THE EFFECT OF HEAT, INHIBITORS AND RIBOFLAVIN DEFICIENCY ON MONOAMINE OXIDASE

By

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PREFACE

In 1928, Hare-Bernheim described an enzyme that catalyzed the oxidative deamination of tyramine to p-hydroxybenzaldehyde, hydrogen peroxide and ammonia. It was subsequently observed that this enzyme, monoamine oxidase (MAO), deaminates many amines, including adrenaline and noradrenaline to the corresponding aldehyde.

With the introduction by Zeller et al. of iproniazid, a potent inhibitor of MAO, new channels were opened to study the role of this enzyme in the metabolism and inactivation of the catecholamines. Some of these catecholamines have been implicated in mental diseases. Since then virtually hundreds of inhibitors of MAO have been discovered and due to these findings a number of applications have been made in recent years to the field of the chemotherapy of mental diseases.

Though many of these inhibitors have been very useful in chemotherapy of mental disorders, our knowledge of the nature and mechanism of action of this enzyme is still very limited. Our knowledge is limited to the recognition of certain types of compound which can act as either substrates or inhibitors. Up to now no adequate theory has been put forward as to the structure-activity relationships amongst substrates or inhibitors, - though many investigators have made speculations.

One of the most characteristic properties of this enzyme is that it is bound to the particulate fraction of the cells and is

very stable. The enzyme has defied many attempts at purification and solubilization. It is thought that the enzyme might be an integral part of mitochondrial membrane. As a result, we lack basic information as to the nature, co-factor requirements and composition of the enzyme. In recent years many investigators have pointed out that there might be more than one form of the enzyme, isoenzymes. Evidence for this has been accumulating steadily. The only positive thing that one can say about the enzyme is that it has an -SH group. Therefore, it has become necessary, if any progress is to be made, to take a new approach to the basic information we are lacking of the enzyme.

Since it was known that the MAO is rather stable on storage, it was thought worthwhile to study the process of inactivation of the enzyme by heat, and to do this at different pH's. The activity of pH curve of heat-inactivated preparations of the enzyme gave two peaks. This result led us to study the properties of these two peaks. With the use of inhibitors of MAO and chelating agents it has been possible to inhibit selectively each of these two peaks. Studies were also carried out to eliminate any artifact which might be responsible for the appearance of a second peak.

TABLE OF CONTENTS

		Page
I.	INTRODUCTION	
	A. Review of Monoamine Oxidase	
	1. Definition	1
	2. Historical	2
	3. Occurrence	4
	4. Properties	7
	5. Cofactor Requirements	10
	6. Procedures for the Measurement of MAO Activity	12
	7. Substrate of Monoamine Oxidase	14
	8. Inhibitors of MAO	17
	9. Stereospecificity of MAO	21
	10. Physiological Role of MAO	22
	B. Nature and Scope of this Investigation	27
II.	EXPERIMENTAL	
	A. Chemicals	
	1. Substrates and Non-Substrates of MAO	28
	2. Inhibitors of MAO	28
	3. Chelating Agents	29
	4. Riboflavin-Deficient and Pyridoxine-Deficient Diets	29
	B. Methods	
	1. Preparation of Rat Liver MAO	29
	2. Preparation of the Specific Gravity Gradient	30

Page

3. Preparation of Riboflavin-Deficient and Pyridoxine-Deficient Animals 31
4. Preparation of Reagents for Protein Estimation.. 31
5. Preparation of Copper-Deficient Rats 32
6. Assay for Measurement of MAO Activity 32
7. Assay for the Measurement of Activity of Partially Heat-Inactivated MAO 34
8. Lowry's Protein Measurement 34

III. RESULTS

1. The Thermal Inactivation of MAO 38
2. The Effect of pH and Thermal Inactivation 40
3. The Effect of Aerobic and Anaerobic Thermal Inactivation of Monoamine Oxidase 46
4. The Effect of Chelating Agents on the Native Enzyme 49
5. The Effect of Chelating Agents on the Partially Heat-Inactivated MAO 54
6. The Effect of Copper-Deficiency on MAO Activity. 56
7. The Effect of Inhibitors of MAO
   (a) *in vitro* 59
   (b) *in vivo* 65
8. The Effect of Inhibitors of MAO on the Partially Heat-Inactivated Enzyme 69
9. The Effect of Riboflavin-Deficiency 71
10. Sucrose Gradient Studies 78

Page

11. The Effect of Pyridoxine-Deficiency 82

12. The Effect of Carbonyl Reagents *In Vitro* and *In Vivo* 83

13. Stabilization of MAO by Substrates, Inhibitors and Other Agents 85

IV. DISCUSSION 88

V. SUMMARY 104

VI. REFERENCES 106

I. INTRODUCTION

A. Review of Monoamine Oxidase

1. Definition

Classification of the amine oxidase is at best beset with difficulties and it is important to define monoamine oxidase in such a manner that misunderstandings are reduced to a minimum. The name given to the individual enzymes depends on the type of substrates which they oxidize, i.e., monoamines by monoamine oxidase, diamines by diamine oxidase, benzylamine by benzylamine oxidase, and spermine by spermine oxidase. Since the substrate specificity displayed by each enzyme is not perfectly sharp, this classification is unsatisfactory (Fouts, Blanksma, Carbon and Zeller (1)). It has been found convenient to use the terms monoamine oxidase (MAO) and diamine oxidase (DAO), since the distinction between the two enzymes has been supported by data on the specificity of inhibitors for these enzymes. Hence it is important in the characterization and identification of these enzymes to employ criteria in addition to substrate specificity. Since in the course of many investigations on the substrate and inhibitor pattern, quantitative and qualitative differences have appeared among oxidases of different origin, the symbol MAO does not represent a single entity, but an immensely large number of closely related catalysts, Zeller (2,3). The members of this group of homologous enzymes (2), are characterized by their insensitivity towards unsubstituted acylhydrazides e.g. semicarbazide and by the same classical equation of oxidative deamination. DAO is completely in-

hibited (4), as are the other amine oxidases (5,6,7) by semicarbazide. Many monosubstituted alkyl- and arylhydrazines inhibit both DAO and MAO (8) while monosubstituted acylhydrazines and hydrazine itself, inhibit DAO (9) exclusively. Monoamine oxidase may therefore be defined as the enzyme which is responsible for the oxidative deamination of such amines as adrenaline, isopropylamine, 3,4-dihydroxyphenethylamine, serotonin, and tyramine. (Monoamine oxidase, oxidoreductase (deaminating 1.4.3.4)).

2. Historical

Schmiedeberg in 1877 discovered that benzylamine given by mouth to dogs was excreted as benzoylglycine. He recognized that this involved the intermediate formation of benzoic acid and thus furnished the first indication that amines could be degraded *in vivo* by deamination. It has since been established that benzylamine oxidase is a distinct enzyme belonging to the semicarbazide-sensitive group of oxidases.

In 1910 Ewins and Laidlaw (10) showed that when tyramine is added to the perfusion fluid of the cat or rabbit liver it could be deaminated to p-hydroxyphenylacetic acid in a 70% yield. Six years later Guggenheim and Loffler (11) in similar experiment demonstrated transformation of phenylethylamine to phenylacetic acid. Hare (12) and later Bernheim (13) described the presence of an enzyme called "tyramine oxidase" (tyraminase) in mammalian tissues. At the same time Blaschko, Richter and Schlossmann (15) and Kohn (16) described

an enzyme which was capable of oxidizing adrenaline. It was concluded independently by Blaschko's group (15), Kohn (16), and Pugh and Quastel (17) that "tyramine oxidase", "adrenaline oxidase", and "aliphatic amine oxidase" were identical. It was Zeller (2) who classified this enzyme as monoamine oxidase on the basis of what was then known about its substrate specificity.

At first it was thought that the deamination process was a result of hydrolytic action,

$$R - CH_2 - NH_2 + H_2O \longrightarrow R - CH_2 . OH + NH_3 \qquad (1)$$

the alcohol formed being further oxidized to the corresponding acid (11). It was Bernheim (13) who suggested the possibility of an oxidative pathway and formulated the reaction as

$$R - CH_2 - NH_2 + O_2 \longrightarrow R - CH = NH + H_2O_2 \qquad (2)$$

$$R - CH = NH + H_2O \longrightarrow R - CHO + NH_3 \qquad (3)$$

The above equations give a true picture of the reaction when catalase is absent. However in crude tissue preparations catalase is usually present and the hydrogen peroxide is broken down as follows:

$$H_2O_2 \longrightarrow H_2O + 1/2\ O_2 \qquad (4)$$

which leaves the overall reaction catalyzed by MAO to be

$$R - CH_2 - NH_2 + 1/2\ O_2 \longrightarrow R - CHO + NH_3 \qquad (5)$$

Therefore one atom of oxygen is used for each molecule of substrate oxidized. It has been shown by Bernheim (13) that the oxygen consumption varies from one to four atoms per mole of substrate depending on the pH of the medium, age, and concentration of the enzyme preparation. However, in the presence of cyanide only one atom of oxygen

per mole of substrate is taken up. This led many investigators (18, 19,20) to add cyanide to the incubation mixture. Creasey (21) has shown the difference in oxygen consumption to be due largely to further oxidation of the aldehyde. Most of these aldehydes, undergo spontaneous oxidation in the presence of air. He concluded that if semicarbazide and cyanide are present in the incubation mixture this would eliminate the endogenous oxygen uptake of curde MAO preparations and thus manometric measurement would be a truly quantitative process.

The formation of aldehydes was demonstrated by Richter (22) by the chemical and crystallographic analysis of the 2, 4-dinitrophenylhydrazones. Using tyramine, Richter repeated Ewins and Laidlaw's experiments mentioned earlier, and recovered the aldehyde quantitatively as the semicarbazide. It was necessary to trap the aldehyde with semicarbazide, since further oxidation to the corresponding acid occurs spontaneously. He also demonstrated that one mole of ammonia is liberated for each atom of oxygen consumed or, in the case of secondary and tertiary amines, one mole of a volatile alkylated amine.

3. Occurrence

Monoamine oxidase has been found in all classes of vertebrates tested and in many invertebrates (15), including the mollusks (23-25); it also occurs in annelids e.g. the earth worm (26,27). Blaschko (28) recently has studied the oxidase activity and its presence in the cockroach Periplaneta americana. Many investigators have also studied amine oxidase in plants. This enzyme is sensitive to semicarbazide and carbonyl reagents.

The enzyme occurs in the tissues of many vertebrates. Bhagvat, Blaschko, and Richter (29) studied MAO in many animals. They found very high activity in the livers of oxen, pigs, rats, and sheep. In all species the kidney levels were also high, with the exception of rats. Very high activity has been found also in the blood vessels, intestine, and stomach of various species. The enzyme is also found in nervous tissue, in the gonads, in smooth muscles, and in smaller amounts in cardiac muscle. The heart also contains MAO activity, but here there is a marked species differences. Other organs such as adrenals, lungs, pancreas, placenta, thyroid, spleen, and uterus all have MAO activity in the mammalian species studied. Stromblad (30) studying MAO activity in man has shown that the parotid and the submaxillary gland represent the richest source of amine oxidase encountered. MAO activity has also been found in the pineal gland, neurohypophysis and brain of albino rats by Barbara Smith (31). Walkes and Coburn (32) have demonstrated the presence of monoamine oxidase in erythrocytes; the enzyme catalyzes the oxidative deamination of serotonin. Haddox and co-workers (33) studying MAO activity in erythrocytes of rheumatics found altered MAO activity in rheumatic patients. The presence of an amine oxidase, oxidizing benzylamine, in normal human serum has been shown recently by McEwen (34), but it seems to be absent from the skeletal muscle.

The Malpighian tubule of the cockroach has the highest MAO activity in that animal. Activity has also been demonstrated in the gastric ceca, the mid and hindgut, the flight muscle, whole head, and the fat body (28).

Pugh and Quastel (14) were the first to study MAO activity in the nervous tissue. They found that the brain was very active in this respect but not as active as the liver. With the finding of MAO activity in the stellate ganglion of the dog, other investigators searched for the enzyme in other structures with adrenergic innervation. In 1952, Robinson (35) showed the presence of MAO in the nictitating membrane and iris of the cat and rabbit. The confirmation of this came as the result of the works of Koelle and his co-workers (36). This is in agreement with the assumption that substances such as tyramine and serotonin and adrenaline are protected from breakdown by the blocking of MAO by iproniazid, an inhibitor of MAO. It is interesting to note that the earlier view on the optical specificity of the enzyme is in contrast to the results obtained by Belleau, Burba, Pindell and Reiffenstein (37). They have been able to show a marked increase in the potency of sympathomimetic amines produced by stereospecific deuterium substitution, an observation which led them to postulate that monoamine oxidase may be the enzyme involved in these isotope effects. They further reported (38) the discovery that when tyramine is labeled asymmetrically with deuterium at the α-carbon position the enzyme exhibits sterospecificity. Their result indicates a three-point contact between the enzyme and substrate. They have further shown that MAO in the nictitating membrane shows the same absolute optical sterospecificity as does the enzyme in rat liver, therefore suggesting similarity between these enzymes from different tissues.

4. Properties

Of all the tissues studied, in rats the liver is the most active source of the enzyme. The most characteristic properties of monoamine oxidase is its association with intracellular organelles, the"insoluble" fraction of the tissue and its action on amines. In determining the MAO activity of various fractions of rat liver homogenate, Hawkins (39) found that about two-thirds of the enzymatic activity is present in the mitochondria and about one-third is present in the microsomal fraction. In 1951, Cotzias (40) studying the same properties, obtained results that confirmed Hawkins' findings. Blaschko (41), working with bovine adrenal medulla, has supported the above findings. He has observed that when freshly prepared particulate fraction, i.e., the particles sedimented at 15,000 XG from the supernatant of homogenate which had originally been centrifuged at about 1000 XG, is layered on a sucrose density gradient, the gradient has amine oxidase activity only in the mitochondrial fraction, the layer that contains both succinic dehydrogenase and fumarase (41). It is generally accepted that the microsomes and mitochondria account for the total activity of the liver homogenates.

With the findings of Hawkins one may ask if monoamine oxidase be considered as a single entity. Werle and Roewer (42) were able to separate enzymes from animal sources capable of oxidizing only aliphatic monoamines and only aromatic amines. They came to the conclusion that there is more than one type of MAO. Alles and

Heegard (43) in their studies tested a large number of substrates with enzyme from liver extracts of different species and found marked species differences as judged by the relative rate of oxidation.

Satake (44) has suggested that MAO may be a mixture of enzymes which have different substrate specificity and each tissue may have a distinct distribution of the mixture. The studies of Wieland (45)with lactic dehydrogenase are a constant reminder that MAO could be a mixture of enzymes which are electrophoretically distinct, similar to the isoenzymes of lactic dehydrogenase.

Gorkin (46), working with rat liver mitochondria, has been successful in partially separating two amine oxidases attacking two different amines. It seems likely that monoamine oxidase may represent a complex system of amine oxidases with relatively narrow substrate specificity (47). In another paper Severina and Gorkin(48) working with two aralkyl amines, tyramine and benzylamine, have been able to inhibit selectively the oxidative deamination of the two amines. The data obtained are explained by assuming the existence of either two different structure-bound amine oxidases in rat mitochondria or of two catalytically active sites on the enzyme. The presence of more than one enzyme has also been suggested by Barbato and Abood (49). In kinetic studies on the inhibition of the oxidation of serotonin and tyramine by rat liver mitochondria, Hardegg and Heilbron (50) have come to the conclusion that the two substrates are probably oxidized by two different MAO. Their results show that two enzymes might be responsible for the oxidation of tyramine. The activity

versus pH curve of enzyme in the presence of phenylcyclopropylamine gave a second maximum at pH 7.0, suggesting the presence of two monoamine oxidases not equally sensitive to phenylcyclopropylamine and iproniazid (49). Youdim and Sourkes (51) in their studies have shown similar results.

The evidence supporting the homogeneity of monoamine oxidase can be quoted as (a) oxidative deamination of representative substrates occurs in all tissues in which amine are oxidized; (b) when two substrates are incubated together at the same time, the oxidation rate of the two substrates is intermediate between the rates of the two amines when each is tested separately. In order to clarify the question of homogeneity, purification of the enzyme or enzymes will have to be carried out.

Ever since its discovery many attempts have been made to purify the enzyme, but up to now, no one has been successful. One of the best purification procedures is that of Ganrot and Rosengren (52). They have been able to purify the enzyme 600-fold with the use of detergent and bile salts. Acetone-dried powders of organs of some species can be prepared and stored for many months without loss of activity. Also preparations in isotonic sucrose (0.25M) can be stored at room temperature without too much loss in activity and at 0° degree for several weeks. The enzyme is inactivated at pH of 5.0 and at pH of 9-10. Davison (53) has found optimum pH for tyramine oxidation to be 7.4.

5. Cofactor Requirements

In spite of the three decades which have elapsed since the discovery of MAO relatively little is known about its chemical constitution. At present there is no evidence of a prosthetic group on MAO. The fact that the enzyme is insensitive to most carbonyl reagents seems to eliminate pyridoxal phosphate as a cofactor. Rats on a pyridoxine-deficient diet showed more or less normal levels of MAO (54). Richter (55) suggested that it may be a flavoprotein, due to the similarity of oxidative reactions of this enzyme to D-amino acid oxidase, a flavin dinucleotide containing the enzyme. Mahler (56) has included this enzyme in his classification of yellow enzymes. The first studies on the cofactor of the enzyme were done by Hawkins (57). Her experiments demonstrated that there was a decrease in the MAO activity in the livers of riboflavin-deficient rats and postulated that the vitamin is involved in the synthesis of the enzyme. Sourkes (58) has been able to confirm these results. Other indirect evidence for the role of riboflavin in the action of MAO comes from the fact that atebrin, an antagonist of flavin enzyme, inhibits the oxidation of adrenaline (59) as well as isoamylamine and tyramine (58).

