Filtration Column G-200*

The powder was suspended in water and allowed to swell for 24 hours. The gel was then washed several times with 0.05 M phosphate (PO_4) buffer, pH 7.4, and allowed to stand for three days at room temperature. The gel was then poured into a large Kimax column (60 x 4.5 cm) partly filled with the same buffer and the particles were allowed to settle to a height of 40 cm. A small amount of Sephadex G-25 was applied to the top of the column to strengthen the surface layer of the gel. A few liters of 0.05 M PO_4, pH 7.4,

were allowed to percolate through the column before applying any material for separation. Fractions of 5 ml eluates were collected with the aid of a LKB 3400 Radi-Rad Fraction collector at 4 - 8°C.

11. *Preparation of DEAE Sephadex A-50 Column*

The procedure was similar to that for preparing the G-200 column. It was not found necessary to treat the gel with NaOH and HCl before use. A smaller column was employed (40 x 2 cm) and the time for swelling of the gel in 0.05M pH 7.4 phosphate buffer was 24 hours. Two liters of 0.05M phosphate buffer, pH 7.4, were allowed to percolate through the column before any sample was applied for separation.

12. *Purification of Monoamine Oxidase*

The method employed was that of Youdim and Sourkes (60). Briefly, the method was as follows: 25% (w/v) liver homogenates in 0.3M sucrose were spun at 600 x *g* in a Model PR-2 International centrifuge (2°C) for 20 minutes to rid the preparation of unbroken cells, cell débris, red blood cells, and nuclei. The supernatant was made up to 0.0125 M with phosphate buffer, pH 7.4, and enough benzylamine added to yield a final concentration of 10^{-3}M. After sonifying this solution for 110 minutes at 3 - 8°C with a Branson (Model No. S-75) sonifier at 8 Kc, cholate (ox bile, Fisher) was added to a 1% (w/v) final concentration. After half an hour, the clear red solution was centrifuged at 160,000 x *g* (R max) for 1 hour in a Beckman model L ultracentrifuge. To the pooled supernatant solutions, enough solid ammonium sulfate (Mann, special

enzyme grade lot #12-2588) was added to obtain a 30% saturated solution. Dilute ammonia was added dropwise to bring the pH to 7.4. After standing for two hours, this solution was centrifuged for 30 minutes at 600 x *g* and the supernatant solutions were pooled. Solid ammonium sulfate was again added to obtain a 55% saturated solution and again brought to pH 7.4 with a few ml of dilute ammonia. After standing overnight at 3 - 8°C, the suspension was centrifuged at 14,000 x *g* for 1 hour and the solid pellets were carefully dissolved in 0.05 M phosphate buffer, pH 7.4. This clear red solution was then dialyzed against 4 liters of 0.005 M phosphate buffer, pH 7.4, for 24 hours to rid the preparation of benzylamine and ammonium sulfate. The dialysate was then centrifuged for 1 hour at 14,000 x *g* to rid the preparation of any insoluble protein. 50 Ml of this protein solution was applied to a G-200 Sephadex column and eluted with 0.05 M phosphate buffer, pH 7.4. The clear yellow solutions containing monoamine oxidase activity were pooled and applied directly to a Sephadex A-50 column and were eluted with 0.05 M phosphate buffer.

13. *Determination of Presence of Flavin*

Flavin determinations were run as prescribed by King *et al.* (124). Enzyme preparations in 4 ml phosphate buffer 0.05 M, pH 7.4, were mixed with 4 ml of ice-cold 20% trichloroacetic acid (TCA) and allowed to remain in a separatory tube on ice for 30 minutes. The solution was then centrifuged at 6,000 x *g* for 30 minutes. The decrease in volume owing to precipitation of protein with TCA was

negligible, as very small amounts of protein were analyzed. The supernatant solution was extracted three times with 15 ml portions of diethyl ether to remove the trichloroacetic acid. The aqueous phase was then adjusted to pH 7.0 with one to two drops of NaOH. The flavin released by the above treatment will be referred to as TCA-extractable flavin.

In order to obtain an index of the total flavin content of some of the enzyme preparations, Pronase (B grade Calbiochem, Los Angeles, Calif.) was employed to degrade the proteins into peptide fragments. 1.5 Mg Pronase, 2 ml of the enzyme sample, and 2 ml of 0.05M phosphate buffer, pH 7.4, were added to a 20 ml beaker and allowed to incubate at 37°C for various time intervals. After incubation, 4 ml of cold 20% TCA was added to the beaker, and the suspension was transferred to a centrifuge tube. After standing for 30 minutes on ice, the tubes were centrifuged, extracted with ether, and neutralized with NaOH as described previously for TCA-extractable flavin. Appropriate blanks and standards were included and run simultaneously with the test solutions. Readings were then taken on the Aminco-Bowman spectrophotofluorometer, excitation wavelength 465 mμ, fluorescence wavelength 530 mμ, and slit system 2:2:2 were employed. Activation and fluorescence scans were also run on TCA-extractable flavin and total flavin released from various enzyme preparations.

14. *Kynuramine Assay for Monoamine Oxidase Activity*

The method of Weissbach *et al.* (77) was followed. The

substrate was kynuramine dihydrobromide, which is oxidatively deaminated; the resulting aldehyde cyclizes spontaneously to 4-hydroxyquinoline. The rate of product formation was determined by the appearance of 4-hydroxyquinoline at 315 - 329 mμ in the Zeiss PMQ II spectrophotometer or the Beckman DK-2 recording spectrophotometer.

General Assay

0.2 Ml of the enzyme preparation from liver, kynuramine, 2.05×10^{-4}M final concentration, 1.0 ml of phosphate buffer, 0.033M final concentration (pH 7 or 8) and water to 3 ml were incubated in 20 ml beakers at 37°C, the blank (A) contained everything except kynuramine. Another blank (B), was employed to which kynuramine was added and immediately followed by 1.0 ml of a 5% (w/v) zinc sulfate solution. The test solutions were pre-incubated for 5 minutes at 37°C, followed by the addition of substrate and further incubation for 20 minutes. At the end of 20 minutes, 1 ml of 5% (w/v) zinc sulfate was added to all beakers to terminate the reaction. These floccular suspensions were then centrifuged in a clinical centrifuge and the supernatant solutions were assayed for 4-hydroxyquinoline formation in the Zeiss PMQ II spectrophotometer at 315 mμ. Blank A was used to set the instrument to 100% transmission and blank B yielded the absorption due to kynuramine (this blank usually read between 0.190 - 0.220 O. D. units).

15. *Spectrophotofluorimetric Assay for Monoamine Oxidase Activity*

A more sensitive assay for MAO activity employing kynuramine with the same procedure as mentioned previously is the fluorimetric assay of Kraml (78). After centrifuging the floccular zinc sulfate ($ZnSO_4$) 1.0 ml of the supernatant solution was added to 1.0 ml of 2.0N NaOH and examined in the Aminco-Bowman spectrophotofluorometer for the production of 4-hydroxyquinoline. The settings are as follows: Excitation maximum, 315 mμ; fluorescence maximum 380 mμ; slit system 1:1:1; sensitivity setting on 10.

16. *Benzylamine Assay for Monoamine Oxidase Activity*

The incubation procedure for benzylamine oxidation was as follows: 0.2 ml of the kidney or liver homogenates were incubated in a total volume of 3.0 ml which contained 1.0 ml of 0.25M phosphate buffer, pH 7.4. The final concentration of benzylamine used was 1.67 x 10^{-3}M. Incubations were carried out at 37°C for varying amounts of time. 1.0 Ml of 5% $ZnSO_4$ was then added and this suspension was centrifuged at 5000 RPM for ten minutes. The supernatant fluid was examined in the Zeiss PMQII spectrophotometer at 250 mμ. The increase in optical density at 250 mμ, which is directly proportional to the amount of benzaldehyde formed, was recorded and used as a measure of the MAO activity.

17. *Plasma Amine Oxidase Assay*

The procedure of McEwen and Cohen (125), developed to measure the activity of human plasma amine oxidase, was followed,

with minor modifications. After incubating 0.6 ml of plasma with 0.05 ml 0.01M benzylamine and 0.70 ml of 0.2M phosphate buffer, pH 7.2, for periods up to 2.5 hours, 0.15 ml perchloric acid was added and the incubation contents were dispersed with the aid of a clean glass rod. 1.5 Ml cyclohexane was then added and the mixture was homogenized again with the aid of a glass rod. After centrifugation, the benzaldehyde and any remaining benzylamine were in the upper (cyclohexane) phase. Readings were conducted at 242 mμ in micro-cuvettes in the Zeiss or Beckman DK-2 spectrophotometers.

The final concentration of benzylamine employed by McEwen was 7.5×10^{-4}M. I used 3.3×10^{-3}M benzylamine (final concentration) for rabbit plasma amine oxidase and 1.67×10^{-3}M benzylamine (final concentration) for rat plasma amine oxidase.

III RESULTS

1. *Investigation of Plasma Amine Oxidase of Rat*

(a) Absence of Benzylamine Oxidase Activity in Rat Plasma

Rat blood was treated with heparin, or citrate, to prevent clotting and spun in a clinical centrifuge to obtain the plasma. As can be seen from Fig. 1, no amine oxidase activity was observed with benzylamine serving as the substrate. When the assay was conducted under 100% O_2 for 2.5 hours, the benzylamine disappeared. If this indicates an enzymic disappearance, the benzene ring might be split, since there was no characteristic spectrum of benzene.

In order to rule out the presence of a dialyzable inhibitor in the plasma which might be preventing the oxidation of benzylamine, the plasma was dialyzed against distilled water (3 - 8°C), before being assayed. No activity with benzylamine as the substrate was observed.

(b) Absence of Kynuramine Oxidase Activity in Rat Plasma

The fluorimetric kynuramine assay of Kraml (78) was employed to determine if rat plasma is capable of oxidizing this amine. When various amounts (0.3 to 1.5 ml) of plasma were incubated for 2 hours at 37°C with kynuramine at pH 8, no activity was observed in ten experiments.

In another experiment, 800 units of catalase were included in the incubation mixture. Plasma, serum and whole rat blood were assayed. No amine oxidase activity was detected in any of these cases.

Legends for the graphs:

Fig. 1

Rabbit plasma, 0.6 ml was incubated with 3.3 mM benzylamine (double-distilled) for 20 minutes. Curve A is the spectrum of the benzaldehyde formed during this incubation.

Curve B is a spectrum of the benzylamine level present in the blank incubation.

Rat plasma, 0.6 ml was incubated with 1.67 mM benzylamine for 2.5 hours. Curve C is the spectrum of the benzylamine level in the blank; this spectrum was identical to those of the incubated test samples, none of which showed any benzaldehyde.

Curve D is the spectrum obtained when the incubation is conducted under 100% oxygen for 2.5 hours showing a disappearance of benzylamine.

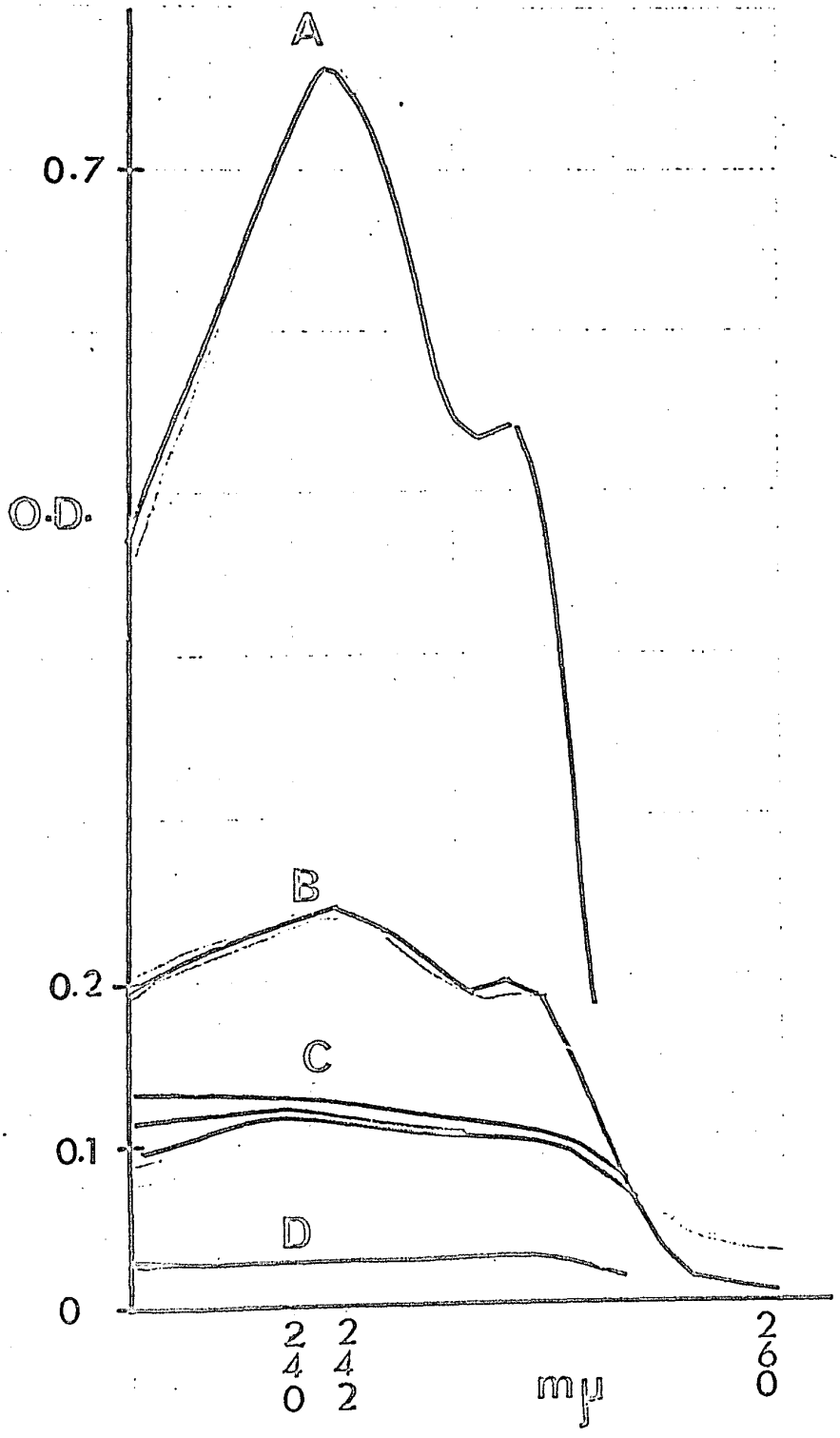


FIG. 1 THE SPECTRA OF BENZALDEHYDE AND BENZYLAMINE, PRESENT IN INCUBATIONS WITH PLASMA AMINE OXIDASE

(c) Rabbit Serum Amine Oxidase

Rabbit blood was treated with heparin and spun in a clinical centrifuge to deposit the cells. As can be seen from Fig. 1 and 2, there was a very active disappearance of benzylamine with a concomitant appearance of benzaldehyde. The reaction was linear with time (cf. Fig. 2) and an unmistakable almond odor was present in the test incubation mixtures.

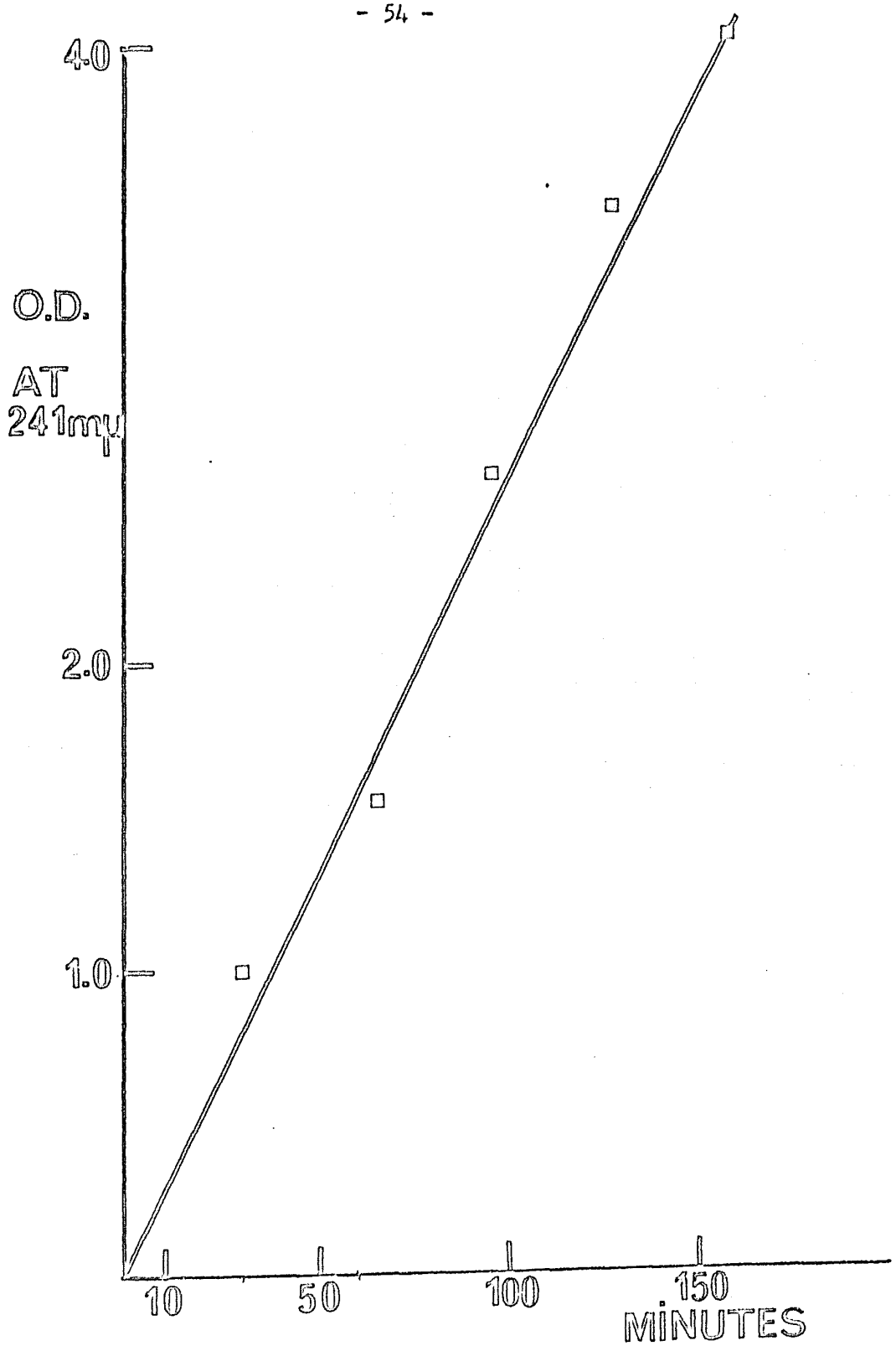


FIG. 2 PRODUCT (BENZALDEHYDE) FORMATION VERSUS THE TIME OF INCUBATION OF THE RABBIT PLASMA WITH BENZYLAMINE

(d) Discussion

Hirsch (23) was the first worker to demonstrate the presence of an amine oxidase enzyme in the serum (or plasma) of cattle and sheep, capable of oxidizing spermine and spermidine. This enzyme was purified one year later by Tabor *et al.* (126) and shown to be capable of oxidizing not only spermine and spermidine but also benzylamine and heptylamine, which are substrates of the particulate MAO enzyme. As this plasma amine oxidase is inhibited by carbonyl reagents, it seems more correct to include it among the DO group of amine oxidases (cf. Table I).

Blaschko *et al.* (127) have studied the plasma amine oxidase of a variety of animals and they concluded that "the ability to oxidize spermine was restricted to the blood plasma of ruminants, (whereas) the ability to oxidize benzylamine and other amines was found not only in ungulates and paenungulates but also in some of the carnivores tested". Specifically, they found that the ferret, dog, and tiger could oxidize benzylamine, whereas the cat, lion, and seal had no oxidase activity in their plasma.

Kobayashi (128) has found DO activity in female rats (Holtzmann strain). With putrescine as the substrate, he was able to demonstrate a continuous rise in the level of this enzyme in pregnant rats until the 14th - 16th day of pregnancy. It is not known if the placenta of the rat serves as the additional DO source for this serum enzyme during pregnancy.

The results presented in this section indicate that male albino Sprague-Dawley rats contain no amine oxidase enzyme in their plasma that is capable of oxidizing kynuramine or benzylamine. Nowhere in the literature, to my knowledge, is there any paper which states that the rat plasma contains such an enzyme. The rat is exceptional in having a very low concentration of MAO and DO in its kidneys. If the kidneys are the site of escape of the amine oxidase enzymes into the blood plasma, an explanation would then be obtained for the results presented.

2. *Cofactor Studies Using a Partially Purified Rat Liver MAO Preparation*

(a) Riboflavin-Like Material

Flavin analysis was conducted on two preparations obtained while purifying rat liver MAO. The two preparations were:
(A) The 30% - 50% ammonium sulfate precipitated protein material which was dialyzed against two liters of 0.005M phosphate buffer, pH 7.4, before being analyzed for its flavin content.
(B) The above preparation was placed on a Sephadex G-200 column and the eluates which contained MAO activity were pooled, precipitated with 55% ammonium sulfate, resuspended in phosphate buffer, 0.05 M and pH 7.4, and dialyzed against two liters of 0.005M phosphate buffer, pH 7.4. The results of the flavin analysis for preparations (A) and (B) are presented in Table III:

TABLE III

RELEASE OF FLAVIN FROM PARTIALLY PURIFIED RAT LIVER MONOAMINE OXIDASE

Treatment	(A)* mμ moles/mg protein	(B)** mμ moles/mg protein
TCA-Extractable Flavin	0.78	0.68
Total Flavin		
Pronase - 1 hr.	1.09	2.05
Pronase - 2 hr.	1.20	1.35
Pronase - 3 hr.	1.39	1.80
Pronase - 4 hr.	1.45	1.49

* specific activity, 400

** specific activity, 900

The specific activity is here expressed in its usual sense, activity per milligram protein. A unit of activity is defined as the amount of enzyme catalyzing a change in absorbance of 0.001 at 315 mμ in 20 minutes and at 37°, using kynuramine as substrate (pH 8).

Activation spectra of the isolated flavin chromophore by simple TCA treatment and Pronase treatment of preparations (A) and (B) are illustrated in Fig. 3.

(b) Metal Analysis

Preparation (B) was further purified in two different procedures. The first was simply to spin some of this material at 160,000 x *g* for one hour and collect the small pellet formed.

This pellet was rinsed with glass distilled water and will be designated as (P). The second procedure involved placing the remainder of preparation (B) on a Bio-gel P-150 column which was prepared as directed by the manufacturers. The dimensions of this column were identical to those given for the Sephadex G-200 column. The MAO activity was eluted with 0.05M phosphate buffer, pH 7.4, and tubes 23 and 24 and 25 which contained increasing concentrations of the enzyme, in that order, were saved. These tubes will be referred to by these numbers. Finally, one tube, 30, from the previous G-200 eluates, which contained MAO activity, was included in the metal analysis recorded in Table IV.

The large volume of material necessary for determining the metal contents of these enzyme preparations precluded flavin analysis.

TABLE IV

IRON AND COPPER CONTENT OF PARTIALLY PURIFIED
RAT LIVER MONOAMINE OXIDASE PREPARATIONS

Preparation*	Activity /0.2 ml	Mg Protein /0.2 ml	Specific Activity**	Purification†	µg Fe /mg Protein	µg Cu /mg Protein
30	0.085	0.26	323	2	2.3	0.6
23	0.015	0.02	750	4	-	2.0
24	0.065	0.07	930	5	3.9	1.9
25	0.125	0.06	2080	12	4.5	0.4
P	0.295	0.12	2460	14	4.2	1.1

* Numbers are explained in the text

** Specific activity of rat liver homogenates, 175

† $\frac{\text{(Specific activity of the preparation)}}{\text{(Specific activity of the homogenate)}}$ = purification

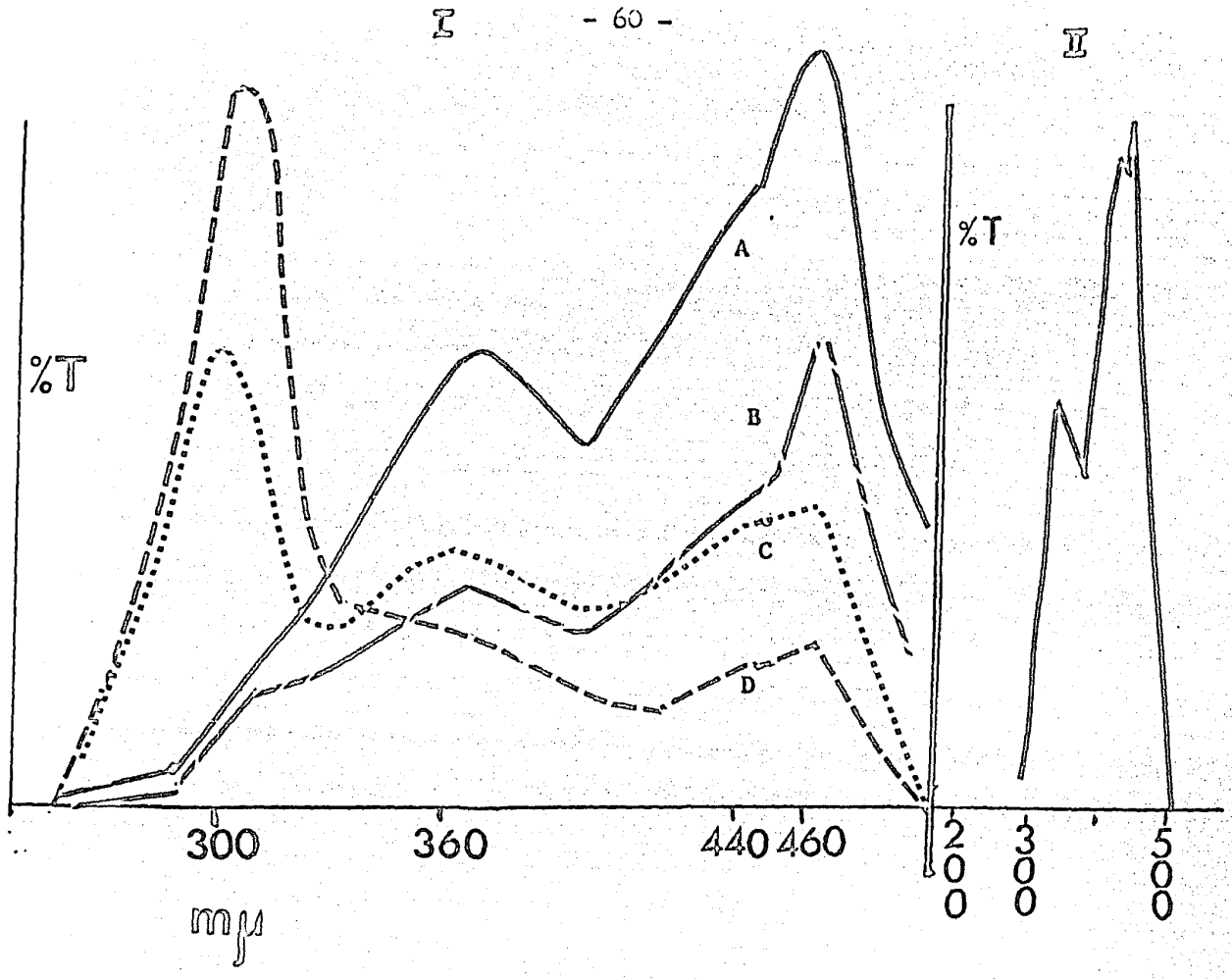


FIG. 3 ACTIVATION SCANS OF PURE FAD AND OF THE FLAVOPEPTIDE RELEASED FROM PARTIALLY PURIFIED RAT LIVER MONOAMINE OXIDASE

Activation scans using a fluoresence of 529 mμ of pure FAD (in water), II, and of the enzyme preparations (A) and (B), I.