In 1942, Friedenwald and Herrmann (60) suggested that MAO possesses a sulfhydryl group which is essential for activity. The enzyme was inhibited by heavy metals such as mercury, arsenic, and silver. The same investigators showed that reversal of inhibition by mercury can be achieved with glutathione and cysteine in the presence of cyanide. However, it was later shown (61) that cyanide

reacts with the organo-metallic reagent used in the above experiments and this accounted for the reactivation. The studies of Lagnado and Sourkes (62) have greatly advanced the role of an -SH group necessary for activity. They found that a number of sulfhydryl compounds such as dimercaprol, thioglycollic acid, cysteine and cystine exert inhibitory effects in vitro. This is in agreement with the fact that the sulfhydryl group functions in the oxidation of the substrate and that an excess of some -SH compound or of a disulfide could inhibit MAO at one stage of the catalytic process. The studies with sulfhydryl compounds provide strong evidence that an -SH group is essential in the activity of the enzyme.

There has been some interest in determining whether the enzyme contains a heavy metal. An extensive survey of the action of many metals on the enzyme (62) has revealed that MAO is activated by a number of ions. In high concentration the compounds inhibited MAO. Gorkin (63) has been able to inhibit reversibly MAO of rat liver and brain mitochondria by various chelating agents such as 8-hydroxyquinoline, cyclohexanediamine tetraacetate and diethyldithiocarbonate. Reversal of inhibition caused by 8-hydroxyquinoline and cyclohexanediamine tetraacetate is achieved by addition of some divalent metals, in particular the ion of zinc and copper. The author concludes that MAO has the properties of a true metalloenzyme. The inhibition with chelating agents can be reversed also by dialysis against distilled water. Green (64) studying the effect of hydrazine derivatives of MAO inhibitors, has postulated that MAO is a copper-containing enzyme

and that inhibition by hydrazine derivatives results from a copper-catalyzed liberation of free radicals in the vicinity of the enzyme's active centre. This is by no means a proof that the enzyme has copper as metal radical.

There have been suggestions that MAO, like other oxidases, may consist of a dehydrogenase linked to a respiratory chain. The only studies on this system have been carried out by Lagnado and Sourkes (65). They have shown the existence of a tetrazolium reducing system in which amines play the role of substrate. Their experiments do not permit a decision as to the nature of electron transport system. They have also shown a requirement for a heat-stable cofactor present in boiled extracts of rat brain in this system, although its identity remains unknown. Whether the enzyme has flavin nucleotide which acts as the primary electron accepter is left unanswered.

6. Procedures for the Measurement of MAO Activity

There are many methods available for the measurement of monoamine oxidase activity. The usual procedure is the manometric assay of Creasey. Any amine can be assayed by this procedure. There are a number of drawbacks in this method: (1) there is lack of sensivity; (2) unless cyanide is added, it is difficult to determine if the oxygen uptake is due to the MAO-catalysed reaction alone; (3) Aebi (66) has demonstrated that relative substrate-enzyme affinities can be varied by varying the oxygen tension. A more accurate method of assaying MAO activity is to measure ammonia liberation but this is hampered by technical difficulties.

Udenfriend (67,68) has developed several methods for specific substrates, such as tyramine and serotonin. Both of these are good substraaes of MAO. The method is based on the extraction of the amine from alkaline solution and then re-extraction into dilute HCl. The acidic solution can then be assayed spectrophotometrically, colorimetrically, or by spectrofluorometry depending on the type of amine and the sensitivity desired.

Green and Haughton's (69) assay consists of trapping the aldehyde, formed by oxidation of amine, as semicarbazone followed by conversion to the 2,4-dinitrophenylhydrazone which is then determined quantitatively by spectrophotometric means. The procedure is not suitable for large-scale incubations due to tediousness.

One of the better assays of MAO is that of Weissbach, et al. (70). The procedure consists of oxidizing kynuramine to the aldehyde, which undergoes non-enzymatic intramolecular condensation to 4-hydroxyquinoline. The reaction can be followed in a spectrophotometer either by measuring the disappearance of kynuramine at 360 mμ and/or the appearance of the product, 4-hydroxyquinoline (315 or 330 mμ).

Gorkin (71) has developed a simple, rapid and accurate method for measuring mitochondrial MAO activity. The method is based on observing benzaldehyde formation during enzymatic deamination of benzylamine at 250 mμ in a spectrophotometer.

Several authors (72,73) have developed very sensitive methods suited for studies on organs with low level of enzyme activity such

as dog's heart, or on small samples of tissues, such as sympathetic ganglion. The methods depended on the measurement of deaminated metabolites of tryptamine-indoleacetic acid.

7. Substrate of Monoamine Oxidase

The enzyme displays a limited substrate specificity; it deaminates a large variety of amines of the general formula $R - CH_2 - NH_2$ where R can be a substituted aryl or alkyl group or even an aminoalkyl chain, and it appears that it can even oxidize a greater variety of diamines than diamine oxidase itself.

The substrate specificity of MAO is affected by the presence of a second amine group as in diamines of the type $NH_2(CH_2)n.NH_2$; the affinity of the enzyme for the lower members of the series (from n=2 to n=6) is decreased. The oxidation of members having methylene groups above 6 increases with chain length. The maximum rate of oxidation is reached with n=13; beyond this, the rate of oxidation decreases with the increase in chain length (74,75,76). It is not well known what is the reason for the disturbing influence of the second amino group. It has been postulated that as the number of methylene group in the chain increases, the orientation of the diamine at the enzyme surface takes a similar configuration to that of the monoamines.

The members of the aliphatic series, $CH_3.(CH_2)_nNH_2$, are substrates for the enzyme. The lowest members are not usually attacked; methylamine is not attacked at all, whereas ethylamine is oxidized slowly by MAO. Cattle liver is an exception. The rate of oxidation

is dependent upon chain length, the reaction velocity increases to a maximum where n=4, n=5, then decreases. The straight chain amine with n=18 is not oxidized by MAO, whereas isoamylamine, a member of the branched chain aliphatic amine is oxidized readily (15,77).

Aromatic amines such as aniline and related substance are not oxidized at all (15). Benzylamine and its para-sulphamide derivative are oxidized very slowly (78) by MAO and the rate of oxidation rises markedly with the homologue, β-phenethylamine. Zeller (79) has pointed out that meta-substituted benzylamines are better substrates than the corresponding ortho- and para-isomers, regardless of the effect of the substitution on the electron distribution within the aromatic ring. The derivatives of β-phenethylamine include many "biogenic amines", such as tyramine, dopamine and noradrenaline. They have become classical substrates.

Randall (80), studying progressive N-methylation, found in a series of substituted phenylethylamines that the primary amines were oxidized more rapidly than tertiary or secondary amine. It has also been shown that if N-substituent increases in size, the rate of oxidative deamination is much lower. Quaternary ammonium compounds are not degraded, though it has been shown that an N-oxide can be oxidized (81).

The phenolic derivatives include compounds which are the most important naturally occurring substrates. These include derivatives of phenethylamine, tryptamine, and histamine. Tyramine, serotonin, 3-hydroxytyramine (dopamine), norepinephrine, and epinephrine

belong to this group. Para-hydroxylation of phenethylamines leads to compounds becoming more readily oxidizable than the parent compound and the meta-substituted analogue is better than the ortho-substituted compound (82). The ortho-methoxy compounds are not as readily oxidizable as the para- or the meta-methoxy compounds. Randall (80) has suggested that chelation with a metal occurs between the amine and ortho-hydroxyl groups, thus interfering with complex formation between the metal, and methylation would inhibit this interaction thus facilitating the formation of substrate-enzyme complex. On the other hand, methylation of the 3-hydroxy group in adrenaline or noradrenaline has no effect on the oxidation rate of metanephrine or normetanephrine produced (76). Hydroxylation of tryptamine at 5 position results in the formation of serotonin which is a good substrate. Substitution at 6 or 7 position produces compounds which are oxidized at a slower rate than serotonin (83). Para-hydroxylation of benzylamine gives results opposite to those in the phenethylamine series (84). A lower affinity for the enzyme results with an increase in the length of the side chain of phenethylamine to phenylpropylamine or phenylbutamine (85). However kynuramine is a good substrate but reduction of the keto group to an alcohol results in much slower oxidation.

Zeller has published many papers (85,86,87,88) on substrate-activity relationships and mechanism. He has emphasized the importance of a "phenethylamine backbone" and in order for the amine to act as a substrate, the presence of two α-hydrogens are required.

Considering that isoamylamine and kynuramine are good substrates whereas mescaline is a poor substrate, Zeller's postulation of "phenethylamine backbone" seems unlikely as a primary requisite for a compound to act as a substrate. It is also objectionable to postulate that a hydrogen α to the amino group of a substrate or inhibitor can enter into covalent bond formation with an ill defined active site during the process of binding on to the enzyme (19). Belleau, Fang, Burba, and Moran (89) using R and S-α-d tyramine clearly have established that the two α-hydrogens are not equivalent for the enzyme. They explain this result by assuming a three-point contact between substrate and the enzyme.

8. Inhibitors of MAO

As already mentioned in the preface, the inhibitors of MAO play an important role in the chemotherapy of mental diseases. The subject has been reviewed by Zeller (90). The inhibitors of MAO can be grouped in two classes:

(a) Reversible Inhibitors.

Amines in which the α-carbon bears a methyl group are not oxidized by monoamine oxidase, but may act as a competitive inhibitor of the enzyme and the inhibition is readily reversible. Such is the case with β-phenylisopropylamine (amphetamine) (91), ephedrine (92), and 2-amino-4-methyl n-butane (93). Blaschko has pointed out that amphetamine is a better inhibitor than ephedrine. The general formula for the side chain of the inhibitor is $-CH_2-CH(NH_2)CH_3$ and is

valid for other amines e.g. 5-hydroxyindole series. As in the case with the substrates β-hydroxylation lowers the affinity for the enzyme. Others in the class of reversible inhibitors include such potent inhibitors as harmaline (94), and harmine (95).

Blaschko and Duthie (74) reported that mono and diguanidines, diisothiourea derivatives, and mono and diamidines also inhibit the enzyme. Octanol (96), cocaine and related anesthetics (97), and 2-aminocyclohexyl-p-tolyl ether (93) represent a variety of inhibitors. Another inhibitor which is of great importance is quinacrine (atebrin). Inhibition by this compound can not rule out the presence of riboflavin prosthetic group. As mentioned earlier many chelating agents such as 8-hydroxyquinoline, o-phenanthroline, diethyldithiocarbamate (63), and thenoyltrifluoroacetone (51) are reversible inhibitors. Inhibition by 8-hydroxyquinoline has been shown to be competitive (64). However it should be noted that many of the above compounds have limited or no inhibition <u>in vivo</u> (98).

(b) Irreversible Inhibitors.

With the discovery in 1952 by Zeller and his group (99) of a potent MAO inhibitor, iproniazid (1-isonicotiny-2-isopropyl-hydrazine) there revived a great interest in monoamine oxidase inhibitors as pharmacological agents. Pletscher's monograph on "monoamine oxidase inhibitors" (100) includes over a thousand references. Zeller's group (101) have established that MAO is inhibited by compounds possessing the structure =N-NH.R, while diamine oxidase is inhibited by hydrazines with the general structure = $N-NH_2$. A few hydrazines inhibit both enzymes effectively such as phenylhydrazine.

The mechanism of MAO inhibition by hydrazines is not well understood. It was thought that before iproniazid can act it is converted into an effective compound, isopropylhydrazine. It was Seiden and Westly who demonstrated the conversion of iproniazid to isonicotinic acid (102). Isocarboxazide 1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl) hydrazine; Marplan a hydrazide inhibitor is converted to benzylhydrazine.

Hydrazine inhibitors have aroused the greatest interest in a symposium (103) on MAO; hundreds of new MAO inhibitors were discussed. Green (104) has investigated benzylhydrazine and phenylethylhydrazine and some of their derivatives and could not arrive at any generalization in the effect of structure on the inhibitory potency of either the arylalkylhydrazines or the hydrazide. Biel and his group (103) have arrived at the same conclusion. Making substitution on the molecule (104) tends to vary the inhibitory effect. The inhibitory power was reduced when the phenyl group is loaded with a large substituent. The substitution of one of the hydrogen atoms attached to the α-carbon has the same effect. Substitution on the phenyl ring with a single small radical group has little effect and slightly increases the inhibitory activity. In order to get a marked inhibitory effect, it is necessary for the enzyme to react with the inhibitor for some time before substrate is added. It has been shown by Davison (105) that there is a decrease in the extent of inhibition when the enzyme and inhibitor were incubated in the presence of nitrogen instead of oxygen. Schwartz has been able to confirm this for iproniazid but not with isopropylhydra-

zine or isocarboxazide (106). Studies with other hydrazines have given similar results to that of Davison. It was observed that some of the compounds studied (107) display profound organ specificity which lends support to the previously mentioned isoenzyme suggestion (cf. page 8).

Maass and Nimmo (107) were the first to report the in vivo and in vitro inhibitory action of 2-phenylcyclopropylamine (Parnate) first synthesized by Burger and Yost (108). In their studies they reported a non-competitive inhibition of rat liver MAO, and a 50% inhibition was obtained at a concentration of 2.8×10^{-6}M. This represents an eleven hundredfold increase in potency over amphetamine and iproniazid respectively. Since then many investigations have been carried out to search for new non-hydrazine inhibitors. It was reported by Zeller (109) that cyclopropylamine is inactive whereas N-methyl and N-dimethyl-2-phenylcyclopropylamine are active (110).

Pargyline (N-benzyl-N-methyl-2-propylamine) was first reported at the Federation Proceedings in 1959 by Taylor and Wykes (111) as an inhibitor of MAO. The authors indicated a 50% inhibition of MAO at a concentration of 9×10^{-7}M, in the same test system, iproniazid inhibited at a concentration of 7×10^{-6}M which is several times lower than usually observed. In another paper (112), these authors state that their compound is seven times as potent as iproniazid whereas 2-phenylcyclopropylamine is 1000 times as potent as determined by Ozaki (95). In a recent paper Swett et al. (113) have studied the effect of various groups on the inhibitory strength of

the molecule. Halogenation of the benzyl radical at ortho or para position is the most effective in increasing the inhibitory activity. Ortho- and para-hydroxylation and alkoxy derivatives cause a decrease in the *in vivo* inhibitory activity.

Recently Ozaki *et al.* (95) reported a thorough investigation of over 80 compounds for *in vitro* inhibition of MAO but they were unable to establish structure-activity relationships. The authors demonstrated that adrenaline inhibited serotonin oxidation whereas Blaschko previously showed that this compound is a substrate (15). One could understand this more fully if one considers that a compound with high affinity for the enzyme, but not oxidized at a rapid rate, can nevertheless act as a substrate competitor for another compound whose affinity and oxidation rate are high.

9. Stereospecificity of MAO

One of the most characteristic properties of the enzyme is the relative lack of optical specificity, in addition to the lack of substrate specificity. The enzyme does not distinguish completely between the inhibitors *d* and *l*-amphetamine (114) or between *cis* and *trans*-2-phenylcyclopropylamine (109). But sometimes a striking stereospecificity is observed as in the case of L-alanine isopropylamine, a very active inhibitor of MAO, whereas the *D* form is almost inactive. However, with serine derivatives stereospecificity is much less pronounced and with the leucine and the phenylalanine isorpopylhydrazine is absent (90). It should be noted that the results obtained with

the above hydrazides are from studies *in vivo*; they are in contrast to the results with amphetamine and phenylcyclopropylamine which are based on *in vitro* studies. It is possible that the *in vivo* stereospecificity can be attributed to selective transport mechanisms or more probably to the specific hydrolysis of only one optical form of derivatives of phenylcyclopropylamine and amphetamine prior to the establishment of the MAO inhibition.

From the above consideration, it is obvious that the stereospecificity reported by Belleau is not an absolute one.

Belleau's preliminary *in vivo* studies (37) have demonstrated a marked increase in the potency of sympathomimetic amines produced by stereospecific deuterium substitution on the α-carbon of tyramine. This suggested that MAO may be the enzyme responsible for oxidation of the substrate even though it is symmetric in its natural form.

10. Physiological Role of MAO

With the discovery of potent inhibitors of the enzyme, new channels were opened to investigate and gain an insight into the role of the enzyme in the physiological processes. It has been stated that the enzyme plays an important role in the function of the brain. Biologically active compounds such as adrenaline, noradrenaline, serotonin, and dopamine are said to be neuro-hormones, formed by enzymatic reactions. It has been shown that these amines are stored in inactive form in the subcellular particles of nervous tissue. Such amines when released into the circulation cause profound pharmacological

effects which would be drastic if the excess is not metabolized. Possibly it is for this reason that an enzyme such as MAO capable of metabolizing the amines is provided.