A Scan of the Pronase treated enzyme preparation (A)

B Scan of the TCA treated enzyme preparation (A)

C Scan of the Pronase treated enzyme preparation (B)

D Scan of the TCA treated enzyme preparation (B)

(c) Discussion

As can be seen from Table III, the TCA-extractable flavin content of both preparations (A) and (B) were almost equal. However, the total flavin released during incubation of the preparations with Pronase differed greatly. Preparation (B) liberated more flavin in the first three hours of incubation than preparation (A). As flavin is very labile when exposed to light for excessive periods of time, the slight fluctuations in flavin content observed for preparation (B), which contained more total flavin, is understandable. If flavin is indeed the prosthetic group of MAO, the flavin content would be expected to increase as the enzyme is further purified. This seems to be the case.

Similarly, if a metal is an integral part of MAO, this metal's concentration should also increase with purification. As can be seen from Table IV, with increasing purification, the amount of iron increases erratically to a constant value, whereas the amount of copper remains variable.

Figure 3 illustrates that the concentration of flavin may equally well be determined from the height of the peak at 460 mμ. These spectra are very similar to that of pure FAD in water which is shown in Part II of Fig. 3.

I included preparation (P) for two reasons. In purifying an insoluble enzyme such as MAO, the question of conformational, structural, and even catalytic changes occurring while solubilizing

and purifying the enzyme always arises. It is indeed apparent that both the soluble and insoluble forms of MAO prepared, not only had similar specific activities but also possessed similar amounts of iron (and copper). It, therefore, seems that the process of solubilization and further purification did not result in any gross changes of this enzyme.

3. *Effect of Chelating Agents on Rat Kidney and Liver Monoamine Oxidase*

(a) The Effect of Various Chelators on Liver MAO

Incubations were conducted as described under Methods. The only innovation was the pre-incubation of all chelators for 15 minutes at 37° with the enzyme before the addition of kynuramine. The fluorimetric assay of Kraml was used because chelators such as *o*-phenanthroline and thenoyltrifluoroacetone absorb very strongly at 315 mμ. To a lesser extent, but equally troublesome, is the absorption by cuprizone, riboflavin, and 8-hydroxyquinoline. No chelator mentioned in this work interfered with the fluorimetric assay.

The inhibition of MAO by various concentrations of metal chelators is shown in Fig. 4. In general, the inhibition of MAO activity was greatly enhanced if incubations were conducted at pH 7, as compared with pH 8. Table V summarizes the effect of metal chelators upon the MAO activity of the rat liver enzyme.

(b) Kinetic Studies Using MAO of Rat Kidney and Liver

Lineweaver-Burk reciprocal plots were calculated for the kidney and liver enzyme. The concentration of 8-hydroxyquinoline was kept constant (5.7×10^{-5}M) and the substrate concentration was varied. As Figures 5 and 6 illustrate, 8-hydroxyquinoline was a competitive inhibitor in the case of the rat kidney enzyme and a mixed inhibitor in the case of the liver enzyme.

The apparent Michaelis constant, K_m, for the liver and kidney MAO enzymes was calculated at 1.0×10^{-4}M and 6.7×10^{-5}M, respectively. Maximum velocities, V_{max}, for the crude kidney enzyme was 0.3 mμ moles 4-hydroxyquinoline/mg protein/min, and for the crude liver enzyme it was 2.8 mμ moles 4-hydroxyquinoline/mg protein/min. The inhibition by excess substrate, kynuramine di HBr, was very striking for the liver and kidney enzymes.

(c) The Effect of Potassium Cyanide

Cyanide was examined for possible inhibitory action on the oxidation of benzylamine by rat liver. The oxidation of benzylamine by rat liver or kidney as opposed to the oxidation of kynuramine is greatly stimulated by oxygen. In the presence of 100% oxygen, the increase in activity is almost two-fold. It was thought that cyanide might inhibit the stimulation afforded by oxygen.

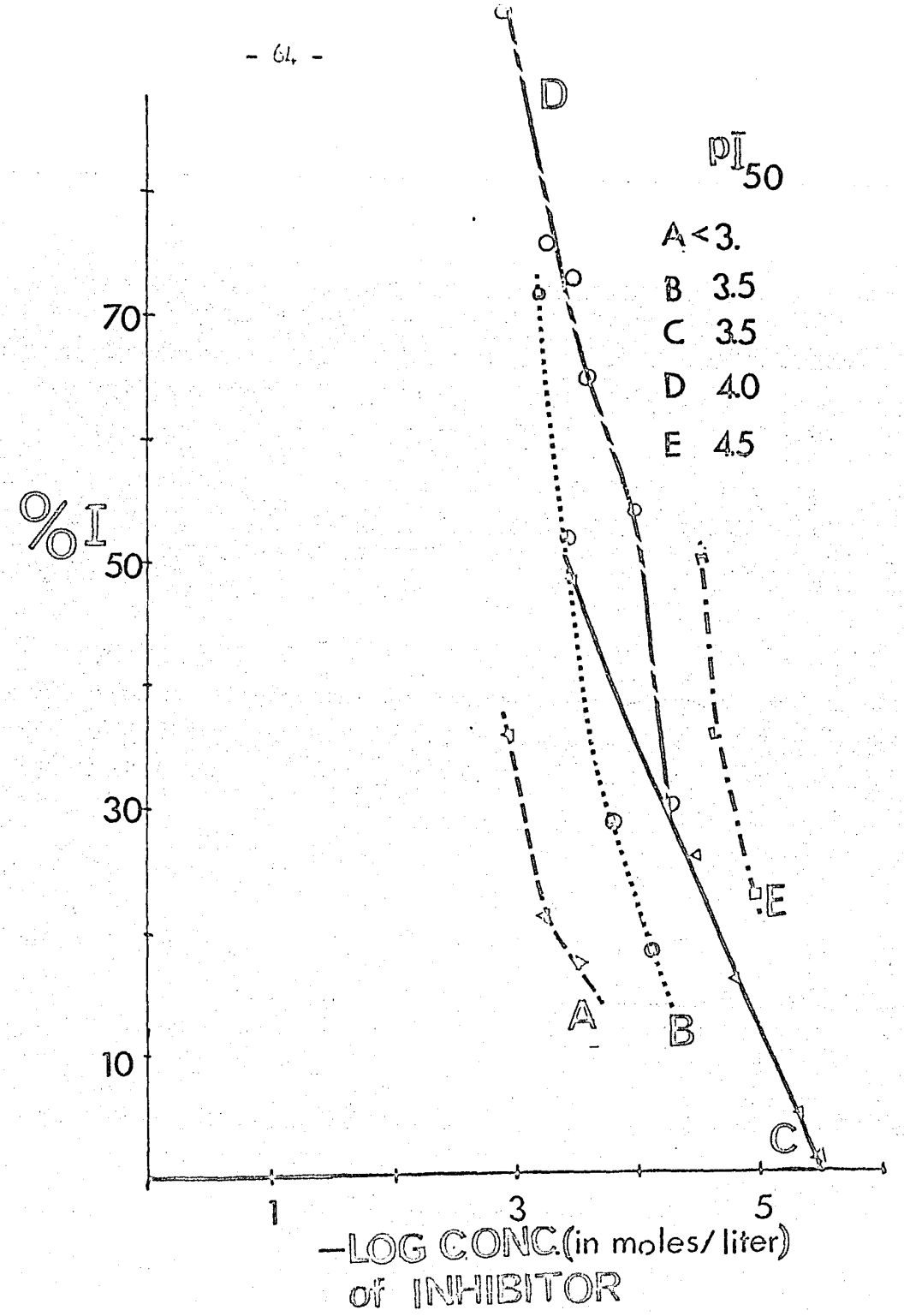


FIG. 4 INHIBITION OF RAT LIVER MAO BY METAL CHELATORS (at pH 7.0)

A, Cuprizone
B, Thenoyltrifluoroacetone
C, Cupferron
D, 8-hydroxyquinoline
E, Riboflavin
I_{50}, The concentration of inhibitors producing 50% inhibition
pI_{50}, $(-\log_{10} I_{50})$

TABLE V

THE EFFECT OF VARIOUS METAL CHELATORS ON LIVER MAO

Inhibitor	M	pH 7	pH 8
None	-	51.0*	78.0
Cupferron	7.0×10^{-6}	50.8 (0)+	78.3 (0)
	1.8×10^{-5}	48.4 (5)	75.4 (3)
	3.5×10^{-5}	43.0 (16)	65.6 (16)
	7.0×10^{-5}	37.7 (26)	52.5 (33)
	4.0×10^{-4}	26.2 (49)	35.3 (55)
TTFA**	8.3×10^{-5}	41.8 (18)	-
	1.7×10^{-4}	36.1 (29)	-
	3.4×10^{-4}	24.6 (52)	-
	6.8×10^{-4}	14.4 (72)	-
Riboflavin	1.2×10^{-5}	29.4 (23)	66.4 (15)
	2.4×10^{-5}	32.8 (36)	54.1 (31)
	3.6×10^{-5}	25.4 (50)	47.5 (39)
DDC***	3.3×10^{-3}	(15)	-
Cuprizone	1.2×10^{-3}	(36)	(20)
o-phenanthrone	1.7×10^{-4}	(22)++	-
	3.0×10^{-5}	(33)	-

* mμ moles 4-hydroxyquinoline formed/10 mg liver/20 min.

** Thenoyltrifluoroacetone

*** Diethyldithiocarbamate

\+ Values in parentheses indicate % inhibition

++ The inhibition here was obtained upon a fourfold purified enzyme preparation

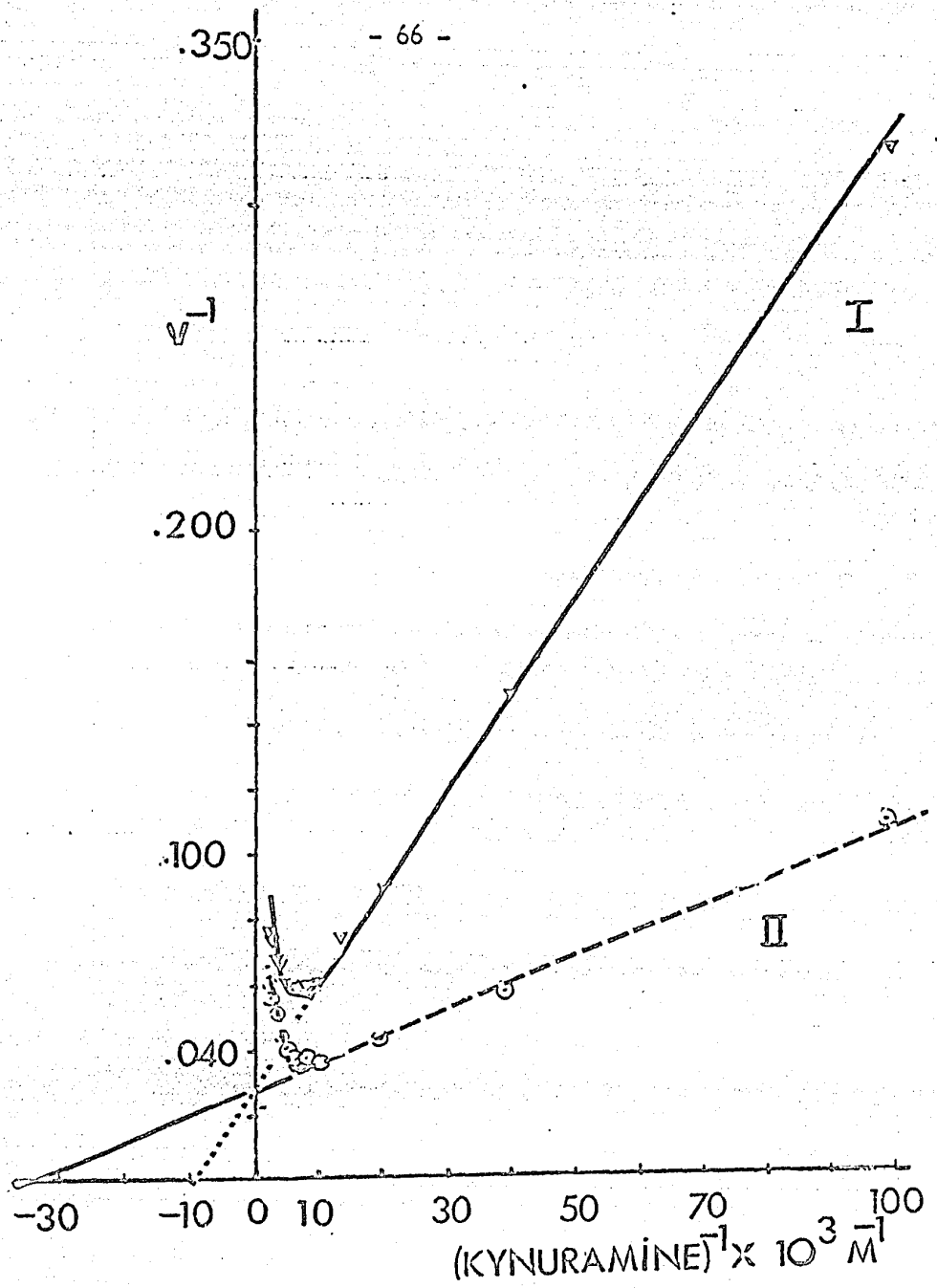


FIG. 5 LINEWEAVER-BURK RECIPROCAL PLOT OF THE KIDNEY MAO ACTIVITY IN THE PRESENCE OF 0.057mM 8-HYDROXYQUINOLINE (I) AND WITHOUT THE INHIBITOR (II)

Ordinate: mμ moles 4-hydroxyquinoline formed /40 mg. rat kidney/ 30 min. (reciprocal)

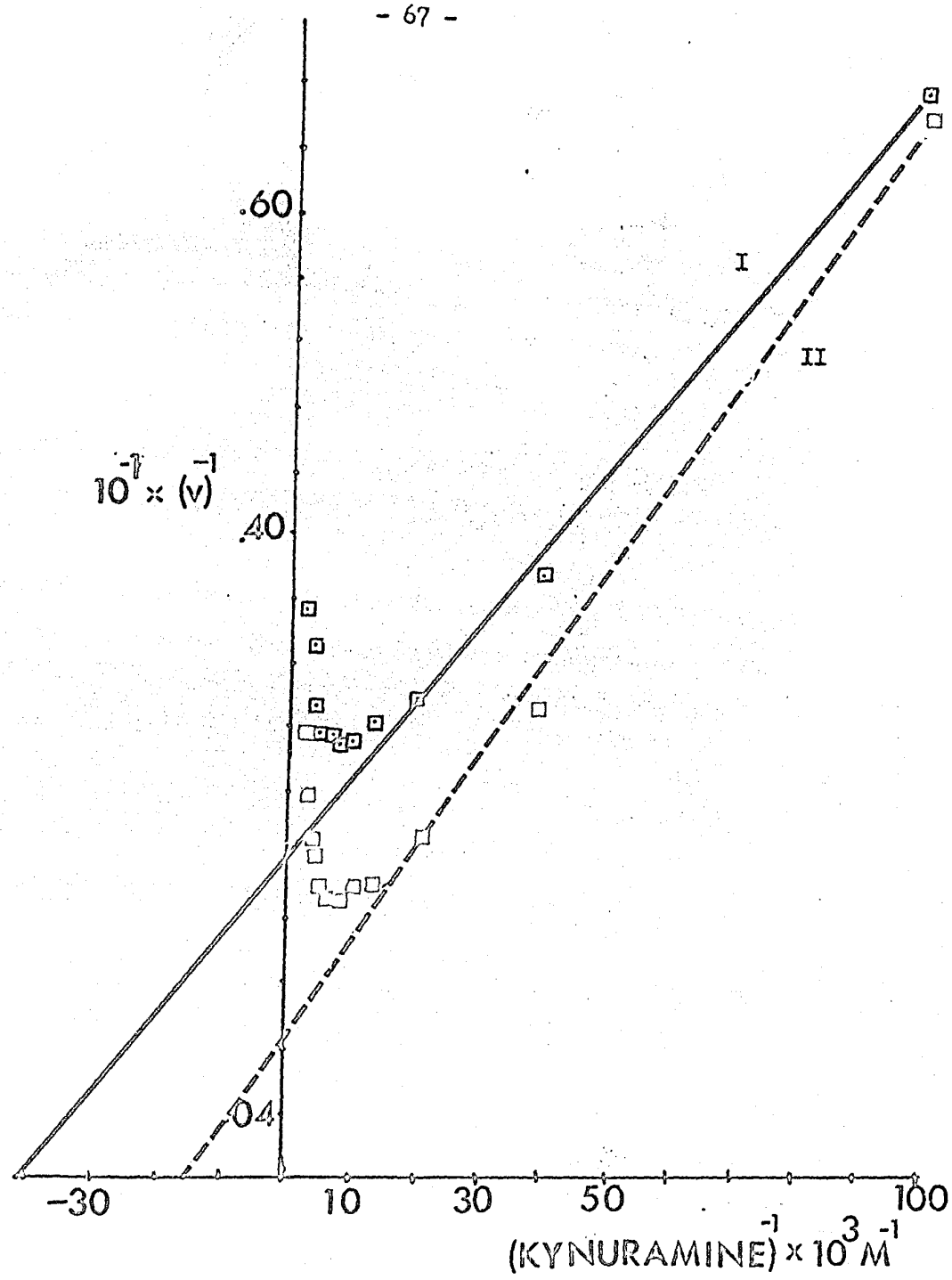


FIG. 6 LINWEAVER-BURK RECIPROCAL PLOT OF THE LIVER MAO ACTIVITY IN THE PRESENCE OF 0.057mM 8-HYDROXYQUINOLINE (I) AND WITHOUT THE INHIBITOR (II)

Ordinate: mμ moles 4-hydroxyquinoline formed /10 mg. rat liver/ 20 min. (reciprocal)

TABLE VI

THE EFFECT OF CYANIDE ON THE BENZYLAMINE OXIDATION BY RAT LIVER MONOAMINE OXIDASE

KCN in M	100% Oxygen Change in O.D. at 250 mμ	Air Change in O.D. at 250 mμ
0	0.590	0.315
0	0.560	0.340
$1.7 \quad 10^{-4}$	0.590	0.365
$8.4 \quad 10^{-4}$	0.630	0.355
$1.7 \quad 10^{-3}$	0.600	0.400

20 Mg of rat liver were incubated for 30 minutes in air and 100% oxygen at 37°C in 1.67×10^{-3}M benzylamine with phosphate buffer, pH 7.4.

The KCN solution was freshly prepared and neutralized to pH 7 with dilute hydrochloric acid. Because cyanide is a carbonyl reagent, the slight increase in activity noted when cyanide is present might be the result of its interaction with the benzaldehyde increasing the extinction coefficient of this compound. Nevertheless, cyanide does not seem to inhibit this system. Table VI records these observations.

(d) Discussion

The *in vitro* effects of metal-binding agents on metallo-enzymes are well known. Cyanide, the classical detector of metals in enzymes, has been replaced by many new and specific metal chelators which have aided greatly in identifying enzymes as metallo-enzymes. Moreover, the use of such specific metal chelators permits a decision as the likelihood of a particular metal's presence.

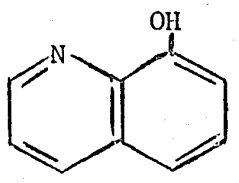
The metal chelators which were tested in the preceding section may be divided into two categories. Thenoyltrifluoroacetone, *o*-phenanthroline and riboflavin chelate strongly with iron. Cupferron, "at pH 7.3, 37°C chelates strongly with iron, weakly with copper" (129). On the other hand, cuprizone and sodium diethyldithiocarbamate chelate strongly with copper and only weakly with iron; 8- hydroxyquinoline is a strong chelator for both iron and copper.

It is evident from the results presented that the *in vitro* inhibition of monoamine oxidase is caused primarily by iron chelators such as cupferron, riboflavin, thenoyltrifluoroacetone, and 8-hydroxyquinoline.

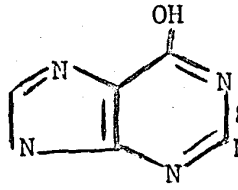
The inhibition caused by 8-hydroxyquinoline was found to be competitive for kidney MAO, further strengthening the possible role of iron in this enzyme. The liver enzyme yielded a mixed type of inhibition, possibly a result of the action of 8-hydroxyquinoline on sulfhydryl groups known to be essential for this enzyme's activity. Barbato and Abood (42) have shown that *o*-phenanthroline is a mixed

inhibitor of the beef liver mitochondrial monoamine oxidase.

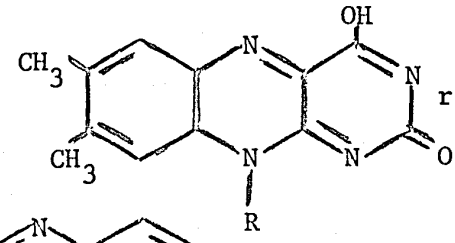
Albert (130) has demonstrated that the basic structure in 8-hydroxyquinoline which gives it the ability to chelate metallic cations is an ionizable hydroxy group peri to a tertiary heterocyclic nitrogen atom.



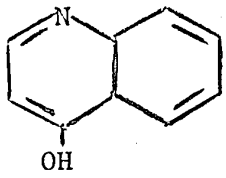
8-hydroxy-quinoline



guanine



riboflavin



4-hydroxyquinoline

The above clarifies the puzzling question, why 4-hydroxy-quinoline, the product of the MAO reaction with kynuramine, does not inhibit this very reaction. Furthermore, this primary structure affords the most potent inhibition of MAO (cf. Fig. 4). FAD does not inhibit MAO activity as well as riboflavin, in fact, only weak inhibition is exhibited by FAD. This might well be the result of other tautomeric forms of the isoalloxazine ring being more favourable for FAD than the one shown above for riboflavin.

High concentrations of kynuramine caused "substrate inhibition" and were not employed in calculations of K_m and V_{max}.

Page and Benditt (131), working with a purified amine oxidase from pig plasma, have determined the K_m value for this enzyme with kynuramine; they obtained a value of 31.3 mM. They also observed inhibition of their purified enzyme with excess substrate, kynuramine. The high K_m associated with the pig enzyme attests to the lower specificity of the pig enzyme with regard to kynuramine as compared to the rat kidney or liver enzymes.

4. *Effect of Iron and Copper Deficiencies and 'Double-Deficiencies' in Rat on Liver and Kidney MAO Activities*

(a) Hepatic MAO Levels of Rats in the First Deficiency Study

The experimental procedures used for the determination of MAO activity have been outlined. All activities have been expressed per milligram (wet weight) of rat liver and kidney. Great care was taken in preparing the tissue homogenates and initially the protein content of these organs was checked to insure that a constant protein content was present for all homogenates. This was experimentally confirmed. Upon assaying the protein content of twenty liver homogenates, 5% (w/v), their protein content was found to be 9.5 mg/ml ± 0.5 (S.E.). For ten samples of rat kidney homogenates, 20% (w/v), the protein content was found to be 21 mg/ml ± 1.0 (S.E.).

In the first deficiency study livers of rats maintained on the deficient diet for 24, 37, 78, 95 and 109 days were analysed for their MAO activity. Control animals were also analysed on the above

dates. The MAO activity for each of the four groups of animals was pooled for all the above dates. No clear decrease in the activity of hepatic MAO was observable for any of the deficient animals. These results are listed in Table VII. Although the doubly deficient animals (those receiving neither copper nor iron), showed a decrease in hepatic MAO, this decrease was not significant (P = 0.2).

TABLE VII

HEPATIC MAO KYNURAMINE ACTIVITY OF 'DEFICIENT' AND CONTROL RATS

State	Mean	±	Standard Error	Number of rats	P
Control	0.442*	±	0.020	(12)	
Doubly Deficient	0.406	±	0.018	(9)	> 0.05
Iron-Deficient	0.441	±	0.096	(8)	> 0.05
Copper-Deficient	0.466	±	0.046	(12)	> 0.05

*Increase in O.D. at 315 mμ/10mg rat liver/20 minutes at pH 8.

(b) Liver and Kidney MAO Levels of Rats in the Second Deficiency Study

In the second deficiency experiment a more extensive series of tests was performed. Not only were the livers of the deficient animals assayed for MAO activity at pH7 and 8, but benzylamine was also used as a substrate for MAO. Also, the kidneys were tested for MAO activity.