There have been many hypotheses as to the role MAO plays in the detoxification of amines. Blaschko (93) finds fault with the suggestion (2) that MAO is responsible for the detoxification of high concentration of amines of bacterial origins in the intestine. He says it is very unlikely that the amine concentration could be so high as to require the amount of enzyme actually present. A "hypertensive crisis" in patients due to the interaction of cheese and certain MAO inhibitors was first reported by Blackwell (115). This crisis occurs only with certain inhibitors such as tranylcypromine (Parnate) and substances related structurely to amphetamine. Asatoor et al. (116) demonstrated that there is a large quantity of tyramine in certain cheeses. Under normal conditions the tyramine is oxidatively deaminated either in the intestine or after absorption. In his studies Blackwell, (117) supported by pharmacological and chemical data, suggested that the substance which causes "hypertensive crisis" was tyramine. Natoff (118) has supported these finding with his studies on the cat. It has been pointed out by Davison (53) that the gut also has high concentration of serotonin (5-hydroxytryptamine) and because of its implication in intestinal mobility, he suggests that MAO is responsible for regulating the concentration of this amine. Interest in the role of monoamine oxidase in the metabolism of the brain serotonin has been stimulated by the findings that inhibitors of this enzyme can increase the level of amine in the brain (119). It is known that serotonin is a good substrate for MAO both in vivo and

in vitro (120). It is of interest to note that Bogdanski and Udenfriend (121) have shown that the distribution of serotonin parallels that of MAO in the dog and cat brain whereas the distribution of catecholamines and MAO in bovine adrenal medulla is different (41). Catecholamines are said to be present mainly in the lower fractions of sucrose density gradients and the enzyme in the upper fractions. Serotonin can be released from its bound form by administration of reserpine thus making the amine available to MAO. Recent studies of Spector (122) have shown that MAO plays an important role in regulating the noradrenaline and serotonin content of subcellular particles. As serotonin is released there is rapid depletion of the "active" amine and this has led Brodie *et al.* (123) to postulate that, just as cholinesterase is responsible for inactivating acetylcholine, monoamine oxidase may play a similar role in destroying an excess of/a neurohormonal transmitter such as serotonin.

There has been a great deal of controversy as to the role of this enzyme in adrenergic mechanism. Both adrenaline and noradrenaline are good substrates for MAO *in vitro*; however there has been some doubt whether monoamine oxidase is the chief physiological inactivator of adrenaline and noradrenaline. Evidence supporting the role of MAO in inactivation of the catecholamines came from the work of Schayer *et al.* (124,125). It was demonstrated that as much as fifty percent of injected C^{14}-adrenaline is metabolized by MAO in rats and could be recovered in the urine. It was concluded that MAO plays a major role in the metabolism of adrenaline. Increase in the level of noradrenaline by inhibition of MAO was shown by Shore *et al.* (126).

Armstrong et al. (127) and later Axelrod (128) and D'Iorio (129) showed that adrenaline, noradrenaline, and dopamine are metabolized by O-methylation and could precede deamination. O-Methylation is now known to be the major pathway of the metabolism of catecholamines. Kopin (130, 131) demonstrated that about 25% of epinephrine is deaminated while about 66% is O-methylated. In a recent paper Kopin and Axelrod (131) have studied the role of MAO in the metabolism of norepinephrine. Evidence is provided to indicate that to a large degree MAO plays a role in the metabolism of more firmly bound stores of norepinephrine whereas norepinephrine released in active form by nerve stimulation is not inactivated by monoamine oxidase. Griesemer et al. (133) found that inhibition of MAO by iproniazid potentiates the response of the nictitating membrane to tyramine but not to adrenaline. This was one of the first important pieces of evidence that MAO did not act directly on the catecholamines under physiological conductances. Belleau, et al. (37) using the nictitating membrane have demonstrated that noradrenaline is not degraded by MAO at the adrenergic effector cell level and they have suggested that the role of the enzyme in adrenergic mechanism can best be described as a protective device for the inactivation of circulating endogenous non-transmitter substances.

Lately Gertner (134) has shown that representative MAO inhibitors block ganglionic transmission in the isolated superior cervical ganglion of the dog. He suggests that the blocking activity is directly related to the MAO-inhibitory activity of these compounds. The explanations put forward for this property are that an active

amine in accumulating at the ganglionic synapse or the normal function of the neuromediator at the ganglionic synapse is interfered with. Implications are that other agents beside acetylcholine are responsible for ganglionic transmission.

Many investigators have pointed out that in the case of lack or dysfunction of MAO, other pathways of amine metabolism could lead to formation of highly active substances capable of producing drastic effects, such as mental abnormalities and other diseases. The evidences for these are obtained from the work done using MAO inhibitors mentioned in the previous sections.

With the highly active and concentrated MAO found in the liver, an important function is assigned to the enzyme, that of destruction of biologically active amines in the circulation. Dawson and Sherlock (135) examining patients with liver diseases characterized by high blood ammonia levels found that iproniazid is able to lower the blood ammonia level effectively.

In conclusion the lack or dysfunction of MAO has been implicated in a number of mental disorders and other diseases. With the use of inhibitors it has been possible to answer the role MAO plays in inactivation of certain physiologically active substances. The above discussion leaves many questions unanswered. But some of the questions as to its role in the metabolism of noradrenaline in the brain, of O-methylated catecholamines, and of metabolism of amines (e.g. of dietary origin) w e r e answered.

B. Nature and Scope of this Investigation

As mentioned earlier in the preface, we lack much basic information such as the nature, cofactor requirements and the composition of the enzyme, whether the enzyme has a metal associated with it and whether an -SH is necessary for the activity. In order to clarify some of these questions, it has become necessary to study these properties from a different point of view.

We have taken advantage of the fact that the enzyme is rather stable to heat. The enzyme stored at zero degree can be kept for 2-3 months without much loss in activity. Heat inactivation studies have shown that the enzyme is irreversibly denatured. Some investigators have suggested that there might be more than one form of monoamine oxidase. Recent studies have shown that the activity-pH curve has a shoulder, suggesting that there might be more than one monoamine oxidase. If this is so, the activity-pH curve of the partially heat inactivated enzyme might show different properties at different pH's.

This investigation consists of studies with chelating agents, inhibitors, riboflavin deficiency, pyridoxal deficiency on the pH-activity curve. Also studied was the stabilization of the enzyme to heat by inhibitors and substrates of MAO.

Using these techniques, it is hoped that subtle differences will provide an interpretation as to whether there is more than one form of monoamine oxidase in the mitochondria.

II. EXPERIMENTAL

A. Chemicals

1. Substrates and Non-Substrates of MAO

Kynuramine dihydrobromide was obtained from Regis Chemical Company, Chicago, Illinois. Ethanolamine was obtained from The British Drug Houses Ltd., Poole, England. Epinine hydrochloride/histamine was obtained from Burroughs Wellcome and Co., dihydrochloride iso-amylamine, iso-butylamine, and benzylamine were obtained from Fisher Scientific Co. Ltd., Montreal. 1,4 Diaminobutane was purchased from Krishell Laboratories Inc., Portland 2, Oregon, U.S.A.

2. Inhibitors of MAO

Harmaline and quinacrine (di) hydrobromide (Atebrin) were obtained from Mann Research Laboratories, New York 6, N. Y. Phenylcyclopropylamine sulfate (Parnate), S.K.F. 385. A (Trans) was obtained from Research Laboratories, Smith, Kline and French Labs., Philadelphia. Iproniazid (Marsilid) was obtained from Hoffmann-La Roche Limited, Montreal. Catron, 2-phenylisopropylhydrazine was purchased from Schering Corporation, Montreal. Mk 485/hydrazino analogue of α-methyl-dopa was obtained from Merck Laboratories, West Point, U.S.A. Pargyline (MO 911) was obtained from Abbott Laboratories, North Chicago, Illinois, U.S.A. Aminoguanidine sulfate was obtained from Eastman Organic Chemicals, Distillation Product Industries, Rochester 3, N. Y. Hydrazine sulfate and semicarbazide hydrochloride were purchased from Fisher Scientific Company, Montreal.

Isoniazid was obtained from Delmar Chemicals, Bulbocapnin HCl. was purchased from L. Light and Co. Ltd.

3. Chelating Agents

Thenoyltrifluoroacetone, o-phenanthroline, α-dipyridyl, and sodium diethyldithiocarbamate were obtained from Fisher Scientific Company, Montreal. D-penicillamine hydrochloride.$1/2H_2O$ from California Corporation for Biochemical Research, Los Angeles, 63, U.S.A.

4. Riboflavin-Deficient and Pyridoxine-Deficient Diets

Were purchased from General Biochemicals, Chagrin Falls, Ohio.

B. Methods

1. Preparation of Rat Liver MAO

Livers were obtained from adult male rats of the Sprague-Dawley strain. The livers were chilled over cracked ice immediately upon removal and forced through a Latapie mincer to remove connective tissue. The liver pulp was weighed and homogenized in a small volume of ice-cold 0.3M sucrose solution in a glass homogenizer with Teflon pestle for 5 minutes at one-minute intervals to allow cooling. The final volume of the homogenate was adjusted so that 1 gm. of tissue was present in 10 ml. of homogenate. This was called a 10% homogenate preparation. Some homogenate was retained for measurement of MAO activity.

(a) Centrifugation Procedure

The method is essentially that of Hawkins (39). The homogenate in 0.3M sucrose was centrifuged at 2000 RPM (580Xg) for 20 minutes to sediment unbroken liver cells, large cell fragments, nuclei, and red blood cells. The sediment was washed once by resuspending it in a 0.3M sucrose solution up to the original volume and by re-centrifuging it at the same speed for 10 minutes. The final sediment was resuspended in the original volume and labelled "the nuclear fraction N."

The supernatant contained the soluble cell components and the particulate elements, mainly mitochondria and microsomes. This was labelled "the first supernatant S^{I}." Some was retained for measurement of MAO activity, the rest was subjected to high speed centrifugation at 11,000 RPM (14,000Xg) for one hour. The sediment was suspended in the original volume to give a 10% preparation and labelled "the particulate fraction P," and the supernatant "the cytoplasmic fraction labelled S^{II}."

2. Preparation of the Specific Gravity Gradient

Tubes were prepared by carefully layering one ml. of each of sucrose solutions in the order of 1.7M, 1.6M, 1.5M, 1.4M and 0.5 ml. 1.3M with the most concentrated solution at the bottom of the tube. The tubes were prepared 12-24 hours before use and held at $0^{o}C$. Just before centrifugation each tube was layered with 0.5 ml of a suspension of large granules, i.e., mitochondria that had been freshly

prepared and washed in 0.3M. sucrose. The tubes were then centrifuged in the Spinco preparative ultracentrifuge, using the Spinco swing-out rotor SW 39L, at 40,000 RPM for 2.5 hours. At the end of this time the position of the layers formed were noted and fractions collected by micro-pipettes. The collected fractions were then frozen and kept at about -4°C until determinations were carried out.

3. Preparation of Riboflavin-Deficient and Pyridoxine-Deficient Animals

Two groups of adult male (Sprague-Dawley) rats were fed commercial riboflavin-deficient and pyridoxine-deficient diets, respectively. At the end of one week the rats of each group were divided into two groups. The control groups received 30 mg. per kg. of diet of riboflavin-5-phosphate dissolved in 5% sucrose solution or a similarly prepared solution of pyridoxine hydrochloride. The experimental groups in both cases received an equivalent amount of 5% sucrose solution. At intervals rats from each group were killed; their livers were removed, mitochondria were prepared, and MAO activity was measured.

4. Preparation of Reagents for Protein Estimation

Reagent A, 2% sodium carbonate in 0.10 N sodium hydroxide. Reagent B, 0.5 per cent copper sulfate (hydrated) in 1 per cent sodium or potassium tartate. Reagent C, alkaline copper solution was freshly prepared. Mix 50 ml. of Reagent A with 1 ml. Reagent B. Discard after one day. Reagent D, carbonate-copper solution is the same as Reagent C except for omission of sodium hydroxide. Reagent E, diluted Folin reagent. Folin-Ciocalteu phenol reagent diluted 2-fold to make it 1 N in acid. Working standard prepared from human serum albumin

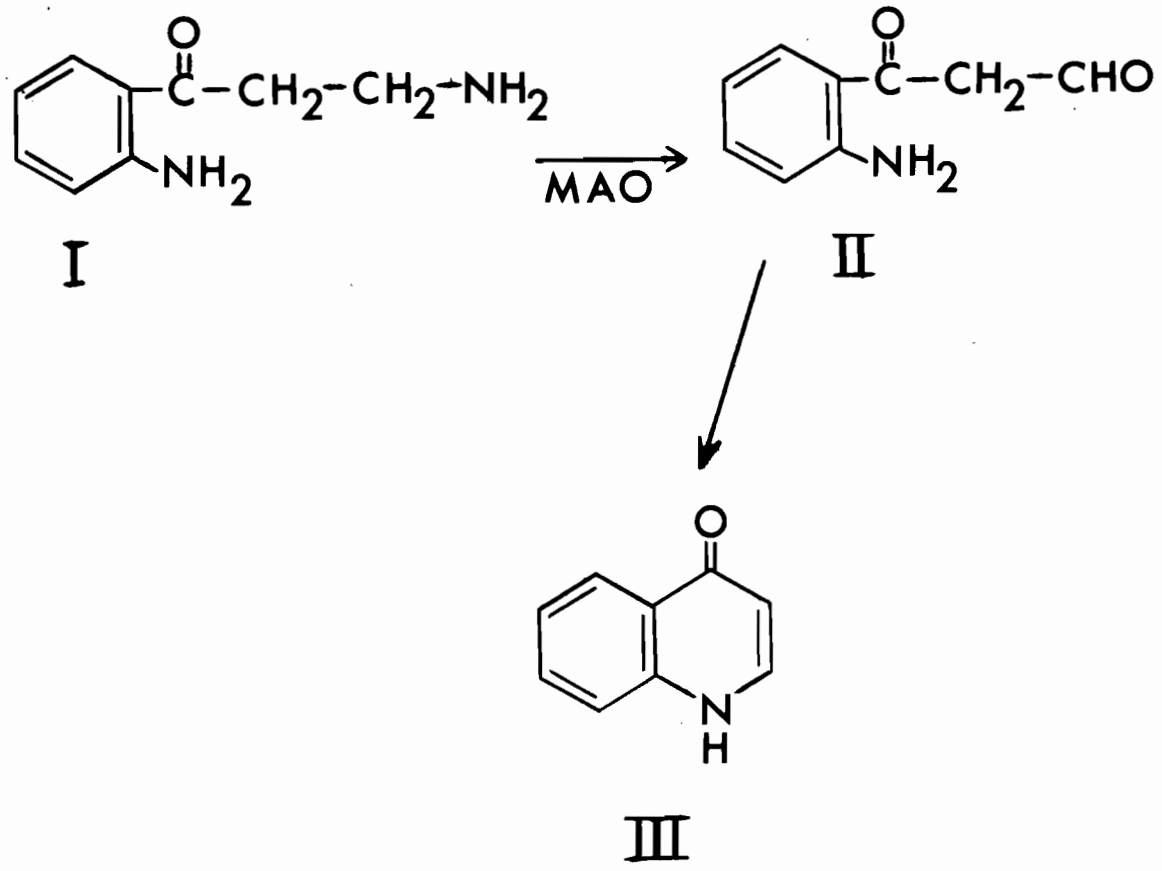
400 γ per ml.

5. Preparation of Copper-Deficient Rats

Young male rats on the deficient diet were divided into two groups after 8 days. The control group received a daily supplement of copper in addition to the daily diet. At periods, rats were killed, their livers removed and MAO activity was measured. The daily copper-deficient diet was composed of evaporated milk (Farmer's Wife) diluted with distilled water in a ratio of 1:1. Each group also received a daily supplement of vitamins ("Ostico" drops). 0.1 ml. of the solution was diluted in 100 ml. distilled water and 0.1 ml. of this solution was given daily to each rat. The composition of the daily supplement of vitamins consisted of Vit. A 10,000 i.u., Bit. D 1,600 i.u., Vit. C 120 mg., Vit. B_6 2.5 mg., Vit. B_1 4 mg., Vit. B_2 2.4 mg., Niacinamide 16 mg., and Na I 0.16 mg/ml. In both the deficient and the control group, each rat received 50 μg Mn^{++} and 400 μg Fe^{+++}. The control group in addition received a supplement of 50 μg Cu^{++} each day.

6. Assay for Measurement of MAO Activity

The method of Weissbach et al. (70) was used. The substrate, kynuramine (I) is oxidatively deaminated by the enzyme to the aldehyde (II) which undergoes intramolecular (non-enzymatical) condensation to 4-hydroxyquinoline (4(1 H)-quinoline, III). The rate of activity can be measured by the disappearance of kynuramine at 360 mμ or appearance of 4-hydroxyquinoline at 315-329 mμ in a spectrophotometer.



All incubations were carried out in 20 ml. beakers. The experimental mixture contained 0.2 ml. 10% mitochondrial enzyme preparation, kynuramine 6.2×10^{-5}M final concentration, 1.0 ml. of phosphate buffer 0.031M final concentration, and water to a total volume of 3.2 ml. A blank was prepared in the same manner, except that kynuramine was omitted. All beakers were preincubated for 5 minutes and a further 20 minutes after the addition of the substrate at 37 C. An initial reading is made at 315 mμ.

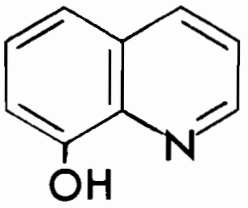
7. Assay for the Measurement of Activity of Partially Heat-Inactivated MAO

For most of the work the enzyme preparation, contained in thin uniform walled test-tubes stoppered with rubber bungs, was immersed in a constant temperature bath, using a "Bronwill" Constant Temperature Circulator. At suitable intervals 0.5 ml. aliquots were removed and immediately placed in 25 ml. beakers and chilled in ice water until activity determined. Then one added 0.5 ml. kynuramine 1.6×10^{-4}M final concentration, 1.0 ml. of phosphate buffer 0.031M final concentration, and water to a volume of 3.2 ml. Blanks were prepared as mentioned previously. Incubations were carried out as mentioned in the previous section.

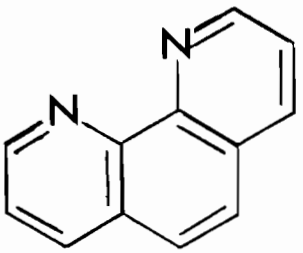
All reactions were terminated in the beaker by adding 1.0 ml. 5% zinc sulphate.

8. Lowry's Protein Measurement

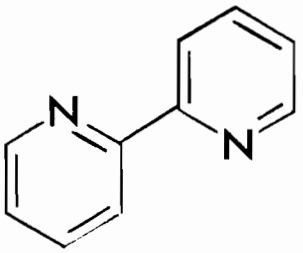
The procedure used was a modification of that of Lowry (136). 0.2 ml. of a solution containing 5-100 γ of protein is added to 1.0 ml. of Reagent C in a 10 ml. test-tube, mixed well and allowed to stand for 10 minutes. 0.1 ml. of Reagent E is added very quickly and mixed as soon as possible. The tubes are left to stand for 30 minutes and then read in a Coleman colorimeter at 690 mμ. A blank containing everything except protein is also prepared. With each estimation a standard curve of human serum albumin 400 γ per ml. is prepared from which the amount of protein can be calculated.



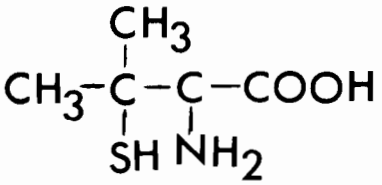
8–Hydroxyquinoline



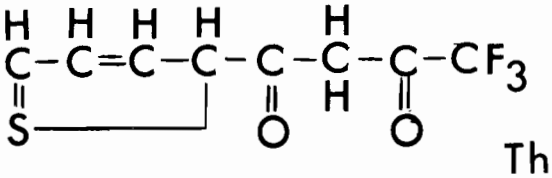
o–Phenanthroline



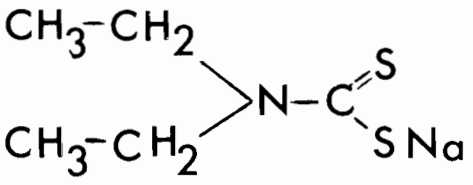
2,2–Dipyridyl



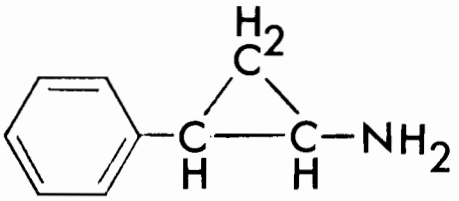
D–Penicillamine



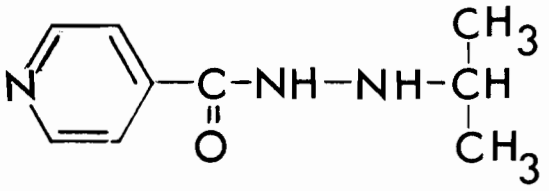
Thenoyltrifluoroacetone



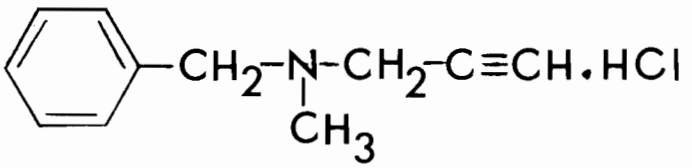
Sodium Diethyl-dithio-carbamate



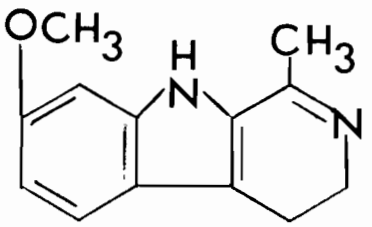
Phenylcyclopropylamine



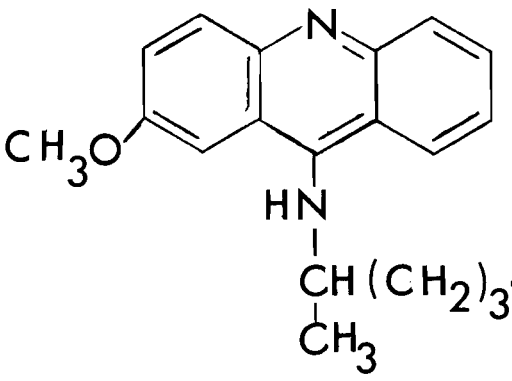
Iproniazid



Pargyline

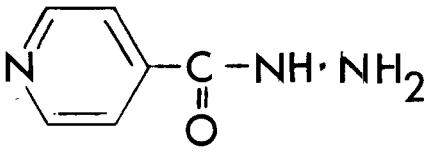


Harmaline



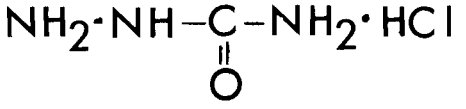
$2HCl, 2H_2O$

Atebrin



Isoniazid

$H_2N \cdot NH_2 \cdot H_2SO_4$ Hydrazine Sulfate



Semicarbazide Hydrochloride

$(NH_2 \cdot NHC : NH \cdot NH_2)_2 \cdot H_2SO_4$ Aminoguanidine Sulfate

III. RESULTS

1. The Thermal Inactivation of Monoamine Oxidase

For the purpose of studying the thermal inactivation of the enzyme as a function of time the enzyme, contained in thin walled test-tubes, 1 in. in diameter, were placed in a heated water-bath heated with a "Bronwill" Constant Temperature circulator. At intervals 0.5 ml., aliquots were removed and placed in 20 ml. beakers, chilled in ice-water until activities were to be measured. Each beaker contained kynuramine, 1.6×10^{-4} M (final concentration), phosphate buffer, 0.031 M (final concentration), and water to a volume of 3.2 ml. A preincubation lasting 5 minutes, was carried out before the substrate was added and then a further 20 minutes incubation with the kynuramine present. Blanks were prepared similarly except that kynuramine was omitted. At the end of 20 minutes the reactions were stopped in the beakers by adding 1.0 ml. of 5% zinc sulfate. All readings in these and in the subsequent experiment were done at 315 mμ.

The enzyme displays a great stability towards heating. For example, after 80 mins.' heating at 50°C, the enzyme had retained about 70% of its activity, at 53°C and 55°C, 50% and 35% respectively of the activity remained. At 60°C most of the activity was lost in the first ten minutes of heating.

The effect of heat on the inactivation of MAO is shown in Fig. 1. The fall in activity does follow a parabolic curve as illustrated by the time course (Fig.1). The curves do not become linear

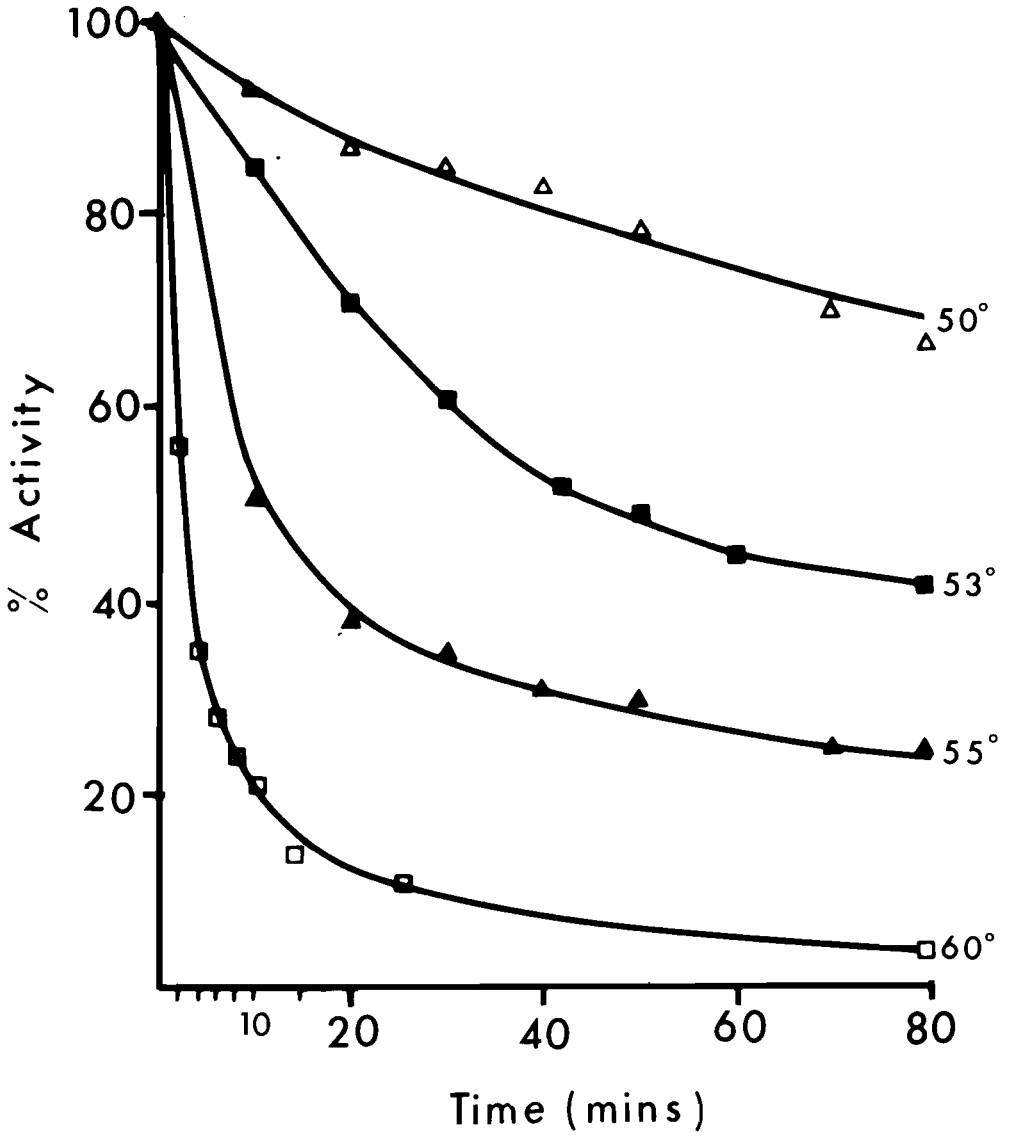


Fig. 1. Time course for the heat-inactivation of monoamine oxidase.

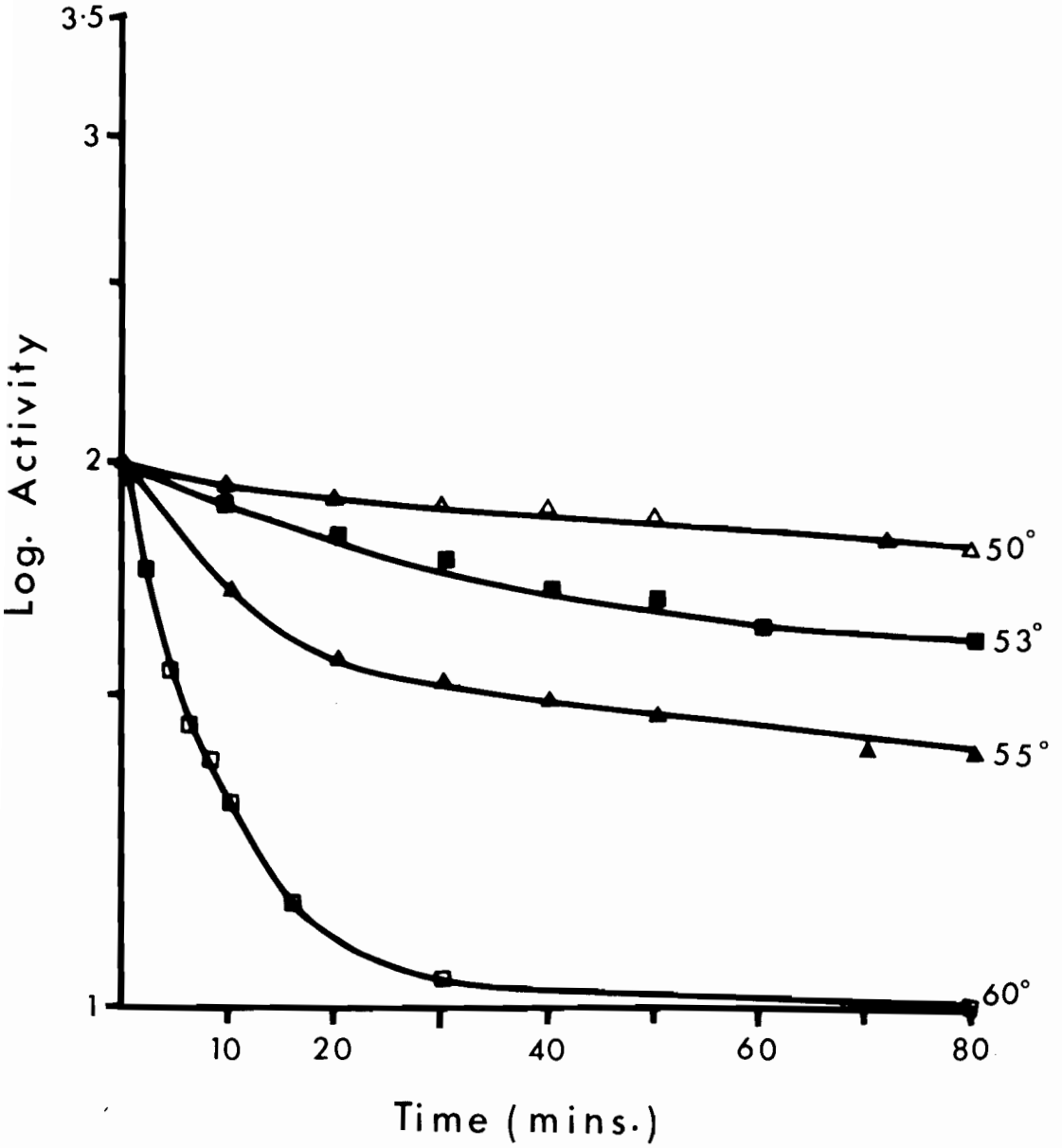
by plotting log. (activity) against time (Fig.2). The time course involves two first-order processes, suggesting (a) the presence of two enzymes, or (b) the formation of a second less active enzyme from the native enzyme both species inactivating independently, or (c) the inactivation is reversible and an equilibrium is reached. However the last explanation is ruled out by the fact that it is possible to inactivate monoamine oxidase preparation completely, by prolonged heating.

2. The Effect of pH and Thermal Inactivation

Using kynuramine (1.6×10^{-4} M final concentration) as substrate, the curve of activity versus the pH of the native enzyme showed a shoulder around pH 6.5-7.0 and a peak at pH 7.0 (Fig.3) (phosphate buffer 0.031 M final concentration) or at pH 8.1 (borate buffer 0.031 M final concentration). The effect of phosphate buffer at various pH's, 5.8-7.9, on the UV. absorbancy of kynuramine was studied. No change was observed. Since a difference in activity was obtained at pH's higher than 7.4 when borate buffer was used, it became necessary to study the effect of borate buffer on the U.V. absorbancy of kynuramine oxidative product, kynurine (4-hydroxyquinoline), at 315-330 mμ. No change was observed at this peak or at 360 mμ.

To study the effect of thermal inactivation of MAO at different pH's, the enzyme and buffer (phosphate buffer 0.066 M final concentration) in a ratio of 1:2 were heated at 53°C for 15 or 30 mins. The conditions for this experiment are described under the section "Methods".

Fig. 2. Time course for the heat-inactivation of monoamine oxidase.



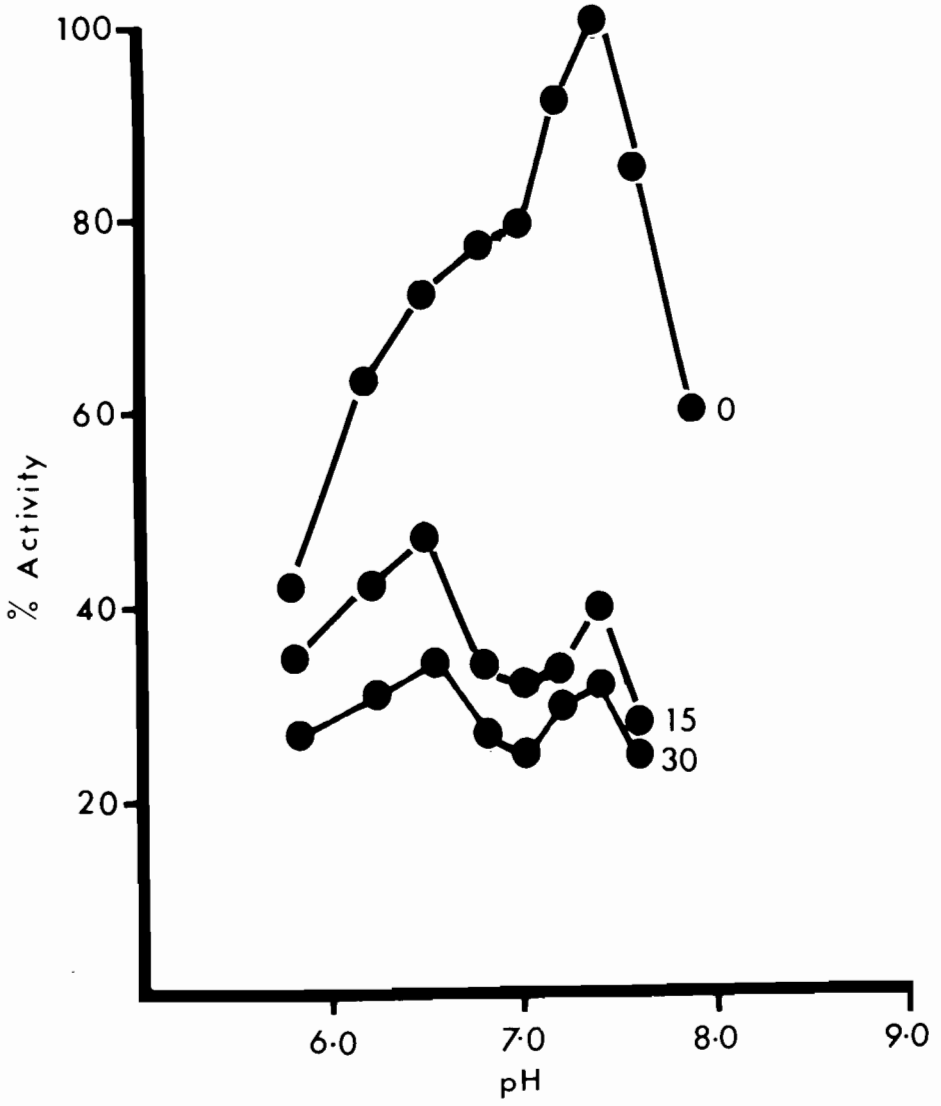


Fig. 3. Activity-pH curve of partially heat-inactivated (53°C, 15 and 30 mins.) and native enzyme.