In the second experiment, rats were killed at 28, 50, 75 and 90 days, and again the activities for each group were pooled. Tables VIII and IX show the effects of deficiencies of iron, copper and iron and copper on the MAO concentrations of rat liver and kidney, respectively. There was a significant decrease in the hepatic concentration of MAO from animals deficient in both elements compared with control animals when the MAO assay was conducted at pH 7. At pH 8, however, the significance is in question. The animals which were deficient in iron showed a marked drop in the hepatic MAO concentration as well; however, this decrease was not significant at either pH due to the large variation in activities for these animals. No decrease in the hepatic MAO levels was observed for the copper-deficient animals.

The kidney enzyme showed a significant decrease in activity for animals which were deficient in iron; those deficient in copper showed no decrease in the activity of MAO when compared with MAO levels in the kidneys of control animals. Paradoxically, the doubly deficient animals showed a decrease in their kidney MAO levels which was not significantly different from that of the control animals.

TABLE VIII

HEPATIC MAO KYNURAMINE ACTIVITY OF 'DEFICIENT' AND CONTROL RATS

State	pH	Mean	±	Standard Error	Number of Rats	P
Control	8	86.4*	±	2.7	(5)	-
	7	54.8	±	4.1	(5)	-
Doubly Deficient	8	71.8	±	4.9	(9)	0.055
	7	43.7	±	1.6	(9)	0.011
Iron-Deficient	8	67.3	±	7.8	(8)	0.086
	7	43.0	±	5.3	(8)	0.15
Copper-Deficient	8	88.3	±	2.3	(3)	-
	7	51.7	±	1.2	(3)	-

*mμ Moles 4-hydroxyquinoline formed/10 mg rat liver/20 minutes

TABLE IX

KIDNEY MAO ACTIVITY (KYNURAMINE)

State	Mean	±	Standard Error	Number of rats	P
Control	25.8*	±	0.1	(5)	-
Doubly Deficient	21.6	±	1.9	(9)	0.12
Iron-Deficient	20.8	±	0.1	(8)	0.001
Copper-Deficient	26.0	±	0.6	(3)	-

*mμ Moles 4-hydroxyquinoline formed/40 mg rat kidney/30 minutes at pH 7.

Table X lists the activities of MAO for the oxidation of benzylamine by hepatic MAO for rats of the second deficiency experiment. The concentration of hepatic MAO from iron and doubly deficient rats showed a pronounced and significant decrease when compared with the levels present in control rats.

TABLE X

HEPATIC MAO BENZYLAMINE ACTIVITY

State	Organ	$\bar{x}$	$\pm$	Standard Error	Number of Rats	P
Control	Liver	0.162*	±	0.025	(5)	-
Doubly Deficient	Liver	0.063	±	0.014	(9)	0.001
Copper-Deficient	Liver	0.117	±	0.027	(3)	0.9
Iron-Deficient	Liver	0.063	±	0.023	(9)	0.02>P>0.01

*Change in O.D. at 250 mμ/10 mg rat liver/30 minutes

The activities of kidney MAO using benzylamine as the substrate were not pooled for the four groups of animals, because on the two dates that the animals were sacrificed the activities of the control rats differed greatly and the results are clearer if the controls of each date are taken as indicating normal activities. Table XI lists the values of the activities for the

four animal groups on the two dates that animals were killed. The decrease of kidney MAO, with benzylamine as the substrate, in the iron and doubly deficient animals killed at 75 days, seems to be as dramatic as that observed in the livers of these animals, which was shown to be significantly different from the hepatic MAO of the control rats; the decrease in the kidney MAO of the doubly deficient animals was highly significant compared with the control animals. The iron-deficient animals were not significantly different from the control animals.

TABLE XI

KIDNEY MAO BENZYLAMINE ACTIVITY OF IRON-DEFICIENT, DOUBLY DEFICIENT AND CONTROL RATS

Diet	21% O_2		100% O_2	P
	50 days	75 days*		
Control	0.220**	0.145	0.320	-
	0.290	0.202	-	-
Copper-Deficient	0.270	0.090	-	-
	0.240	-	-	-
Iron-Deficient	0.180	0.050	0.145	0.1
	0.185	0.077	-	-
Doubly Deficient	0.215	0.051	-	-
	0.220	0.052	0.160	0.01
		0.047	-	-
		0.045	-	-

* Duration of the deficiency

** Change in O.D. at 250 mμ/40 mg rat kidney/50 min. (pH 7.4) each value was obtained from a single rat.

Table XI also has values of MAO activity which were determined in the presence of 100% O_2 and in the presence of air (21% O_2). Both of these activities are depressed in the iron and doubly deficient animals compared with the control values.

Fig. 7 summarizes the results obtained in the second deficiency experiment for the hepatic MAO of iron and doubly deficient animals using kynuramine and benzylamine in assaying the activity.

(c) Activation Experiments with Hepatic MAO of Iron and Doubly Deficient Rats

The decrease in the activity of hepatic MAO of iron and doubly deficient rats was so striking at times that a variety of substances suspected of potential cofactor or prosthetic group potential were employed in attempting to activate these homogenates. All such compounds were prepared freshly and were incubated for 15 minutes at 37°C with the 'deficient' homogenates before the addition of substrate. Table XII includes the concentration and identity of the various suspected activators, on MAO activity which were tested with benzylamine serving as the substrate for MAO. In the air-incubation no increase in the activity of the 'deficient' homogenate was observed with the compounds tested. However, in the oxygen-stimulated incubation it seemed that FAD was indeed acting as an activator of MAO. Another experiment was therefore conducted

Legend for Fig. 7

The ordinate is % of the control animals' activity (with benzylamine and kynuramine serving as substrates).

The abscissa gives the number of days the animals were on the deficiency diet. The upper shaded area refers to the level of the kynuramine activity, while the lower shaded area refers to the benzylamine activity.

In both the kynuramine and benzylamine activities the copper-deficient animals maintained 85 - 115% of the control animal's activity.

open square - double-deficient rats

closed square - iron-deficient rats

TABLE XVI

INHIBITION BY CUPRIZONE OF RAT LIVER MAO FROM THE FOUR GROUPS OF ANIMALS OF THE DEFICIENCY EXPERIMENT

Cuprizone	0	0.3 (A)	1.2	0 (B)	1.2	0 (C)	1.2
Controls	0.310[1]	0.256 (17)[2]	0.200 (36)	0.497[3]	0.411 (18)	0.288[1]	0.145 (50)
				0.492	0.374 (22)	0.320	0.174 (46)
Doubly Deficient	0.276	0.235 (15)	0.155 (44)	0.402	0.304 (22)	0.325	0.176 (46)
						0.293	0.160 (45)
Copper-Deficient	0.310	0.276 (11)	0.200 (36)	0.467	0.371 (21)	0.268	0.151 (44)
				0.454	0.356 (22)	0.250	0.135 (46)
Iron-Deficient	0.290	0.245 (16)	0.170 (41)	0.427	0.342 (20)	0.310	0.157 (49)
						0.298	0.173 (42)

(A) Livers of the 78 day deficient animals of Experiment 1

(B) Livers of the 95 day deficient animals of Experiment 1

(C) Livers of the 28 day deficient animals of Experiment 2

1 Change in O.D. at 315 mμ/10 mg liver/20 minutes at pH 7

2 Figures in parentheses represent % inhibition

3 Change in O.D. at 315 mμ/10 mg liver/20 minutes at pH 8

(Table XIII) to verify this observation. As previously, no activation of MAO was observed in the air-incubation; however, in the oxygen-stimulated incubation, the deficient homogenates showed higher activity, in the presence of FAD.

TABLE XII

COMPOUNDS TESTED FOR A POSSIBLE ACTIVATING EFFECT OF MAO

Substance Tested	x 10^{-5}M	Incubation under	
		21% O_2	100% O_2
-	-	0.110*	0.209
Hemin**	3.5	0.060	0.145
$FeSO_4.7H_2O$	12.0	-	0.230
$ZnSO_4.7H_2O$	10.0	0.115	0.250
$CuSO_4.5H_2O$	35.0	0.125	0.175
$CoSO_4$	24.0	0.103	-
FAD	3.7	0.145	0.285

* Change in O.D. at 250 mμ/10 mg rat liver/20 minutes (pH 7.4)

** Dissolved in 25% (v/v) ethanol

As kynuramine oxidation is not stimulated in the presence of oxygen, FAD was tested only in the air-incubation. Table XIV lists the results of this experiment. None of the metals tested in combination with FAD was able to stimulate the activity of the deficient homogenates. FAD itself was also not able to increase the activity of the deficient homogenates.

TABLE XIII

STIMULATION OF HEPATIC MAO FROM 'DEPLETED' HOMOGENATES AS DEMONSTRATED BY THE OXIDATION OF BENZYLAMINE IN AN ATMOSPHERE OF 100% O_2

FAD	21% O_2	100% O_2
0	0.225*	0.315
0	0.235	-
37 µM/liter	0.195	0.380
74 µM/liter	0.200	0.325
0	0.235**	0.420
0	0.205	0.440
37 µM/liter	0.225	0.550
74 µM/liter	0.205	0.420

* Homogenate was obtained from an iron-deficient rat, units as in Table XII

** Homogenate was obtained from a doubly deficient rat

A 5% (v/v) solution of boiled whole rat blood was prepared fresh. Addition of 0.05 - 0.2/ml of this solution, to the benzylamine or kynuramine assays, failed to bring about an increase in the activity of the deficient homogenate, whether pre-incubation was conducted in air or nitrogen.

The only *in vivo* attempt to reactivate MAO was with three animals from the second deficiency experiment which were deficient for a period of 11 weeks. These animals were fed on a diet containing iron and copper for two weeks, and their hepatic MAO activity is recorded in Table XV. Although the oxidation of benzylamine returned to normal levels in these rats, the ability to oxidize kynuramine at pH 8 was significantly lower in these animals compared with the controls. At pH 7, kynuramine activity was normal in these animals.

TABLE XIV

THE EFFECT OF FAD, COPPER, AND IRON ON THE HEPATIC MAO KYNURAMINE ACTIVITY OF AN IRON-DEFICIENT RAT

Compound	$FeSO_4 \cdot 7H_2O$	FAD	$CuSO_4 \cdot 5H_2O$	Activity[1]
moles/liter x 10^{-5}	1.6	0.7	-	29.5
	2.3	1.3	-	27.0
	-	0.7	3.0	27.8
	-	1.3	3.0	26.2
	8.2	3.7	-	24.6
	1.6	3.7	-	23.8
	-	3.7	3.0	22.2
	-	-	-	29.5
	-	-	-	29.5

[1] mμ Moles 4-hydroxyquinoline/20 minutes/mg protein

TABLE XV

HEPATIC MAO ACTIVITY OF DOUBLY DEFICIENT RATS
THAT WERE FED IRON AND COPPER

State	pH	Mean	±	Standard Error	Number of Rats	P
	7	55	±	4.1	5	
Control	7.4	0.162*	±	0.025	5	
	8	86	±	2.7	5	
	7	48	±	3.3	1	0.3
Repleted	7.4	0.166	±	0.022	1	
	8	61	±	6.0	1	0.005

* Activities expressed as in Table VIII

(d) Discussion

The results of the first deficiency study showed a decrease in the hepatic MAO of doubly deficient animals compared with the controls. This decrease was not significant. For the second deficiency experiment, a more sensitive assay was employed (fluorimetric method) with which it was hoped experimental variations would be minimized. The kynuramine oxidation by rat liver was found to be significantly lowered compared with the controls, in double deficiency, when incubations were conducted at pH 7 and 8. This enzymatic activity was not significantly lowered, however, in iron-deficiency. In the kidney, the results were the reverse- significant lowering of the activity for iron-deficient animals, but not for doubly deficient animals. Copper deficiency had no effect on the activity of kidney or liver.

The oxidation of benzylamine by rat liver was significantly depressed compared with the control activities in both iron and doubly deficient animals. Although in the kidney, the decrease in activity with iron deficiency was not significant, only a small number of animals was utilized. If more observations were utilized for control benzylamine activity, the differences might become significant (if n = 7 for controls). Thus, it seems that the oxidation of benzylamine (cf. Fig. 7) was depressed to a greater extent than the oxidation of kynuramine, and that in both liver and kidney, and in both iron and double deficiencies, the benzylamine activity was significantly lower than that of the controls. In liver and kidney, copper deficiency failed to lower the benzylamine activity.

Kynuramine activity of the liver was significantly lowered only in the doubly deficient animals, whereas in the kidney iron deficiency, and not a double-deficiency, caused a significant decrease. This anomalous finding will be left unexplained; presumably it stems from the greater standard error associated with the doubly deficient animals.

The results which were similar for both benzylamine and kynuramine oxidations were: (a) copper deficiency did not affect either of these activities in kidney or liver, (b) although the severity of the depletion of benzylamine and kynuramine activity differed, the trend of doubly deficient animals having a greater significant difference than iron-deficient animals was observed for both activities, i.e., double-deficiency caused an additional depletion of enzymatic activity over that observed in iron deficiency. This might indicate that copper possibly is important for the utilization of iron incorporation into the MAO enzyme. As was pointed out in the Introduction, the importance of copper in the utilization of iron is a well recorded phenomenon.

The striking difference in the oxidation of benzylamine compared with kynuramine, i.e., the effect of oxygen, and the effect of FAD in stimulating this oxygen - stimulated reaction in iron and doubly deficient livers, (cf. Table XIII), suggests an additional pathway for the oxidation of benzylamine. Flavin, might function as a cofactor by linking the monoamine oxidase reaction in the mitochondria with some parts of the cytochrome system; this pathway

possibly functions in the benzylamine oxidation and is not important in the oxidation of kynuramine. In this light, it might be the loss of an intermediate compound(s) functioning as a cofactor which depresses the activity of benzylamine so much more than that of kynuramine.

In Section 2 of the Results, evidence was advanced for the presence of iron in the purified MAO preparations. Evidence for flavin as a prosthetic group was also advanced in that section. It was, therefore, desirable to try to reactivate MAO from 'deficient' homogenates with iron or FAD. Also, the combination had to be tested for it is possible that without iron, the FAD molecule might fail to be incorporated. These studies failed to show any stimulation of MAO from deficient animals. Possibly, the apoenzyme might not be synthesized in iron and double deficiency states.

The ability to restore benzylamine and kynuramine activities by supplementing doubly deficient rats with copper and iron was indeed a necessary requirement, in order to substantiate the role of a metal in MAO. This requirement was clearly established for the benzylamine oxidation; however, the kynuramine oxidation was not restored to normal in assays conducted at pH 8, although it was restored to normal in assays conducted at pH 7. Homologous MAO enzymes might therefore be involved, possibly some requiring longer periods of time to be synthesized when the cofactor is restored. Iron, because of its many functions in the body, might also be selectively distributed; it would be logical to assume that much of the iron would first be utilized in hemoglobin synthesis.

5. *Effect of Chelating Agents on Liver and Kidney MAO of Iron and Copper-deficient Rats*

(a) Effect upon the Liver MAO

The chelators mentioned in this section were freshly prepared and were pre-incubated with homogenates for 15 minutes at 37°C before the addition of substrate. The only substrate used in these chelator studies was kynuramine, because benzaldehyde cannot be determined in the usual manner (by its absorption at 250 mμ), as all the chelators studied absorbed strongly at this wavelength. The homogenates of the deficient animals were allowed to be repeatedly frozen and thawed to insure that the mitochondria were adequately ruptured, thereby allowing the chelators better diffusability.

Table XVI lists the results obtained using cuprizone. The inhibition of hepatic MAO by cuprizone is greatly increased if the enzyme and cuprizone are incubated at pH 7 as compared with pH 8. In the presence of 1.2 mM cuprizone, the average per cent inhibition (%I) was 41, 41, 42, and 42 for the control, doubly deficient, copper-deficient, and iron-deficient homogenates respectively (pH 7).

(b) Effect Upon the Kidney MAO

Table XVII demonstrates this same uniformity of %I over a wide range of concentrations with 8-hydroxyquinoline. Except for the lowest concentration employed, MAO from the deficient or control animals's kidney was inhibited about equally.

TABLE XVI

INHIBITION BY CUPRIZONE OF RAT LIVER MAO FROM THE FOUR GROUPS OF ANIMALS OF THE DEFICIENCY EXPERIMENT

Cuprizone	0	0.3 (A)	1.2	0 (B)	1.2	0 (C)	1.2
Controls	0.310[1]	0.256 (17)[2]	0.200 (36)	0.497[3]	0.411 (18)	0.288[1]	0.145 (50)
				0.492	0.374 (22)	0.320	0.174 (46)
Doubly Deficient	0.276	0.235 (15)	0.155 (44)	0.402	0.304 (22)	0.325	0.176 (46)
						0.293	0.160 (45)
Copper-Deficient	0.310	0.276 (11)	0.200 (36)	0.467	0.371 (21)	0.268	0.151 (44)
				0.454	0.356 (22)	0.250	0.135 (46)
Iron-Deficient	0.290	0.245 (16)	0.170 (41)	0.427	0.342 (20)	0.310	0.157 (49)
						0.298	0.173 (42)

(A) Livers of the 78 day deficient animals of Experiment 1

(B) Livers of the 95 day deficient animals of Experiment 1

(C) Livers of the 28 day deficient animals of Experiment 2

1 Change in O.D. at 315 mμ/10 mg liver/20 minutes at pH 7

2 Figures in parentheses represent % inhibition

3 Change in O.D. at 315 mμ/10 mg liver/20 minutes at pH 8

TABLE XVII

INHIBITION BY 8-HYDROXYQUINOLINE OF RAT KIDNEY MAO
FROM THE FOUR GROUPS OF ANIMALS OF THE DEFICIENCY EXPERIMENT

8-hydroxyquinoline mM	0	0.058	0.113	0.29	0.39	0.58	1.13
Control	21[1]	10 (52)[2]	9 (57)	7(67)	5 (76)	5 (76)	1 (95)
	33	23 (30)	15 (55)	12(64)	10 (70)	8 (76)	2 (94)
Iron-	31	24 (23)	14 (55)	-	10 (68)	7 (77)	1 (97)
Deficient	25	14 (44)	10 (60)	8(68)	7 (72)	5 (80)	1 (96)
Copper-	28	19 (32)	12 (57)	12(57)	8 (71)	7 (75)	1 (96)
Deficient	24	16 (33)	10 (58)	9(63)	7 (71)	5 (79)	0 (100)
Doubly	27	16 (41)	11 (59)	9(67)	8 (70)	6 (78)	0 (100)
Deficient	31	28 (10)	13 (58)	11(65)	10 (68)	8 (74)	1 (97)

Rat Kidney of animals from the second deficiency series killed at 28 days served as enzyme source

1 mμ moles 4-hydroxyquinoline/40 mg kidney/30 minutes (pH 7)

2 All figures in parentheses indicate % inhibition.

Each value listed was obtained from one animal.

(Similarly for Tables XVIII and XVIV).

TABLE XVIII

INHIBITION BY 8-HYDROXYQUINOLINE AND CUPRIZONE OF RAT KIDNEY MAO FROM THE FOUR GROUPS OF ANIMALS OF THE DEFICIENCY EXPERIMENT

Cuprizone, mM	0	0.75	1.00	0	0
8-Hydroxyquinoline, mM	0	0	0	0.058	0.113
Control	26.1[1]	20.3 (22)[2]	20.0 (23)	18.9 (28)	13.9 (47)
	25.1	19.7 (22)	20.0 (20)	18.4 (27)	13.3 (47)
Copper-Deficient	27.2	21.3 (22)	21.3 (22)	21.1 (22)	15.6 (43)
	25.3	22.2 (12)	21.3 (16)	21.4 (15)	15.1 (40)
Iron-Deficient	20.2	19.7 (3)	18.0 (11)	18.0 (11)	13.9 (31)
	22.3	19.7 (12)	18.0 (19)	17.2 (23)	13.3 (40)
Doubly Deficient	23.8	18.9 (21)	18.7 (21)	18.0 (24)	13.3 (44)
	25.4	21.3 (16)	19.8 (22)	18.4 (28)	13.9 (45)

Rat kidney of animals from the second deficiency series killed at 50 days served as the enzyme source

1 and 2 are identical in content to the numbers employed in Table XVII.

Table XVIII contains additional data on the inhibition by cuprizone and 8-hydroxyquinoline of rat kidney MAO for rats maintained on a deficient diet for 50 days. These results only strengthen the previous ones, which also did not show a difference in %I of deficient rats when compared with control rats.

Table XIX presents similar data for the liver enzyme from deficient and control rats. No difference in %I was observed with cuprizone or 8-hydroxyquinoline.

There was no difference in the inhibitory activity of 8-hydroxyquinoline and cuprizone for liver and kidney MAO; both these tissues were inhibited to an almost equal extent with these chelators. 1.2 mM cuprizone and 0.113 mM 8-hydroxyquinoline inhibited kidney and liver MAO, from the deficient and control animals, from 35 to 50%. The lower concentration of 8-hydroxyquinoline, yielded the same %I as the higher concentration of cuprizone; this trend was true for the liver and kidney MAO.

TABLE XIX

INHIBITION BY 8-HYDROXYQUINOLINE AND CUPRIZONE OF RAT LIVER MAO FROM THE FOUR GROUPS OF ANIMALS OF THE DEFICIENCY EXPERIMENT

Cuprizone, mM	-	0.6	1.2		
8-hydroxyquinoline, mM	-			0.058	0.113
Control	60	43 (28)	37 (28)	43 (28)	30 (50)
	52	38 (27)	33 (37)	35 (33)	28 (46)
Copper-Deficient	50	38 (24)	30 (40)	34 (32)	27 (46)
	51	35 (31)	28 (45)	34 (33)	28 (45)
Iron-Deficient	53	41 (23)	31 (42)	34 (36)	27 (49)
	50	38 (24)	31 (38)	39 (22)	29 (42)
Doubly Deficient	43	34 (21)	27 (37)	28 (35)	18 (58)
	43	36 (16)	28 (35)	25 (42)	- (-)

Rat liver of animals from the second deficiency experiment killed at 50 days served as the enzyme source

All numbers have the identical meanings as listed in Table XVIII.

The ratio, activity at pH 7/activity at pH 8 was calculated for the MAO of liver and kidney from control and deficient animals. Table XX contains these calculations. Although this ratio was unaffected for the liver enzyme, there was a good deal of variation for the kidney enzyme. There was a drop in the numerical value of this ratio for all the deficient animals, especially the iron-deficient animals, possibly suggesting the existence of more than one type of MAO.

TABLE XX

RATIO OF THE ACTIVITY AT pH 7/ACTIVITY AT pH 8 USING KYNURAMINE FOR THE KIDNEY AND LIVER MAO OF THE FOUR GROUPS OF RATS

	Activity at pH 8		Activity at pH 7		$\frac{\text{Activity at pH 7}}{\text{Activity at pH 8}} \times 100$	
Control	6.8 [1]	84 [2]	4.8 [1]	56 [2]	70 [3]	67 [4]
Copper-Deficient	8.2	79	5.1	51	60	64
Iron-Deficient	7.7	81	4.1	52	54	64
Doubly Deficient	7.7	67	4.6	43	59	64

These rats were killed at 50 days in the second deficiency experiment

[1] mμ moles 4-hydroxyquinoline/mg protein/30 minutes, using rat kidney

[2] mμ moles 4-hydroxyquinoline/mg liver/20 minutes

[3] ratio for the kidney enzyme

[4] ratio for the liver enzyme

6. *Absorption of Iron in Copper-deficient, Iron-deficient and Normal Rats*

(a) General Observations of Iron and Copper Deficiency in the Rat

Rats becoming deficient in iron and copper begin losing their body hair even after only two weeks on the deficiency diet. The loss of hair becomes much more pronounced as the deficiency progresses. The reddish colour of their eyes is progressively lightened, indicating anemia. In severe iron and copper deficiency the testicles of the rats are excessively enlarged and their penes are constantly erect. These observations are also true for the doubly deficient animals. Iron and doubly deficient rats, at times, have hearts which weigh twice those of the control animals. The livers of the iron and doubly deficient rats are much smaller and paler than those of the copper-deficient or control animals. The stomachs of iron and doubly deficient rats are much larger than those of normal rats.

The effect of iron, copper and double-deficiencies on the weight gains of these animals is demonstrated in Fig. 8. The smaller weight gain of the iron-deficient animals was not observed in the second deficiency experiment. These results are listed in Table XXI.