The activity-pH curve showed reduced activity at all pH's, but the fall in activity at pH's 7.0-7.9 was greater than below pH 7.0 (Fig.3). This resulted in the disappearance of the shoulder and the appearance of new secondary peak at pH 6.5, the primary peak remaining at pH 7.4. Table I and II show the effect of various pH's on the activity of the native and partially heat inactivated enzyme.

The time course for the effect of pH and thermal inactivation showed first-order kinetics. The conditions for these experiments were the same as those already described in the previous section. Activity fell rapidly at all pH's but more at pH 7.0 (Fig.4). At the end of 30 mins. the observed activity at pH 7.0 was below that of pH 6.5 or 7.4.

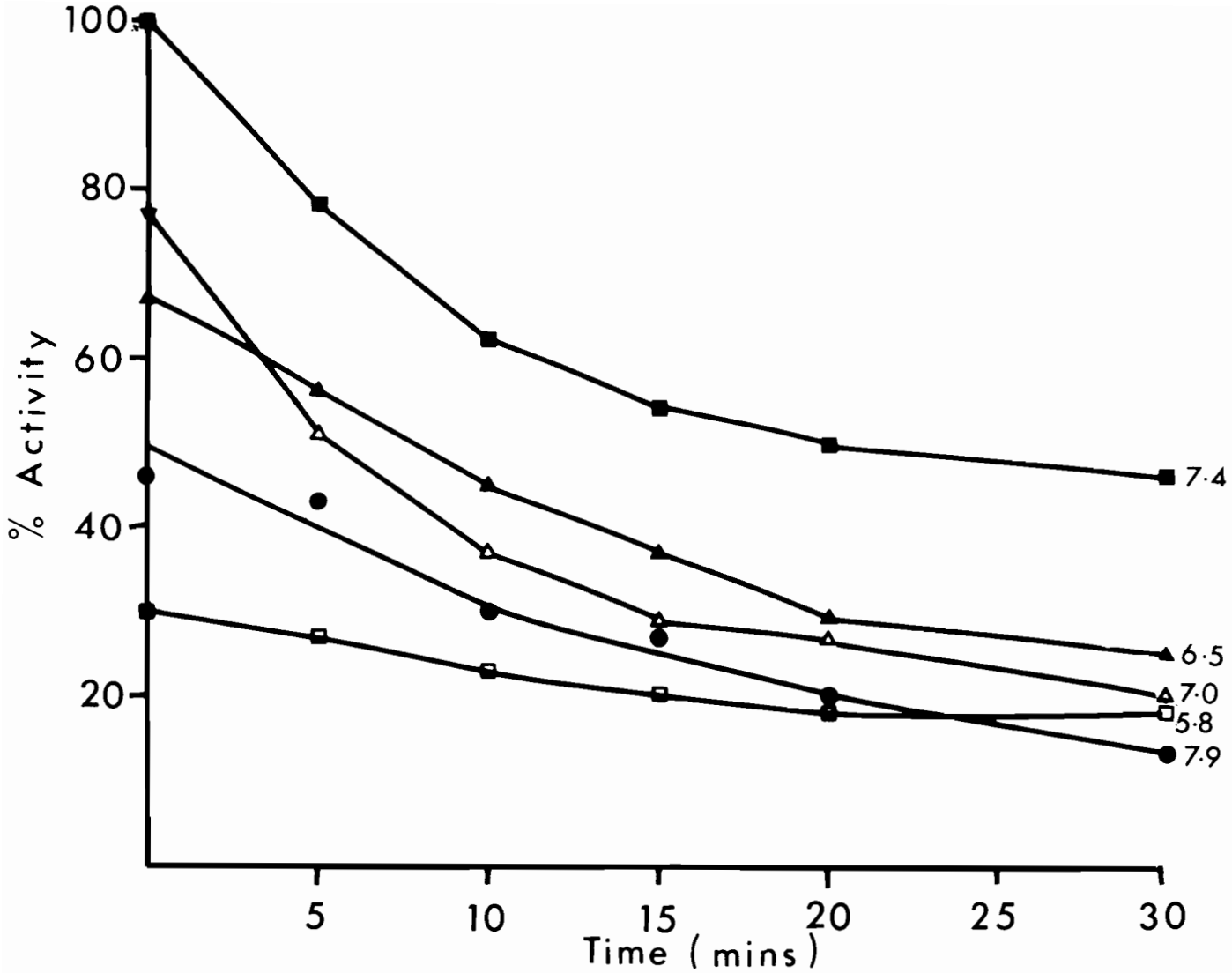


Fig. 4. Time course for heat-inactivation (55° C) at different pHs.

Table I. The Effect of Heat (53°C) on the pH-Activity Curve.

pH	Time (minutes)					
	0*		15*		30*	
5.8	0.51[1.]	(41)[2.]	0.42	(33)	0.34	(27)
6.2	0.74	(62)	0.53	(42)	0.40	(32)
6.5	0.95	(75)	0.61	(48)	0.43	(34)
6.8	1.00	(79)	0.43	(34)	0.35	(28)
7.0	1.04	(82)	0.39	(31)	0.33	(26)
7.2	1.19	(94)	0.43	(34)	0.38	(30)
7.4	1.26	(100)	0.49	(39)	0.42	(33)
7.6	1.13	(88)	0.35	(28)	0.32	(25)
7.9	0.80	(63)	-----		-----	

1. Optical density for the first 20 mins. incubation with the substrate (1.6×10^{-4}M).

2. Figures in the bracket represent per cent of activity found at pH 7.4.

* Time of heating at 53°C.

Similar curves were obtained when the enzyme-buffer preparation was heated at 50° and 55°C. Table II shows the effect of heating at 50°C for 30 to 60 minutes. The effect of heating at this temperature was not as drastic as heating at 53°C. At this temperature the activity lost at pH 6.5 was 20 and 35 per cent for 30 and 60 minutes heating, but at pH 7.4 there was a much greater loss in

activity, 44 and 56 per cent for 30 and 60 minutes hearing respectively. But the greatest fall in activity occurs at pH 7.0;

Table II. The Effect of Heating (50°C) on the pH-Activity Curve.

pH	Time (minutes) Control 0		30		60		Loss in activity 30	60
6.2	0.52[1.]	(52)[2.]	0.42	(42)	0.36	(36)	19	30
6.5	0.60	(60)	0.48	(48)	0.39	(39)	20	35
6.8	0.70	(70)	0.46	(46)	0.28	(28)	34	40
7.0	0.72	(72)	0.35	(35)	0.25	(25)	51	65
7.2	0.84	(84)	0.50	(50)	0.40	(40)	40	52
7.4	1.00	(100)	0.56	(56)	0.44	(44)	44	56
7.9	0.85	(85)	0.56	(56)	0.36	(36)	34	58

1. Optical density for the first 20 mins. incubation with the substrate (1.6×10^{-4}M).

2. Figures in the bracket represent per cent activity in terms of activity at pH 7.4.

* Per cent activity in terms of the control.

51 and 65 per cent of the activity is lost in 30 and 60 minutes respectively.

3. <u>The Effect of Aerobic and Anaerobic Thermal Inactivation of Monoamine Oxidase</u>

On many occasions it has been reported that monoamine oxidase possesses a sulfhydryl group which is necessary for the activity.

If this is true then, when the enzyme is heated in the presence of oxygen, it should show less activity than when heated in the presence of nitrogen.

The enzyme suspension, contained in thin-walled test-tubes, was heated at 50°C for 30 mins. One test-tube acted as the control. This tube was heated as it sat in the bath. Into three other test-tubes, nitrogen (which had been passed through an alkaline solution of hydroquinone) to remove any oxygen present in the nitrogen, air and oxygen, respectively, were bubbled through with the use of gas dispersion tubes. At the end of this 30 mins. period, the test-tubes were removed and cooled immediately in an ice bath. Their activities at various pH's were then determined, as described under "Methods" Section 6.

Table III. The Effect of Aerobic and Anaerobic Thermal in Activation (50°C, 30 mins.)

Gases	pH				
	6.2	6.5	7.0	7.4	7.9
Control	0.06*	0.11	0.19	0.31	0.44
Nitrogen	0.05	0.09	0.14	0.23	0.35
Air	0.04	0.07	0.11	0.15	0.27
Oxygen	0.04	0.06	0.09	0.13	0.21

* Increase in optical density for the first 20 mins. incubation with the substrate (kynuramine, 6.2×10^{-5}M).

As shown in Table III, the loss in activity of the enzyme heated in the presence of nitrogen was not as great as the loss when the enzyme

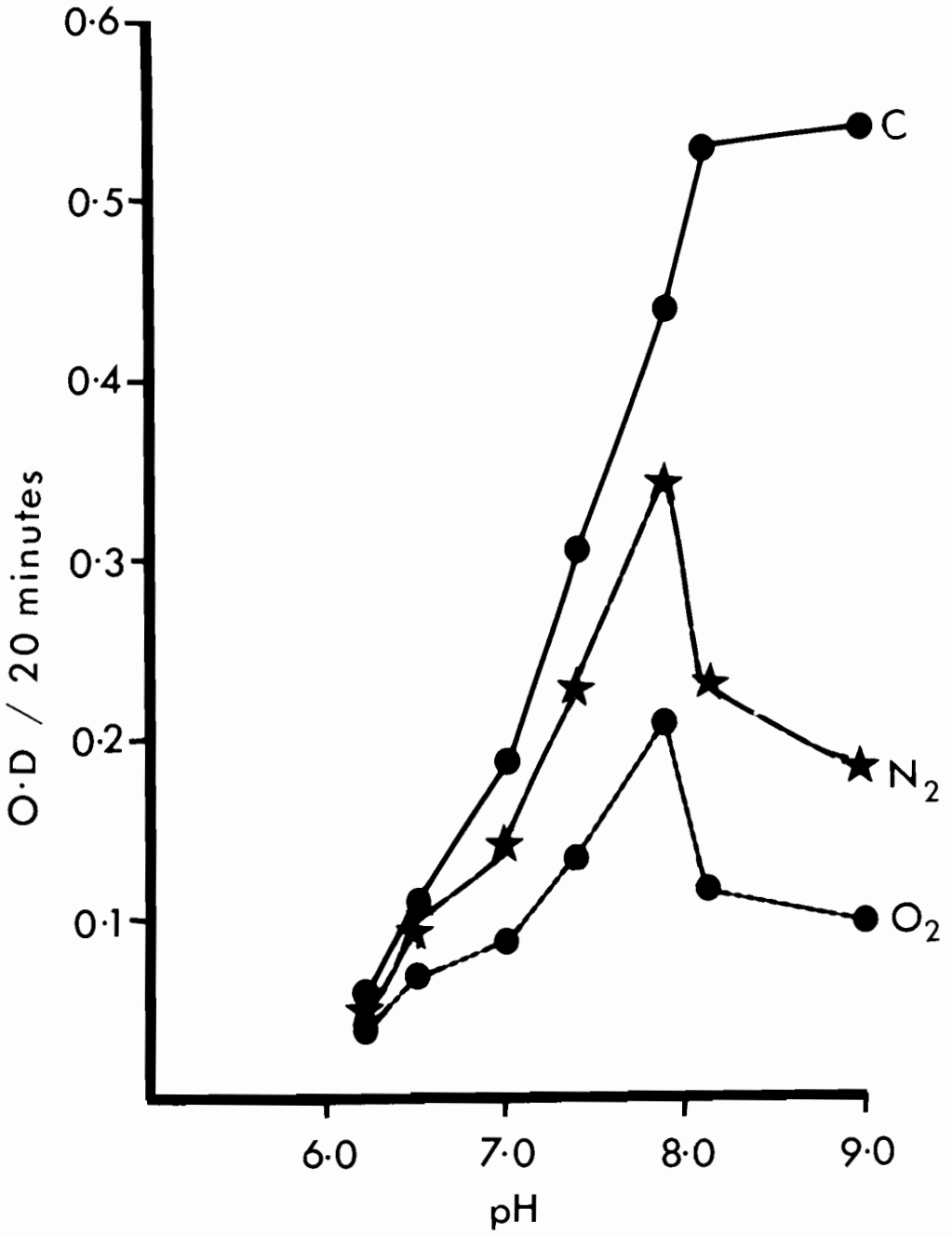


Fig. 5. The effect of aerobic and anaerobic thermal inactivation (50 C, 30 mins.) on the activity-pH curve of MAO. The control (C) is heated at the same temperature and for the same period.

was heated in the presence of oxygen or air. Fig. 5 shows the activity-pH curves of the enzyme heated in the presence of various gases. Though activity in all cases is lower than the control, the curves of nitrogen, oxygen, and air show the same pattern of activity as the control.

At pH 7.4 the enzyme heat in the presence of nitrogen had lost 25% of its activity, while in the presence of air or oxygen it lost 50% and 58% respectively. More than twice the activity was lost in the presence of oxygen than with nitrogen.

4. The Effect of Chelating Agents on the Native Enzyme

Previous investigations indicate that MAO activity can be reduced by treatment with a chelating agent. Certain analagous enzymes such as plasma amine oxidase (Yamada and Yasunobu (137) and Blaschko (138)) and diamine oxidase (Mann (139)) have been shown to contain metal component. Gorkin (63), who studied the effect of chelating agents on monoamine oxidase, concluded that the MAO was a metal enzyme. In this work I first studied the effect of o-phenanthroline and found that it inhibited the activity at pH 6.5 by 60%, and at pH 8.1 by 8%, with a concentration of 9.4×10^{-5}M (Table IV). A number of other chelating agents such as 2,2-dipyridyl, 8-hydroxyquinoline, thenoyltrifluoroacetone, diethyldithocarbamate and D-penicillamine were used. Of all these agents studied, 8-hydroxyquinoline was the most effective (as reported previously by Gorkin). At a concentration of 6.2×10^{-5}M it had an inhibitory effect of 52%, 33% and 4%

at pH's 6.5, 7.0, and 8.1 respectively (Fig.6). Thenoyltrifluoroacetone, an iron chelator, had a similar effect, but at a slightly higher concentration (Fig.7).

The effect of other chelating agents is also shown in Table IV. D-penicillamine and diethyldithiocarbamate gave different results. D-penicillamine, at a concentration of 6.2×10^{-5}, inhibited at pH 8.1 by 22%, this is slightly more than the 10% inhibition obtained at pH 6.5 (Fig. 8). A number of investigators have pointed out that the enzyme, like some other amine oxidases, might have copper as cofactor. For this reason we have used diethyldithiocarbamate, a chelating agent used in estimation of copper. The results from this investigation were negative. At a concentration of 1.6×10^{-5}M there was no inhibition, while at a concentration of 3.2×10^{-5}M there was slightly more inhibition at pH 6.5 than at 8.1 (Table IV).

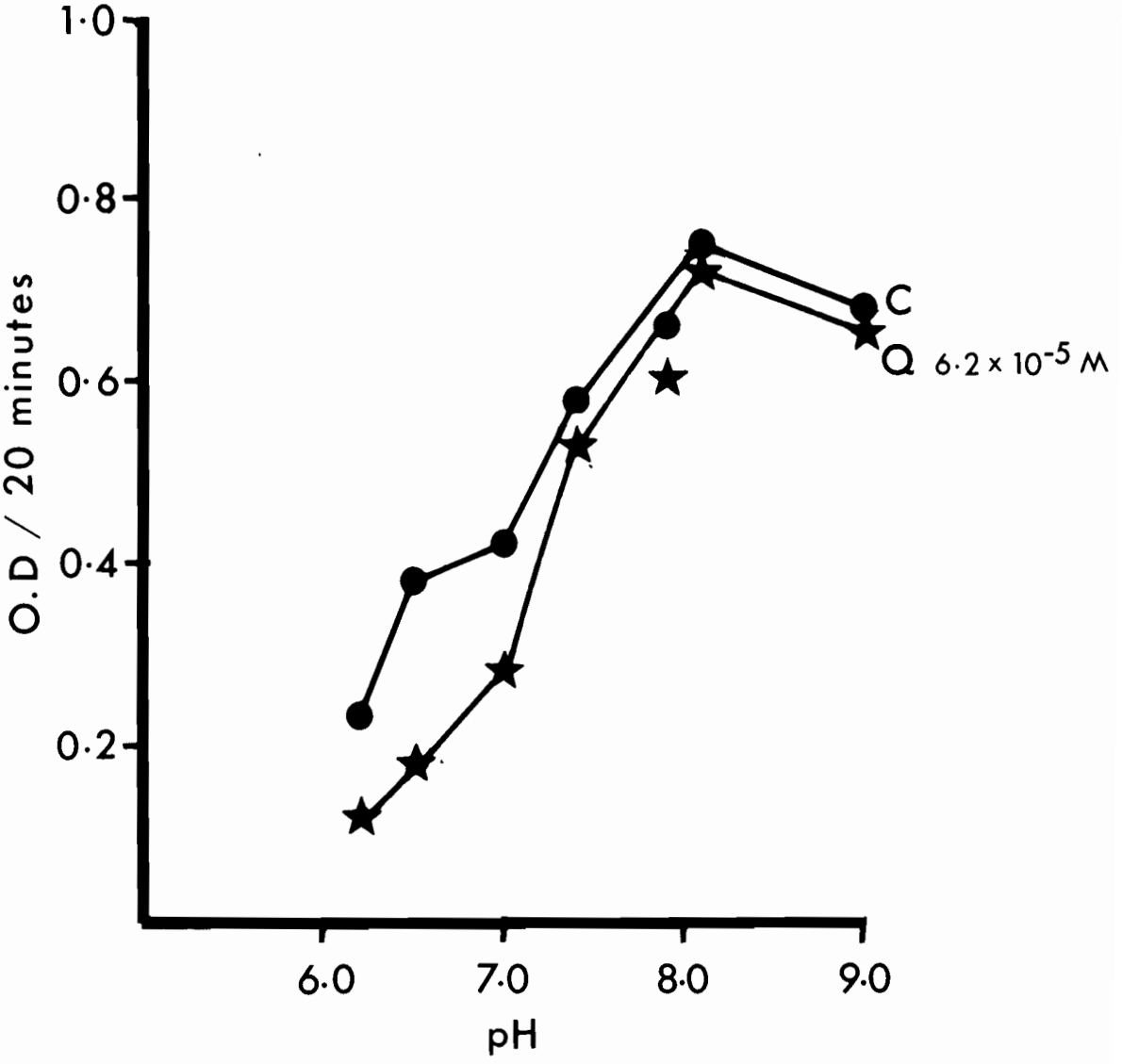


Fig. 6. Activity-pH curve of enzyme alone (C) and enzyme plus 8-hydroxyquinoline (Q). Kynuramine (6.2×10^{-5} M) is the substrate.