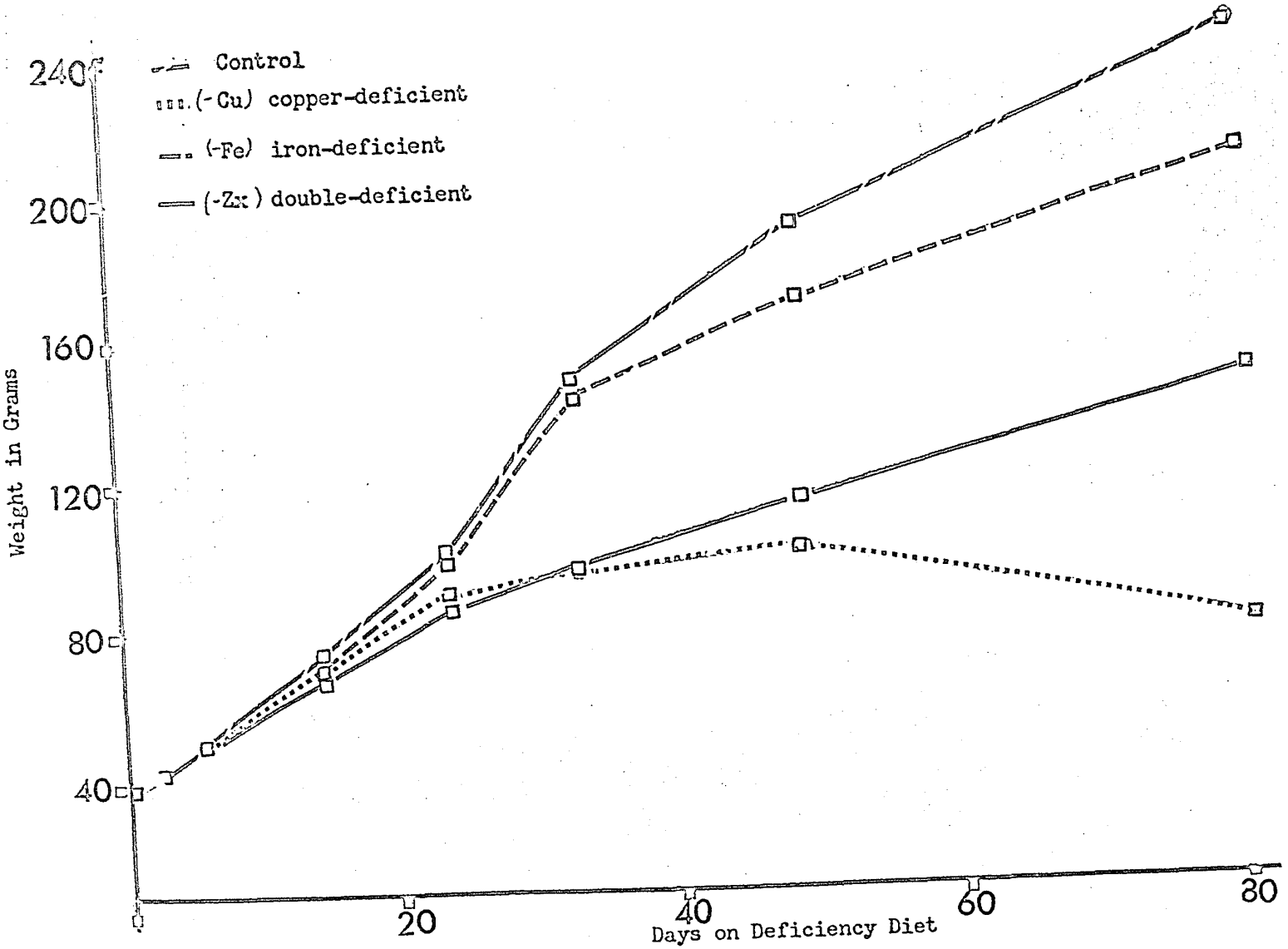


FIG. 8 WEIGHT VERSUS TIME GRAPHS FOR THE ANIMALS OF THE FIRST DEFICIENCY EXPERIMENT

TABLE XXI

WEIGHTS OF THE ANIMALS USED IN
THE SECOND DEFICIENCY EXPERIMENT

Days on Diet	28	50	75	90
Control	108*(2)**	200 (2)	358 (2)	375 (1)
Iron-Deficient	88 (2)	129 ± 3.5†(8)	182 (2)	231 (3)
Copper-Deficient	123 (2)	198 (2)	198 (2)	-
Doubly Deficient	106 (2)	134 ± 21†(14)	129 (4)	250 (3)

* Averages

** Number of animals

† S. E.

(b) Levels of Hepatic Iron in the Four Animal Groups Studied

Table XXII contains the pooled hepatic iron levels of the rats used in the first and second deficiency experiment. The hepatic iron concentration of the control, doubly deficient and iron-deficient animals remained fairly constant, from the fourth week on the various diets, till termination of the experiment. The doubly deficient animals and iron-deficient animals, however, had only 50% of the concentration of hepatic iron of the control animals. Fig. 9 illustrates the accumulation of iron in the liver of copper-deficient animals; this accumulation is directly proportional to the number of days the animal has been on the diet.

TABLE XXII

POOLED HEPATIC IRON LEVELS OF THE ANIMALS USED
IN THE FIRST AND SECOND DEFICIENCY EXPERIMENTS

Days on Diet	0	24	28	37	50	66	75 (8)**	90 (5)	109
Controls	42±1.3 (5)*	60±29 (2)	40±4 (2)	60±29 (2)	81±11.0 (2)	71±6.0 (2)	56±7.6 (4)	53±1.9 (2)	115±6.7 (3)
Doubly Deficient		36±3.6 (2)	18±2.5 (2)	30 (1)	30±2.9 (2)	21 (1)	28±2.1 (5)	34±4.1 (4)	44 (1)
Iron-Deficient		19±2.5 (2)	24±6 (2)	22±2.1 (2)	36±5.0 (2)	-	33±1.7 (4)	27±3.5 (5)	-
Copper-Deficient		57 (1)	108±16 (2)	-	196±8.7 (2)	170 (1)	210±14.6 (3)	172±13.6 (2)	281±42.5 (4)

* S.E., all values are in µg Fe/gm wet weight of perfused liver

** The values for the rats of 75 and 78 days in the two deficiency studies were pooled in this group and considered as 75 days. Similarly for the 90 day animal column.

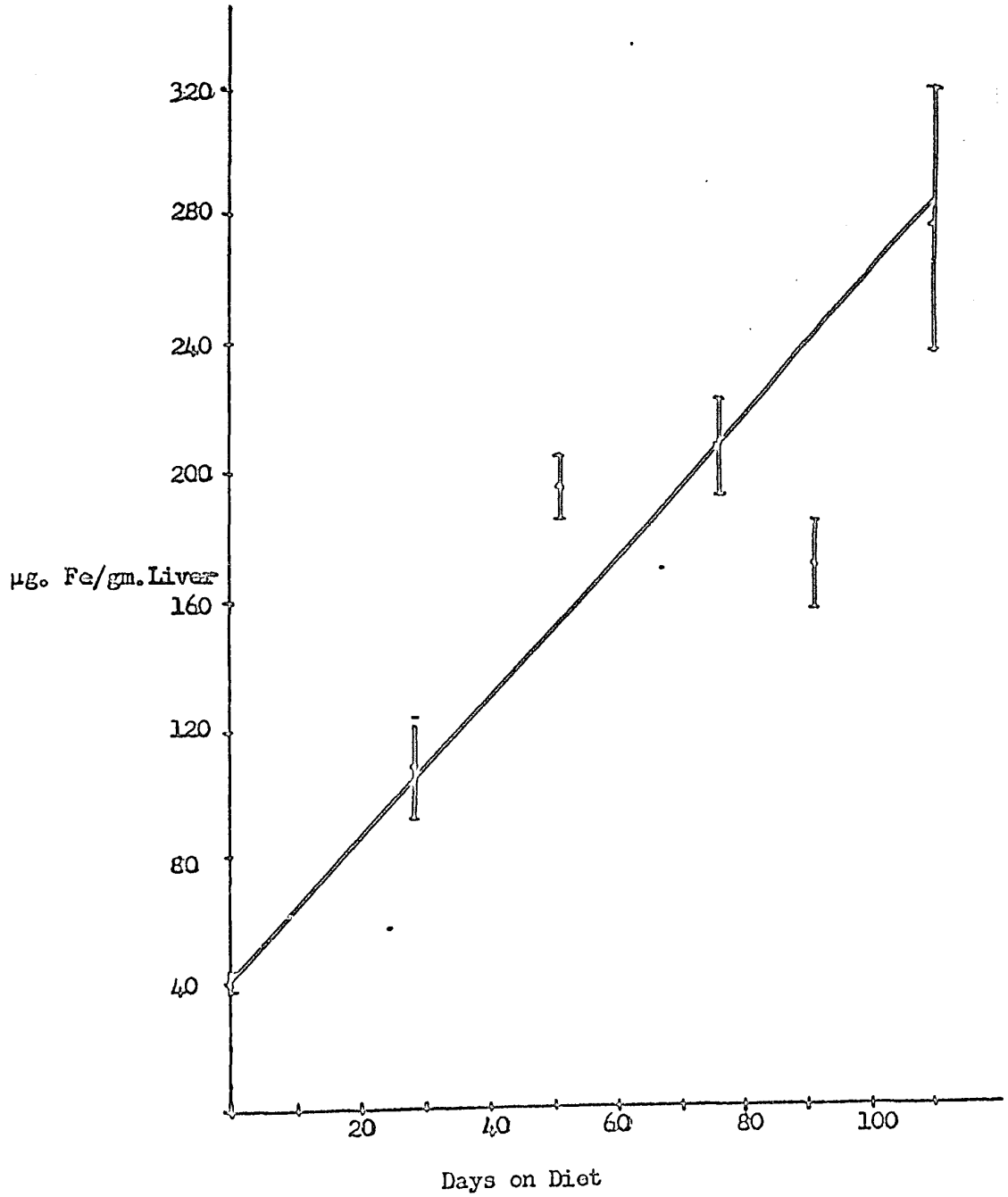


FIG. 9 THE ACCUMULATION OF IRON IN THE LIVERS OF RATS MAINTAINED FOR VARYING PERIODS OF TIME ON A DIET DEFICIENT IN COPPER

I - Standard error

The two single points represent the value of one animal

The values of Table XXII for copper-deficient animals was used to plot this graph

(c) Hemoglobin Levels

Hemoglobin values for the animals of the first and second deficiency experiments were performed and the results are listed in Table XXIII. The most doubtful significant decrease in hemoglobin concentration was that of the copper-deficient animals of the first experiment. These values were therefore utilized for calculating their significance, comparing them with the controls of that group. As can be seen in Table XXIII, the results were indeed significant.

TABLE XXIII

HEMOGLOBIN LEVELS OF THE ANIMALS OF THE FIRST AND SECOND DEFICIENCY EXPERIMENTS

	Days on Diet (Experiment 1)					Days on Diet (Experiment 2)			
	37	66	78	95	109	28	49	77	91
Controls	16.9[1]	14.5	13.5	14.4	14.0	8.8	13.0	13.8	16.0
	15.6	13.7	13.7	14.0	14.1	9.0	13.7	14.0	-
	-	-	-	-	14.7	-	-	15.2	-
Iron-	5.3	6.8	8.1	7.3	-	7.0	5.0	4.3	6.4
Deficient	8.1	5.1	-	-	-	4.3	3.9	5.9	4.0
	-	-	-	-	-	-	-	4.3	5.7
	-	-	-	-	-	-	-	4.0	-
Copper-	-	12.4	13.0	12.6	12.5	11.2	11.7	12.3	-
Deficient	-	12.7	12.8	12.3	12.5	10.6	11.5	-	-
	-	-	-	-	11.5	-	-	-	-
	-	-	-	-	11.8	-	-	-	-
Doubly	5.3	5.3	5.9	11.0	7.3	4.4	3.6	6.2	3.2
Deficient	5.3	3.6	-	-	-	6.0	2.7	6.3	3.5
	-	-	-	-	-	-	-	6.4	2.8
	-	-	-	-	-	-	-	7.0	-

1 in gm %

Controls	14.5	0.50 (11)	
Copper-deficient	12.4	0.59 (10)	$0.02 < P < 0.05$

(d) Discussion

Primarily, the level of iron (and copper) in the livers of the four groups of animals studied was of interest for correlating these values with the animal's MAO values, for if the MAO level fell in any of these groups, it would be important to show a concomitant fall in the metal suspected of causing this fall. As was demonstrated, only animals in iron- and double-deficiencies showed a lowered level of MAO, and as has just been presented, in both of these groups, the hepatic level of iron was 50% that of the controls*. Although the copper levels in the doubly deficient animals fell, no decrease was observed in the hepatic MAO of these animals.

Hemoglobin values for both deficiency experiments were obtained primarily to substantiate the presence of a deficiency of copper and iron in the animals utilized. The long recognized role of copper in hemoglobin formation was demonstrated as well.

The finding of increased levels of iron in the livers of copper-deficient rats is in agreement with the earlier work of Elvejhem and Sherman (86), who used a different experimental design; namely, a therapeutic experiment. They made animals doubly deficient by feeding them a milk diet to the point of severe anemia, and then followed the therapeutic values of copper and iron in restoring a

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* Mr. K. Lloyd of this laboratory has shown that the level of copper in the copper-deficient animals and the doubly deficient animals was 80% and 65% respectively, compared with the level of copper in the control animal's liver.

normal hemoglobin level. In my experiments with iron and copper deficiency, the design utilized allowed the single effects of either an iron deficiency, or a copper deficiency, to be determined due to their prophylactic design. An increase in the iron content of the liver of sheep grazing on copper-deficient soil has also been observed (113). Bunn and Matrone (120) have found that rats supplemented with copper, have a lower level of hepatic iron than those not receiving copper. Gubler and co-workers (87, 96, 111, 112) have not supported Elvehjem's conclusions regarding increased iron stores in copper-deficient animals (85); in fact, these workers find decreased levels of iron in the livers of copper-deficient swine. Recent work casts some reason on the anomolous behaviour of the pig. Nacht *et al.* (112) have concluded, "that the uptake of iron by the intestinal mucosa of the copper-deficient animal is normal or increased, but that the subsequent transfer to the blood or other tissue is impaired". This point of transfer, the small intestine, then is the site of the block for iron absorption in the pig; in the rat, and in the sheep, the iron is absorbed through the small intestine and the block is between the storage organs (liver and spleen) and the blood stream.

IV. GENERAL DISCUSSION

The various amine oxidases are classified at present, not only by the substrates they act upon and by the inhibitors which prevent their action, but also by the cofactors which they require. It is apparent from a review of the more recent literature that the diamine oxidase from the kidney contains copper and no FAD (28), that the purified beef and pig plasma amine oxidases contain copper and pyridoxal phosphate (24, 25), and that the mitochondrial monoamine oxidase contains FAD. The two most recent papers on the mitochondrial MAO from beef liver and kidney report 0.015 and 0.014 per cent copper respectively in the purified enzyme (132, 68), asserting the earlier observation of Youdim and Sourkes (43), who found a low copper content in the purified rat liver enzyme, 0.034 per cent. Furthermore, Youdim and Sourkes found four times as much iron in their purified enzyme as copper.