Table IV. The Effect of Chelating Agents on the Activity-pH Curve of the Native Enzyme.

In all the experiments the beakers containing the enzyme and chelating agent preparation were preincubated for 20 mins. at 37°C in a Dubnoff shaker before the addition of kynuramine (6.2×10^{-5}M). Blanks were prepared in the similar manner except that kynuramine was omitted.

Chelating Agents	pH	Control 6.5	Control 7.0	Control 8.1	Experimental 6.5	Experimental 7.0	Experimental 8.1
8-Hydroxy-quinoline,							
6.2×10^{-5}M		0.38[1]	0.42	0.75	0.18(52)[2]	0.28(33)	0.72(4)
3.1×10^{-4}M		0.38	0.56	0.93	0.05(87)	0.18(68)	0.56(40)
6.2×10^{-4}M		0.34	0.48	0.85	0 (100)	0.08(98)	0.34(58)
Thenoyltri-fluoroacetone,							
1.6×10^{-4}M		0.28	0.34	0.56	0.12(56)	0.12(64)	0.51(10)
o-Phenan-throline,							
9.4×10^{-5}M		0.37	0.45	0.67	0.15(60)	0.33(27)	0.61(8)
2,2-Dipyridyl,							
6.2×10^{-5}M		0.38	0.53	0.62	0.29(23)	0.44(16)	0.60(2)
1.6×10^{-4}M		0.38	0.53	0.62	0.22(43)	0.42(21)	0.53(15)
Diethyldithio-carbamate,							
1.6×10^{-4}M		0.37	0.46	0.74	0.35(0)	0.60(0)	0.78(0)
3.2×10^{-4}M		0.43	0.51	0.74	0.30(30)	0.39(24)	0.68(8)
D-Penicill-amine,							
6.2×10^{-5}M		0.29	0.39	0.75	0.26(10)	0.36(6)	0.58(22)

1. Optical density for the first 20 mins. incubation with the substrate.
2. Figures in the bracket represent per cent inhibition.

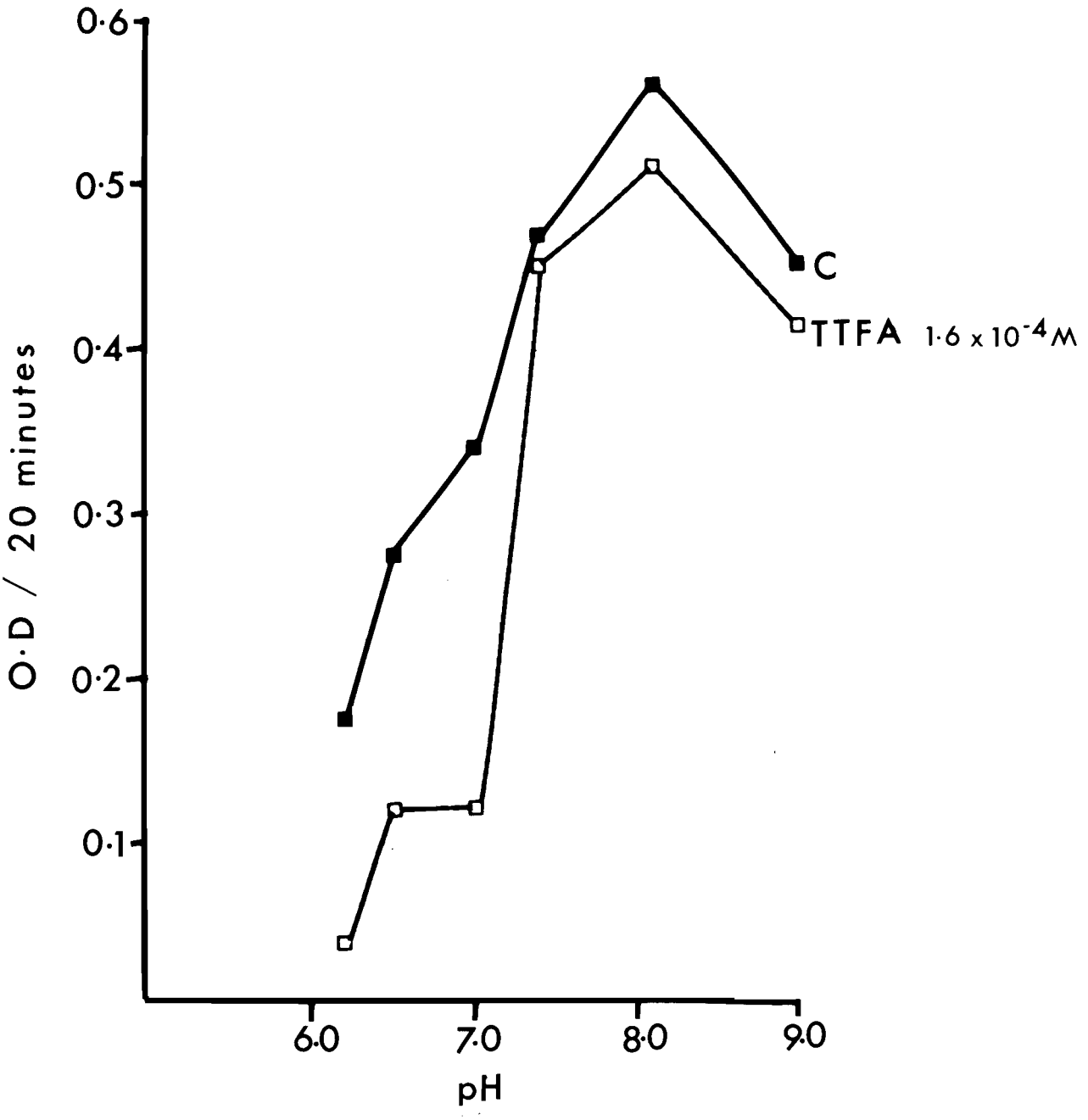


Fig. 7. Activity-pH curve of enzyme alone (C) and enzyme plus thenoyltrifluoroacetone (TTFA). Kynuramine (6.2×10^{-5} M) is the substrate.

Table V. The Effect of Chelating Agents on the Partially Heat Inactivated (50°C, 30 mins.) Enzyme

In all experiments the flasks containing the enzyme and chelating agent preparation were incubated for 20 mins. at 37°C in a Dubnoff shaker before the addition of kynuramine (1.6×10^{-4}M). Blanks were prepared in a similar manner except that kynuramine was omitted.

Chelating Agents	pH	Control 6.5	Control 7.0	Control 7.4	Experimental 6.5	Experimental 7.0	Experimental 7.4
8-Hydroxy-quinoline,							
1.6×10^{-4}M		0.36[1.]	0.19	0.26	0.16(57)[2.]	0.15(21)	0.24(8)
Thenoyltri-fluoroace-tone,							
1.6×10^{-4}M		0,40	0.26	0.34	0.19(54)	0.19(29)	0.30(12)
1.6×10^{-4}M		0.68*	0.45	0.53	0.34(50)	0.37(19)	0.42(19)

1. Activity per mg. protein in terms of Optical Density for the first 20 mins. incubation with the substrate.

2. Figures in the bracket represent per cent inhibition.

* Optical density for the first 20 mins. incubation with the substrate.

5. <u>The Effect of Chelating Agents on the Partially Heat-Inactivated MAO</u>

The relative inhibition obtained in these experiments was not very large and since (as mentioned earlier) the partially heat-inactivated enzyme gave two peaks, further experiments were carried

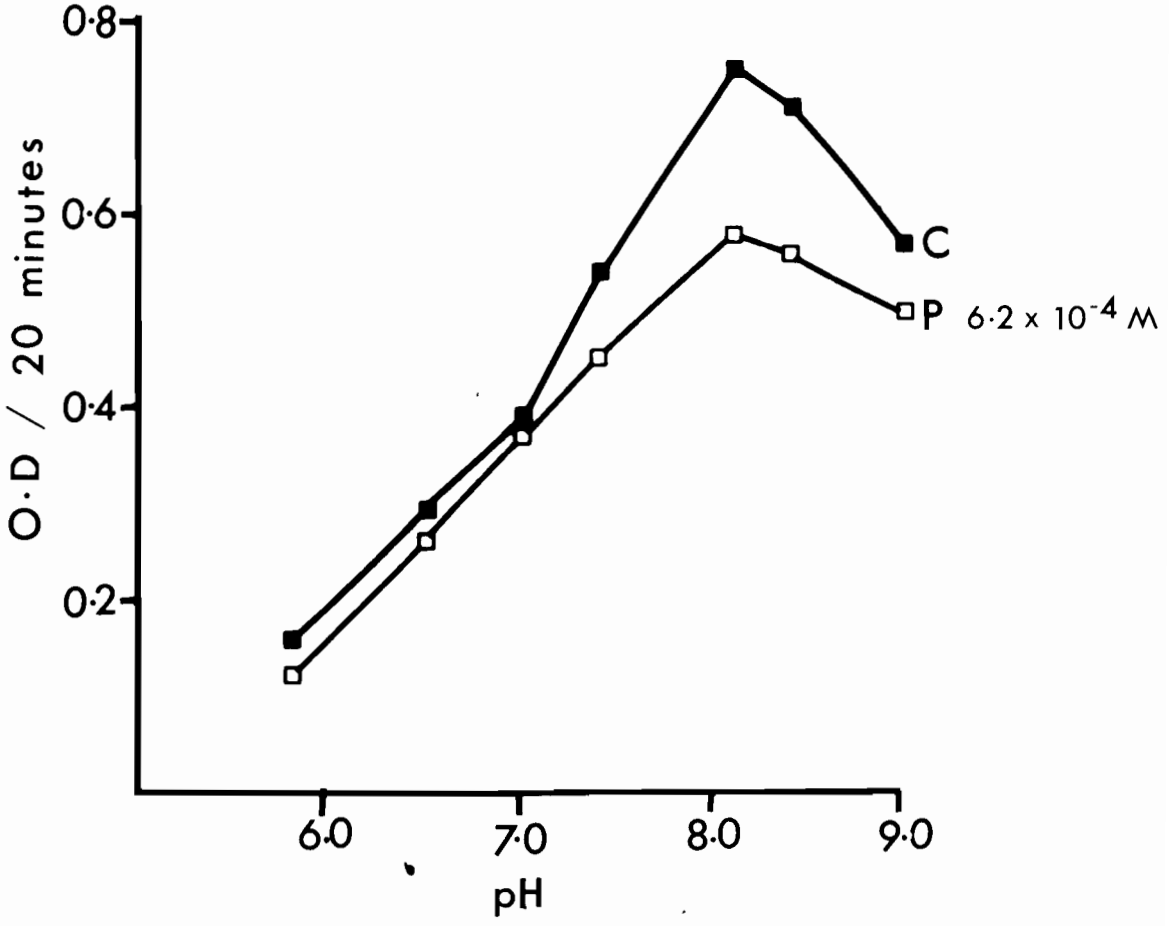


Fig. 8. Activity-pH curve of enzyme alone (C) and enzyme plus D-penicillamine (P). Kynuramine (6.2×10^{-5} M) is the substrate.

out with a preparation of the enzyme that had been heated at 50° for 30 mins. The results obtained in these series of studies are shown in Table V and are in accordance with those obtained with the native enzyme, i.e., the greatest inhibitions occurred at pH 6.5. Figs. 9 and 10 show the effect of 8-hydroxyquinoline and thenoyltrifluoro-acetone on the activity-pH curve of the partially heat-inactivated enzyme. The inhibitory effect of these two chelators could be reversed by dialysis against distilled water for 3 hours. As can be seen in Figs. 9 and 10 activity reverted to the original.

6. The Effect of Copper Deficiency in MAO Activity

The procedure outlined ("Methods") for the production of copper-deficient rats proved satisfactory. Even after the first 8 days the livers showed a drop in copper content. After 15 days the decrease in copper concentration was even more prominent. After 44 days on the copper-deficient diet, the liver of the deficient animals showed a decrease of 75% in the copper content.

It is interesting to note that the copper deficiency did not impair the MAO activity as did the riboflavin deficiency. Even after 44 days on the deficient diet there was no effect. In fact, there was an increase in the activity by 55% at the end of 44 days on the deficient diet as shown in the Table VI.

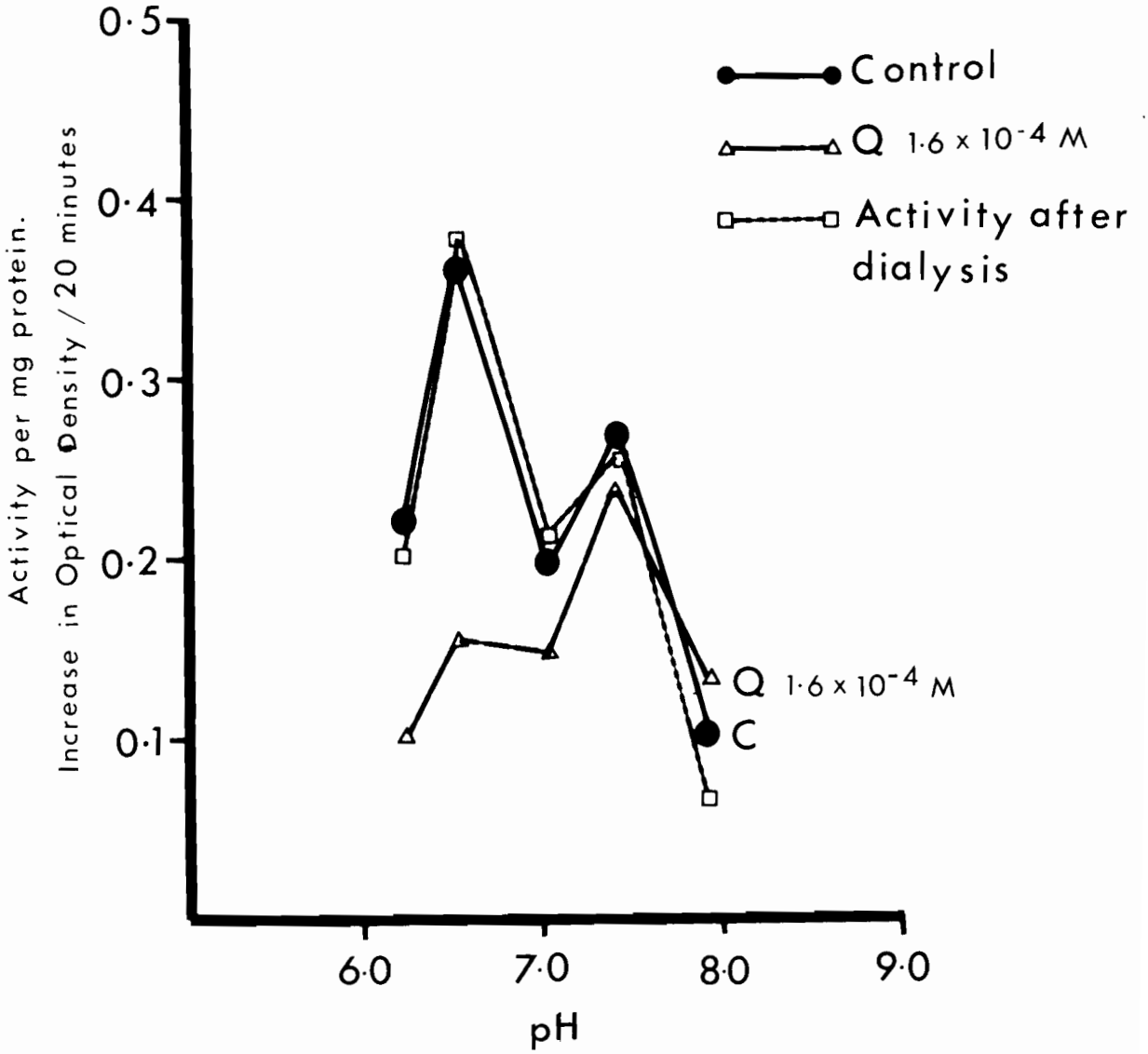


Fig. 9. The effect of 8-hydroxyquinoline (Q) on the activity-pH curve of partially heat-inactivated (50°C, 30 mins.) enzyme. (C) is the control. Substrate is kynuramine (1.6×10^{-4} M).