The question which workers studying mitochondrial MAO have yet to clarify is the aspect of function, that is, is the metal (copper or iron) in amine oxidase functional? The problem is a very important one, because MAO is prepared from mitochondria which contain many lipoprotein membranes capable of nonspecifically binding metals. The occurrence of a metal in purified samples of MAO does not alone indicate any catalytic function for this metal. The study of metal chelators therefore becomes important, for if specific iron or copper chelators inhibit MAO in small concentrations, then the

function of the metal in MAO becomes apparent. The work of Youdim (51), and the work reported here, certainly demonstrate the specific effects of iron chelators in the inhibition of MAO.

The design of the deficiency experiments described earlier enables the following conclusions regarding the importance of iron and copper for the activity of mitochondrial MAO. Iron deficiency lowers the hepatic concentration of MAO; copper deficiency does not cause any change in the MAO concentration of liver and kidney; a deficiency of iron and copper causes a significant decrease in the concentration of liver and kidney MAO which is more pronounced for the hepatic MAO than that occurring in iron deficiency. Before continuing this discussion, the role of riboflavin should be mentioned as this vitamin has been detected in MAO by various workers (132, 68, 51). It is the presence of riboflavin and (possibly) iron which will have to be discussed.

Hawkins' (62) nutritional studies with riboflavin-deficient rats pointed to a connection between riboflavin and MAO. After a dietary deprivation of riboflavin lasting two weeks, the hepatic levels of MAO in 'deficient' animals fell to 50% of the values of MAO levels in control animals' livers. Riboflavin, when fed to 'deficient' animals, required several days of feeding to restore normal levels of MAO in these animals' livers. In the presence of inositol, riboflavin was more effective in restoring the MAO activity to normal levels. The addition of FAD to 'deficient' liver homogenates did not result in

any activation of their MAO activity. On the other hand, D-amino acid oxidase activity was restored rapidly by feeding riboflavin or by the addition of FAD to these 'deficient' homogenates.

Ebisuzaki and Williams (133) also rendered rats riboflavin-deficient and studied the choline oxidase activity of such 'deficient' animals. After the animals had been on the deficient diet for two months, the workers observed about a 36% decrease in the hepatic choline oxidase activity of such animals, when compared with the activity of normal animals. When FAD was added *in vitro* (not FMN), the choline oxidase activity of the 'deficient' liver homogenates was greatly activated.

The latter experiment implicates FAD in the activity of choline oxidase; the former experiment does not implicate FAD in the activity of MAO, and in fact, Hawkins thought the vitamins riboflavin and inositol were essential for the biosynthesis of the MAO enzyme. It was the experiments of Distler and Sourkes (134) which suggested a possible function for riboflavin with regard to MAO activity, for they showed that riboflavin deficiency rendered the hepatic MAO more susceptible to inhibition both *in vivo* and *in vitro*. Hepatic MAO of iron and doubly deficient rats, however, was not rendered more susceptible to *in vitro* inhibition by metal chelators.

Work with the purified liver MAO enzyme showed some peculiar differences from other flavoenzymes. Nara (67) and Youdim (51) were agreed that the native enzyme exhibited no fluorescence, and that only by proteolytic digestion could a peptide be released which exhibited

the spectral properties of a flavin-like compound. Nara's and Youdim's enzyme preparations had a typical FAD-like spectrum if the oxidized minus the reduced (dithionite) curve was plotted. Nara (67, 61) has also demonstrated the disappearance of the peak at about 460 mμ when the enzyme and substrate are incubated anaerobically.

It is known that many enzymes which contain flavin hold their flavin component with varying degrees of tenacity. An understanding of the reason for such differences in the binding of flavin by various enzymes is important in understanding the cofactors of MAO.

The only metalloflavoprotein enzyme which requires enzymic digestion for the release of its flavin is succinate dehydrogenase (135). The remainder, both simple flavoproteins and metalloflavoproteins, can be induced to lose their flavin content by suitably lowering the pH of the medium and raising the ionic strength of the medium. Mahler *et al.* (136) were able to remove all the flavin from NAD (CoI) cytochrome c reductase by treatment of the enzyme with trichloroacetic acid (TCA). Moritani *et al.* (137) were able to dissociate reversibly the flavin from amino acid demethylase by the simple process of adding hydrochloric acid to pH 3.2. Upon centrifugation of the precipitated protein no activity was demonstrable. However, upon addition of FAD, 54% of the enzymic activity of the untreated enzyme was recovered. Ratner *et al.* (138) in purifying glycine oxidase, noted that during purification, the activity of the enzyme decreased. This decrease was traced to the acidic ammonium sulfate step which removed

the flavin coenzyme. Furthermore, under appropriate conditions, using TCA, the enzyme could be reversibly separated from the flavin component, *viz.*, FAD. The principles underlying the various conditions necessary for the release of flavin from a protein must ultimately stem from the nature of the bonds between flavin and protein.

In 1954, Mahler and Elowe (136) elucidated the binding sites they thought most probable for the enzyme they had then purified, NAD cytochrome c reductase. Twelve years later, Mahler (139) offers no further clues to the nature of flavin binding in flavoenzymes.

Briefly, flavin binding sites fall into six groups:

(1) Hydrogen bonding of the protein groups to the nitrogen and oxygen functions of the isoalloxazine ring.

(2) Hydrogen and apolar bonding to the ribityl side chains.

(3) Ionic interactions through the phosphate groups of the nucleotide and the cationic sites of the protein.

(4) Van der Waals forces between the isoalloxazine ring and aromatic regions of the protein.

(5) Covalent bonds between the 'free' nitrogen atoms of the isoalloxazine ring and the terminal groups of certain amino acids; covalent bonds between the hydroxyl groups of the ribityl side-chain with acidic groups of dicarboxylic amino acids.

(6) Ligand binding through a metal (copper or iron) which is already attached to the protein.

By raising the ionic strength and lowering the pH, the flavin will become protonated at the expense of bonds of types 1-4, and if these are the only bonds uniting flavin and protein, the flavin group will be released. However, if bonds of type 5 and 6 are present as well, the flavin will still be attached to the protein. Only by proteolytic digestion will the latter bonds be ruptured. This situation seems to exist for succinate dehydrogenase and monoamine oxidase, the former enzyme having been proven to contain iron, and the latter, from this author's work and that of Youdim, implicating the presence of iron.

Therefore, the metalloflavoproteins which lose their flavin readily, *viz.*, TCA treatment, do not possess a covalently linked flavin moiety. The reactivation of such depleted enzymes, either by *in vivo* depletion (deficiency diet) or *in vitro* depletion (chemical removal of the flavin or metal group), should be feasible with the proper conditions. However, the reactivation of an enzyme which is linked covalently to its flavin is not possible under *in vitro* conditions unless energy is supplied for the formation of such bonds. The case for MAO then becomes clearer; this enzyme may be a metallo-flavoprotein as are so many other of the enzymes isolated from the mitochondrion catalysing oxidation and dehydrogenation. The great number of *in vitro* reactivation experiments which were unsuccessful certainly attest to the covalent bonding of iron, flavin, and protein in some combination. Furthermore, the great specificity of riboflavin in inhibiting MAO certainly casts some light upon the nature of the

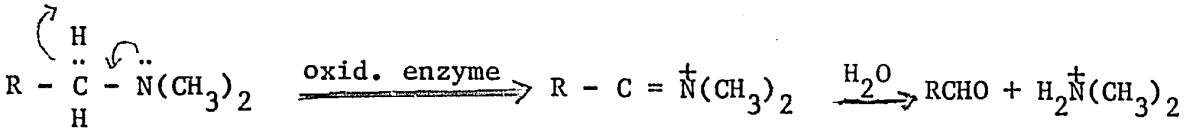
active site; a site which complements the binding sites of riboflavin, a known chelator of iron (130).

What form of iron exists in the MAO enzyme? The existence of non-haem iron (NHI), would certainly be plausible when one considers that all the metaloflavoproteins isolated to date, with but one exception, L-lactate Dehydrogenase, contain NHI (139). The inhibition of MAO by sulfhydryl reagents such as organic mercurials would certainly be understandable, as the iron atoms in NHI proteins are linked in an unusual fashion to cysteine residues, forming 'acid labile' sulfide. The spectral properties of these proteins are not unlike those reported for MAO.

The effect of copper deficiency in the iron-deficient animals utilized in my experiments was to further lower the levels of MAO in these animals' livers. Although these results are consistent with the presence of a haem group in MAO, they do not contradict the possible presence of NHI in MAO.

Smith *et al.* (140) investigated the possibility that MAO might act by a direct oxygenation mechanism. Experiments utilizing O^{18} and tritium ruled out such a possibility. Experiments with H_2O^{18} showed that the oxygen in the aldehyde came from water and not from molecular oxygen, consistent with the protonation mechanism proposed by Richter (12). These workers, furthermore, stated that "the reaction might be pictured, then, as involving removal of either a hydride ion or a comparable group by one equivalent transfers as occurs with known metalloflavin enzymes." Their original equation is reproduced

below:



(4)

Axelrod *et al.* (141) studied the level of succinic dehydrogenase in riboflavin-deficient rats. They observed that the hepatic level of this enzyme in 'deficient' animals fell to 67 per cent of the level present in control animals. Their conclusion was that "clearly one or more components of the succinoxidase system of rat livers are affected by the dietary intake of riboflavin." A similar conclusion is appropriate for the deficiency studies reported in this thesis concerning MAO. The benzylamine oxidation by hepatic MAO of iron- and doubly-deficient animals was at times reduced to nil. However, the kynuramine oxidation of hepatic MAO of iron and doubly deficient animals never fell to such undetectable levels. The possibility therefore exists of homologous MAO enzymes with varying susceptibilities to iron depletion, *in vivo*. Possibly, iron as well as FAD is required for the oxidation of some substrates, and other substrates might be oxidized slowly if the enzyme lacks iron yet still has FAD.

Answers to such queries must be obtained with purified MAO preparations. Nevertheless, *prima facie* evidence has been advanced for the presence of iron in MAO.

V. SUMMARY

The absence of an amine oxidase in rat serum and plasma capable of oxidizing kynuramine and benzylamine has been reported in this thesis for the first time.

Cofactor requirements of the rat liver mitochondrial enzyme, MAO, have been studied with the use of rats which were fed diets low in copper, iron, and both metals. Only those animals which were deficient in iron and iron and copper showed a significant decrease in their hepatic MAO activity compared with control animals. Chelator studies utilizing MAO of 'deficient' and control homogenates of liver and kidney failed to reveal any increased sensitivity of the 'deficient' homogenates to iron or copper chelators when compared with normal homogenates. However, the iron chelators were shown to inhibit the liver MAO in lower concentrations than that required for inhibition by copper chelators.

Studies with partially purified rat liver MAO have revealed that iron and covalently linked flavin are present in this enzyme.

The well recognized phenomena of increased hepatic iron stores in animals which are copper-deficient has also been reported with the use of a novel deficiency experimental design.

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APPENDIX

A third deficiency experiment, similar in design to the previous two was undertaken. In this third experiment, the levels of hepatic MAO and iron were analyzed in rats maintained for 39 days on the deficiency diet

TABLE I

HEPATIC MAO KYNURAMINE ACTIVITY

Group	Mean	±	Standard Error	Number of Rats	Significance
Control	10.9*	±	0.3	(3)	
Iron-Deficient	8.7	±	0.6	(3)	0.05>P>0.02
Copper-Deficient	10.7	±	0.3	(3)	
Doubly Deficient	8.5	±	1.0	(3)	0.05>P>0.02

* mμ Moles of 4-hydroxyquinoline formed/mg protein/20 minutes (pH 7)

TABLE II

BENZYLAMINE MAO ACTIVITY AND HEPATIC IRON LEVELS
FOR RATS OF THE THIRD DEFICIENCY EXPERIMENT

Rat No.	Group	Air Incubation	Oxygen Incubation	µg Fe/gm Liver	Total Fe/Liver (µg)
E2	Control	0.096*	0.234*	59**	395**
E3		0.086	0.221	38	333
E7		0.079	0.189	45	361
A6	Iron-Deficient	0	0.126	28	55
A3		0.039	0.192	36	118
A4		0.024	0.165	27	64
C5	Copper-Deficient	0.069	0.223	44	257
C6		0.123	0.271	88	631
D2		0.125	0.429	253	1,417
F2	Doubly Deficient	0	0.067	36	69
B1		0.066	0.149	30	78
B7		0.107	0.284	16	41

* Change in O.D. at 250 mµ/mg protein/20 minutes (pH 7.4)

** Wet (perfused) weight was used in these calculations

The results presented for the third deficiency experiment support the conclusions previously recorded for the second deficiency experiment a) Iron-deficient and doubly deficient rats have lower levels of hepatic MAO compared with copper-deficient and control rats (Table II). b) Copper-deficient rats were also shown to have increased hepatic iron levels (Table II), as was also demonstrated previously.