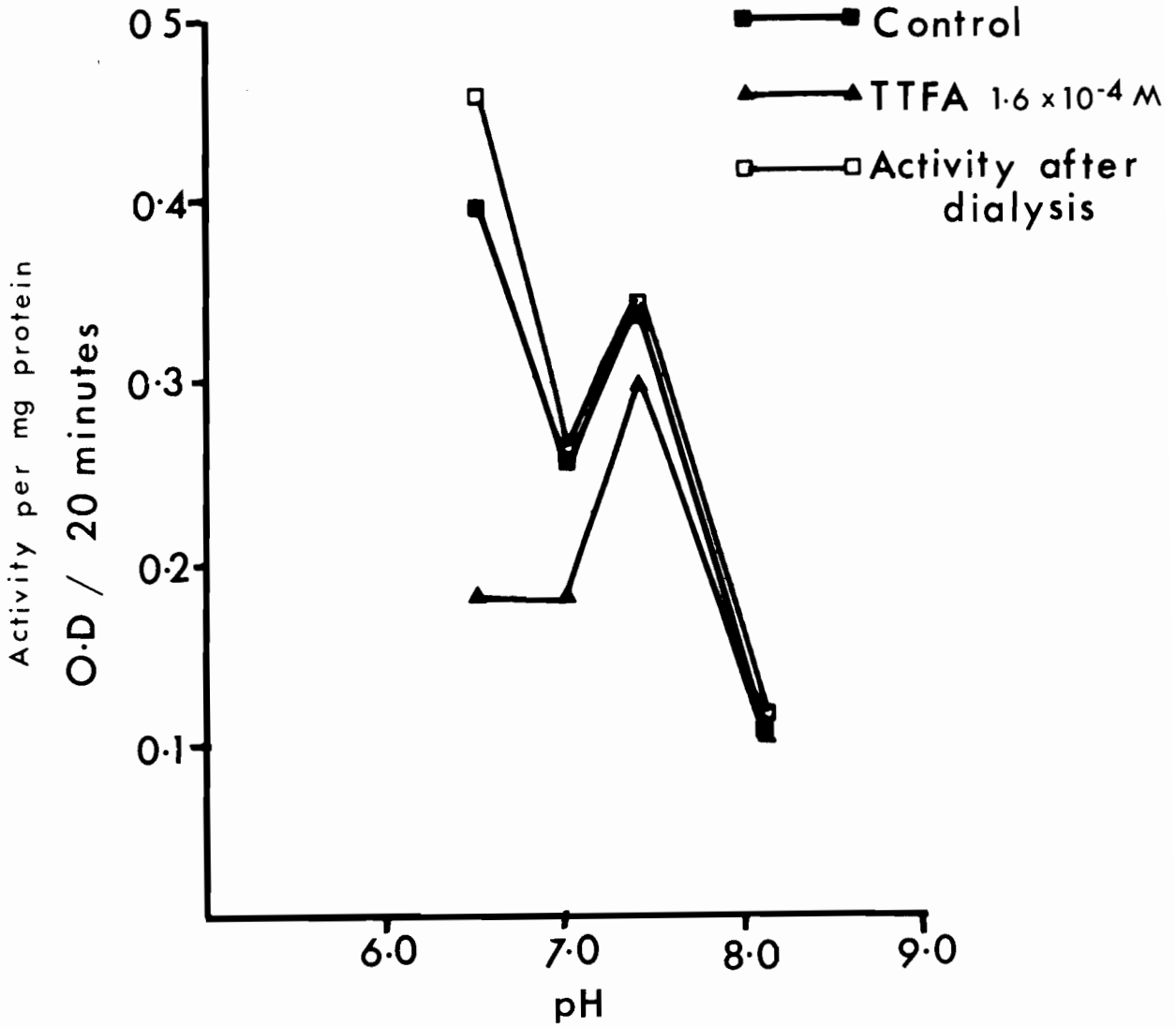


Fig. 10. The effect of thenoyltrifluoroacetone (TTFA) on the activity-pH curve of partially heat-inactivated (50°C, 30 mins.) enzyme. Substrate is kynuramine (1.6 X 10^{-4} M).

Table VI. The Effect of Copper Deficiency on MAO Activity at Various pH's

Days on diet	pH	Supplemented 6.5	7.0	8.1	Deficient 6.5	7.0	8.1
15		0.57[1.]	-	1.04	0.62(110)[2.]	-	1.18(113)
23		0.51	0.64	1.07	0.58(113)	0.73(114)	1.21(113)
44		0.40	0.50	0.98	0.62(155)	0.76(157)	1.40(143)

1. Increase in optical density for the first 20 mins. incubation with the substrate.

2. Figures in the brackets represent per cent activity, as compared to the control.

7. The Effect of Inhibitors of Monoamine Oxidase

(a) In Vitro

As mentioned in the review of MAO, there are different classes of inhibitors of MAO. We have used inhibitors from these different classes in order to study how they affect the activity versus pH curve. In order to obtain maximal effect of inhibitors of monoamine oxidase at any particular concentration, the inhibitors were preincubated with the enzyme before addition of the substrate. Phenylcyclopropylamine, a non-hydrazine inhibitor of MAO, inhibited at all pH's, but to different extents. In fact, at a concentration of 3.1×10^{-8}M, activity at pH 8.1 was reduced by 26%, but on the acid side of pH, by only 15% (see Table VII). As the concentration of inhibitors was

increased, there was a rapid fall in activity at the higher pH's. This resulted in the appearance of a second peak at pH 6.5 and a broad original peak at pH 8.1 (Fig.11).

Another highly potent non-hydrazine inhibitor, pargyline, gave results which were in contrast to those obtained with phenylcyclopropylamine. In fact, inhibition by pargyline (Fig. 12) resulted in the disappearance of the shoulder at pH 6.5-7.0. This result was similar to that obtained with some of the chelating agents. Pargyline at 3.1×10^{-8}M final concentration was a more powerful inhibitor than phenylcyclopropylamine at 6.2×10^{-8}M concentration.

Inhibition of MAO by hydrazine derivatives was similar to that of phenylcyclopropylamine. Neither iproniazid nor phenylhydrazine was as effective as catron (β-phenylisopropylhydrazine). All had their greatest effect on the alkaline side of the pH-activity curve. The results of these and other inhibitors are shown in Table VII. With iproniazid, at a concentration of 1.6×10^{-7}M, the pH-activity curve gave two peaks. A similar result was obtained with phenylhydrazine (Table VIII) but inhibition by β-phenylisopropylhydrazine at 6.2×10^{-7}M final concentration resulted in the pH-activity curve having only one peak at pH 6.5. At this concentration, near complete inhibition was obtained at pH 8.1 but only 25% inhibition was observed at pH 6.5 (Fig.13).

Harmaline, a reversible inhibitor of monoamine oxidase, gave results which were dissimilar to those obtained with chelating agents or the hydrazine derivative inhibitors. The activity-pH curve

Fig. 11. Activity-pH curve of enzyme alone (C_1, C_2) and enzyme plus phenylcyclopropylamine (PCP_1, PCP_2) at various concentrations. Kynuramine (6.2×10^{-5} M) is the substrate.

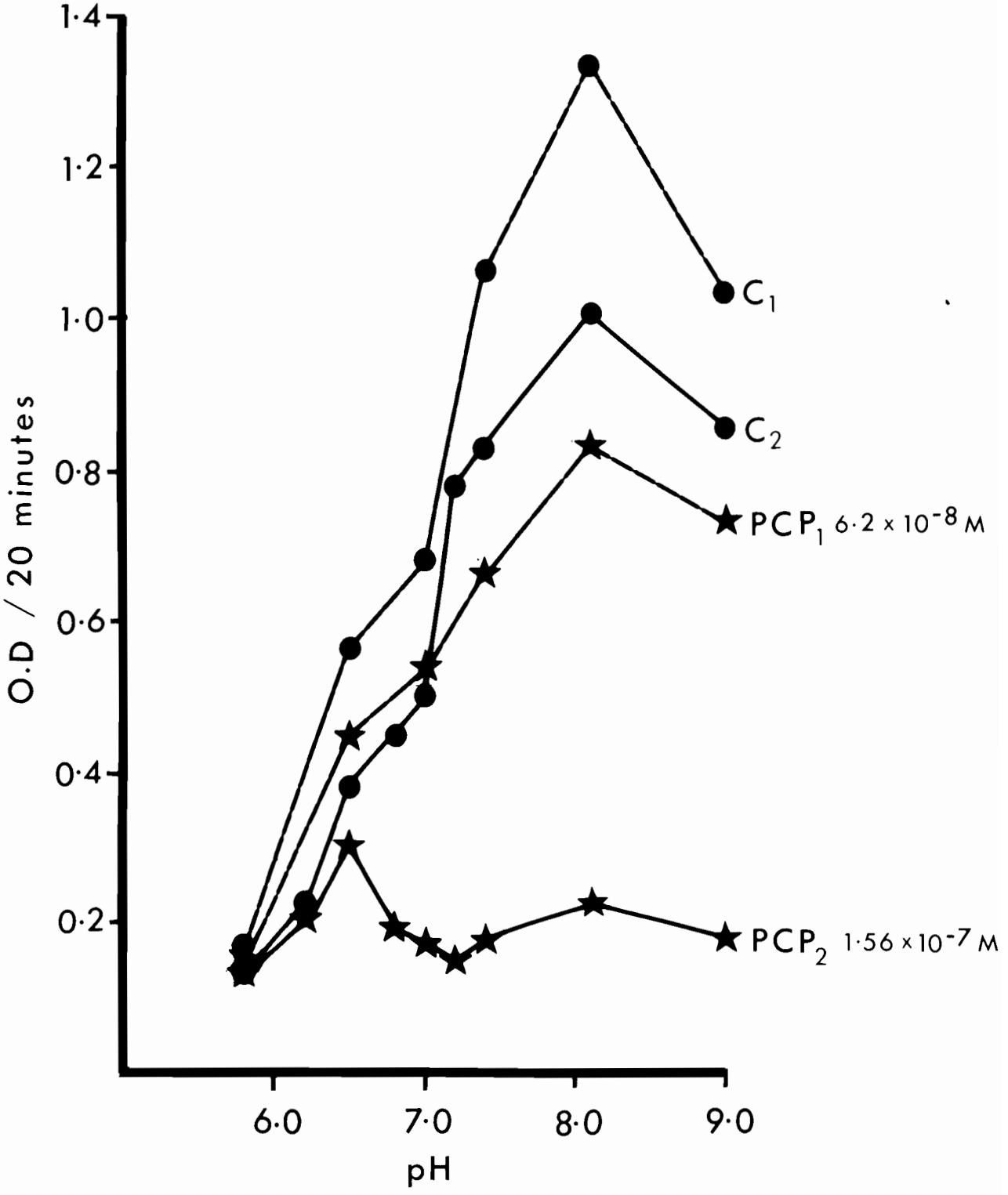


Table VII. The Effect of Monoamine Oxidase Inhibitors on the Activity-pH Curve of the Native Enzyme.

The conditions for these experiments are similar to those outlined in Table IV.

Inhibitors	Control				Experimental			
	6.5	7.0	7.4	8.1	6.5	7.0	7.4	8.1
Phenycyclopropylamine,								
3.1×10^{-8}M	0.57*	0.69	1.07	1.34	0.50(15)**	0.65(7)	0.78(22)	0.99(26)
6.2×10^{-8}M	0.57	0.69	1.07	1.34	0.45(15)	0.54(22)	0.67(38)	0.84(37)
1.6×10^{-7}M	0.38	0.51	0.83	1.01	0.30(22)	0.18(65)	0.17(80)	0.23(77)
Iproniazid,								
3.2×10^{-6}M	0.28	0.34	0.52	0.70	0.24(14)	0.33(3)	0.42(19)	0.52(26)
6.2×10^{-6}M	0.23	0.39	0.50	0.66	0.19(17)	0.28(28)	0.34(32)	0.35(47)
1.6×10^{-5}M	0.28	0.34	0.52	0.70	0.23(18)	0.15(56)	0.20(62)	0.25(64)
6.2×10^{-5}M	0.40	0.49	0.65	0.93	0.0 (100)	0 (100)	0 (100)	0 (100)
Pargyline,								
3.1×10^{-9}M	0.36	0.42	0.62	0.73	0.25(31)	0.33(19)	0.42(33)	0.56(23)
6.2×10^{-9}M	0.36	0.42	0.62	0.73	0.18(50)	0.25(40)	0.32(48)	0.43(41)
3.1×10^{-8}M	0.31	0.36	0.55	0.66	0.05(84)	0.07(82)	0.15(73)	0.34(48)
6.2×10^{-8}M	0.31	0.36	0.55	0.66	0.03(91)	0.05(86)	0.14(76)	0.18(73)
1.6×10^{-7}M	0.30	0.43	0.66	0.76	0 (100)	0.01(98)	0.02(91)	0.10(87)
2-Phenylisopropylhydrazine,								
6.2×10^{-7}M	0.40	0.50	0.70	0.78	0.31(27)	0.25(50)	0.19(73)	0.14(83)
6.2×10^{-6}M	0.38	0.45	0.60	0.70	0.11(73)	0.03(94)	0.03(96)	0 (100)
1.6×10^{-6}M	0.38	0.45	0.60	0.70	0 (100)	0 (100)	0 (100)	0 (100)

* Optical density for the first 20 mins. incubation with the substrate.

** Figures in the bracket represent per cent inhibition.

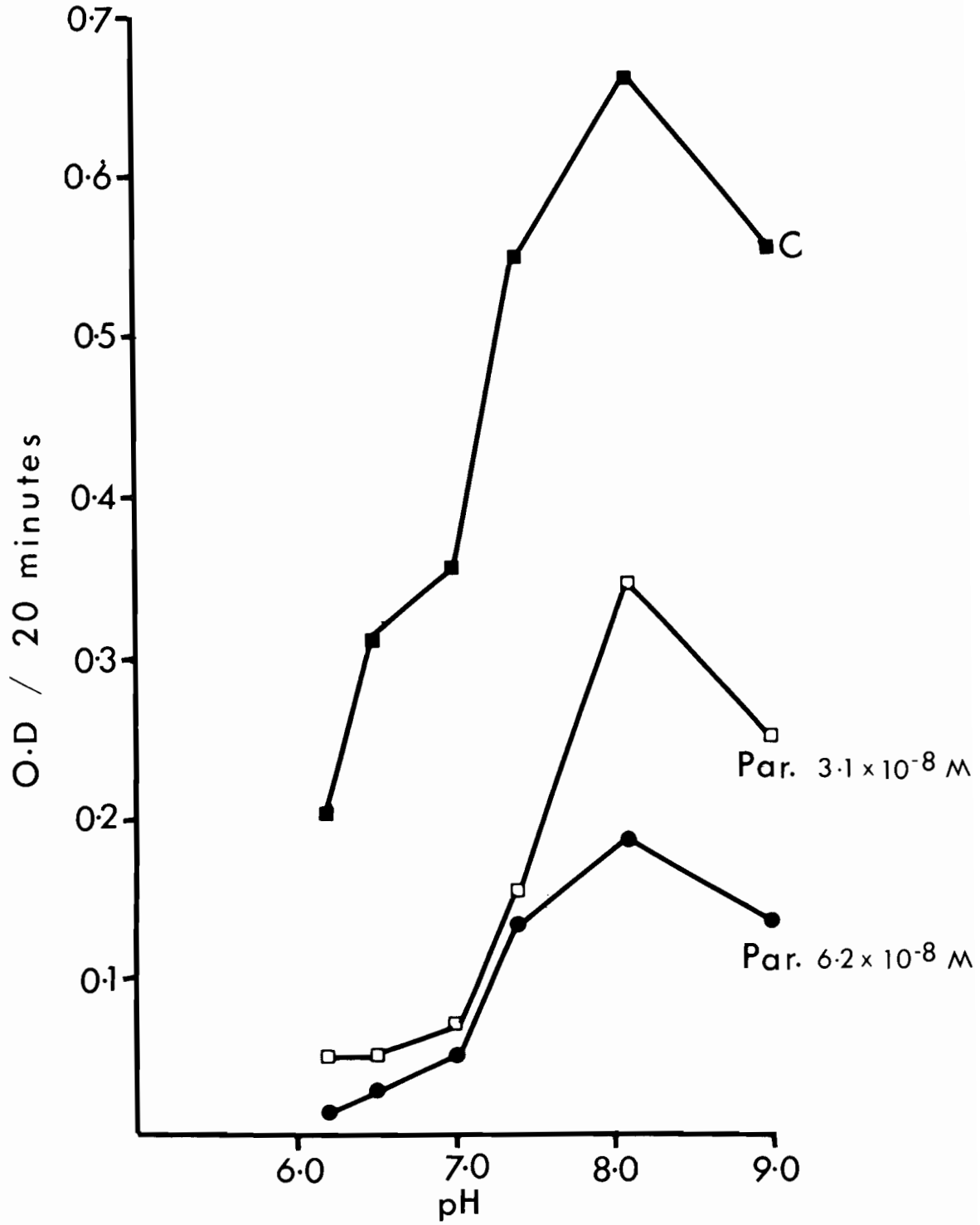


Fig. 12. The effect of pargyline (Par) at various concentrations on activity-pH curve of the enzyme. (C) is the control. The substrate is kynuramine (6.2 X 10^{-5} M).

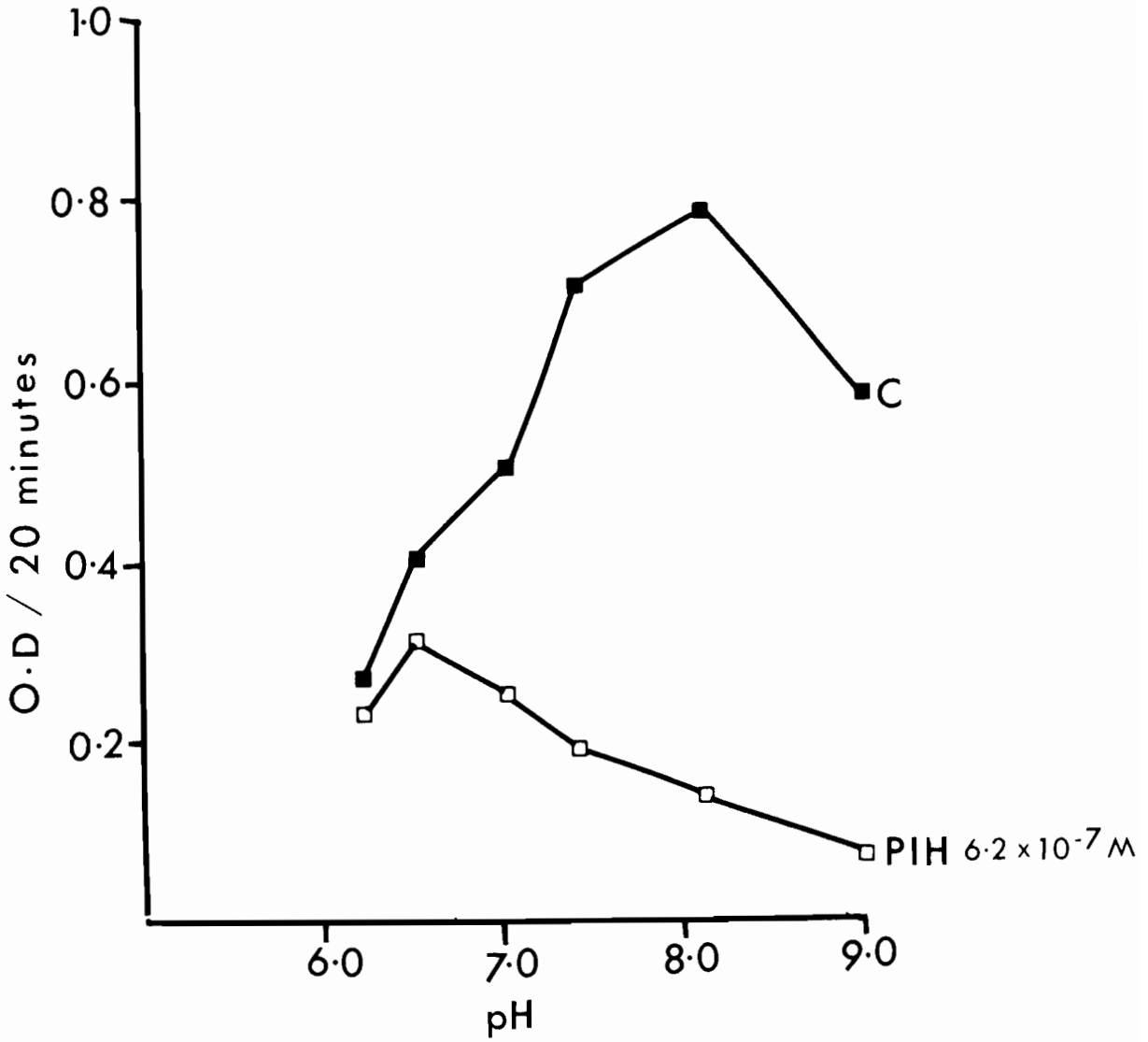


Fig. 13. Activity-pH curve of enzyme alone (C) and enzyme plus 2-phenylisopropylhydrazine (PIH). The substrate is (6.2 X 10^{-5} M) kynuramine.

of the enzyme in the presence of harmaline (Table VIII) and was similar to that of the enzyme alone. 1.6×10^{-4}M harmaline produced inhibition ranging from 36 to 60%.

Atebrin, is of interest for its inhibitory activity against monoamine oxidase. Inhibition by this agent is presumtive evidence for the presence of a riboflavin prosthetic group. The results of this experiment are shown in Table VIII. A 46% inhibition was obtained at pH 8.1 with 1.5×10^{-5}M atebrin, but at pH 6.5 and 7.0, apparently weaker inhibitions were obtained. The effect of aminoguanidine and bulbocapnine, inhibitors of diamine oxidase, were also studied. Neither of these inhibitors had any effect in the concentration tested on the activity of MAO.

(b) The In Vivo Effect of Parnate, Iproniazid and Pargyline

Most of the inhibitors used for the in vitro experiments are also effective in vivo. For these series of experiments, male rats (Sprague-Dawley) weighing 150-200 gm. were injected with the inhibitor four hours before sacrifice. Livers were then removed and MAO activity was determined at various pH's. The in vivo results confirmed the results obtained with the in vitro experiments.

Table VIII. The Effect of Inhibitors on the Activity-pH Curve of Native Enzyme.

The conditions for these experiments are similar to those outlined in Table IV.

Inhibitors	Control				Experimental			
	6.5	7.0	7.4	8.1	6.5	7.0	7.4	8.1
Phenylhydrazine,								
6.2×10^{-5}M	0.34*	0.44	0.61	0.78	0.18(48)**	0.10(78)	0.17(72)	0.18(76)
Harmaline,								
1.6×10^{-4}M	0.44	0.58	0.78	0.95	0.28(36)	0.35(40)	0.37(53)	0.38(60)
Atebrin,								
1.6×10^{-5}M	0.40	0.55		1.4	0.34(15)	0.48(10)		0.72(46)
MK 385								
6.2×10^{-5}M	0.28	0.40		0.76	0.26(7)	0.35(12)		0.60(22)
Bulbocapnine								
1.6×10^{-5}M	0.33	0.43	0.	0.66	0.38(0)	0.45(0)		0.66(0)
Aminoguanidine,								
1.6×10^{-4}M	0.46	0.62		0.74	0.46(0)	0.62(0)		0.74(0)

* Increase in the optical density for the first 20 mins. incubation with the substrate.

** Figures in the bracket represent per cent inhibition.

Table IX. The *In Vivo* Effect of Inhibitors on the MAO Activity at Various pH's

In all experiments the inhibitor was injected intraperitoneally 4 hours before sacrifice. The control animals received in the same manner an equivalent amount of 0.9% saline solution. The dose of inhibitor used is given in mg/kg body weight.

Inhibitors	Control			Experimental		
pH	6.5	7.0	8.1	6.5	7.0	8.1
Phenylcyclopropylamine,						
0.25 mg/kg	0.42*	0.55	1.0	0.32(24)**	0.31(44)	0.53(45)
0.50 mg/kg	0.38	0.54	0.95	0.22(43)	0.17(69)	0.26(73)
1.0 mg/kg						
Iproniazid,						
0.75 mg/kg	0.42	0.55	1.00	0.31(26)	0.36(35)	0.58(42)
1.50 mg/kg	0.48	0.62	1.03	0.12(75)	0.09(86)	0.20(79)
Pargyline,						
0.50 mg/kg	0.36	0.45	0.74	0.17(51)	0.26(42)	0.66(11)
1.00 mg/kg	0.36	0.45	0.74	0.09(75)	0.16(64)	0.49(34)

* Increase in the optical density for the first 20 mins. incubation with the substrate.

** Figures in the brackets represent per cent inhibition.

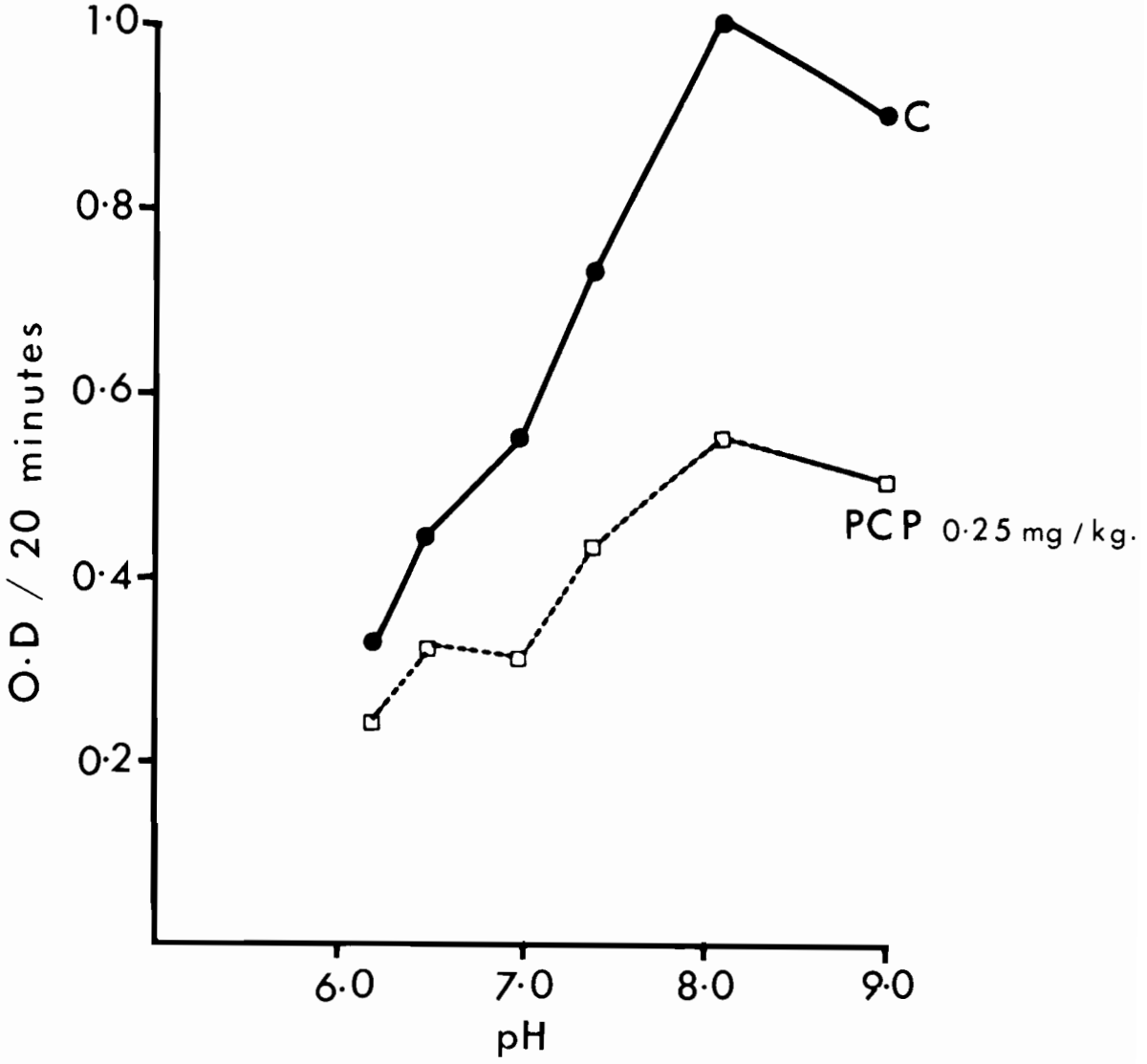


Fig. 14. The <u>in vivo</u> effect of phenylcyclopropylamine (PCP) on the activity-pH curve of MAO. (C) is the control. The dose of inhibitor (mg. / kg. body weight) was given 4 hours before the animal was sacrificed.

Phenylcyclopropylamine was the most effective of the inhibitors used in the in vivo experiments, while pargyline was the most effective in the in vitro ones. The results of the above experiments are given in Table IX. Phenylcyclopropylamine 0.25 mg/kg body weight inhibited the enzyme at pH 8.1 by 45% and at pH 6.5 by 24% (Fig.14). Complete inhibition was produced with 2 mg/kg body weight. Iproniazid gave similar results. As in the in vitro experiments, pargyline reduced the activity more at the lower pH's (Fig.15). Pargyline at 0.5 mg/kg caused the shoulder around pH 6.5-7.0 to disappear. When the concentration of pargyline was increased to 1.0 mg/kg body weight, nearly complete inhibition was obtained at pH 6.5 (Fig.15). From these results and those obtained in the in vitro experiments it would appear that phenylcyclopropylamine and pargyline act differently to inhibit the enzyme.

8. The Effect of Inhibitors of MAO on the Partially Heat-Inactivated Enzyme

Since the partially heat-inactivated enzyme gave two peaks and since we obtained considerable inhibition at pH 6.5 with a number of chelating agents, I thought of repeating these experiments using MAO inhibitors. Using the partially heat-inactivated (50°C, 30 mins.) enzyme, I was able to inhibit the activity at pH 8.1 with 1.6×10^{-7}M phenylcyclopropylamine (Fig.16) up to 50%. Pargyline, at a concentration of 6.2×10^{-8}M, inhibited 48% at pH 6.5 and 11% at pH 7.4. This result was very similar to that obtained with 8-OH quinoline (Fig.8). The effect of pargyline on the heated enzyme is shown in Fig. 17. I have therefore been able to inhibit selectively with two

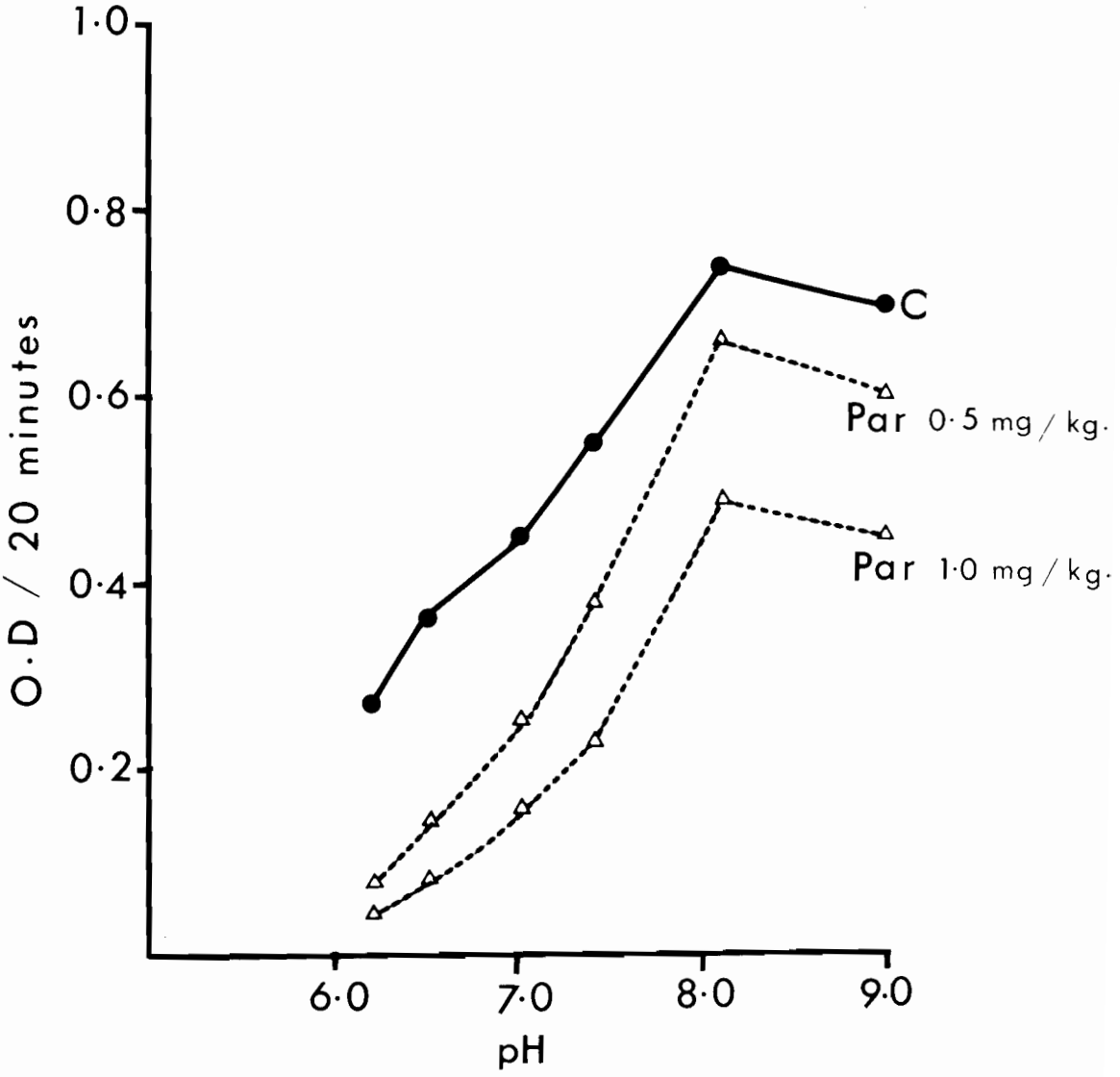


Fig. 15. The <u>in vivo</u> effect of pargyline (Par) on MAO activity at various pHs. (C) is the control. The dose of inhibitor (mg./ kg. body weight) was given 4 hours before the animal was sacrificed.

different non-hydrazine inhibitors, phenylcyclopropylamine and pargyline. Attempts to reverse the inhibitory action of these two inhibitors by dialysis against water failed.

Table X. The Effect of Inhibitors of MAO on the Partially Heat-Inactivated (50°C, 30 mins.) Enzyme

The conditions for these experiments are similar to those in Table V.

Inhibitors	Control			Experimental		
pH	6.5	7.0	7.4	6.5	7.0	7.4
Phenylcyclopropylamine, 1.6×10^{-7}M	0.42	0.31	0.47*	0.34(19)**	0.17(45)	0.22(53)
Pargyline, 6.2×10^{-8}M	0.48	0.24	0.36	0.25(48)	0.16(33)	0.32(11)

* Increase in optical density for the first 20 mins. incubation with the substrate.

** Figures in the brackets represent per cent inhibition.

9. The Effect of Riboflavin-Deficiency

If monoamine oxidase is in some way dependent upon adequate riboflavin nutrition of the animal, it would be expected that the enzyme prepared from the liver of riboflavin-deficient rats would oxidize a smaller proportion of kynuramine than would rats supplemented with the vitamin. The results of one set of experiments are shown in Table XI.A.

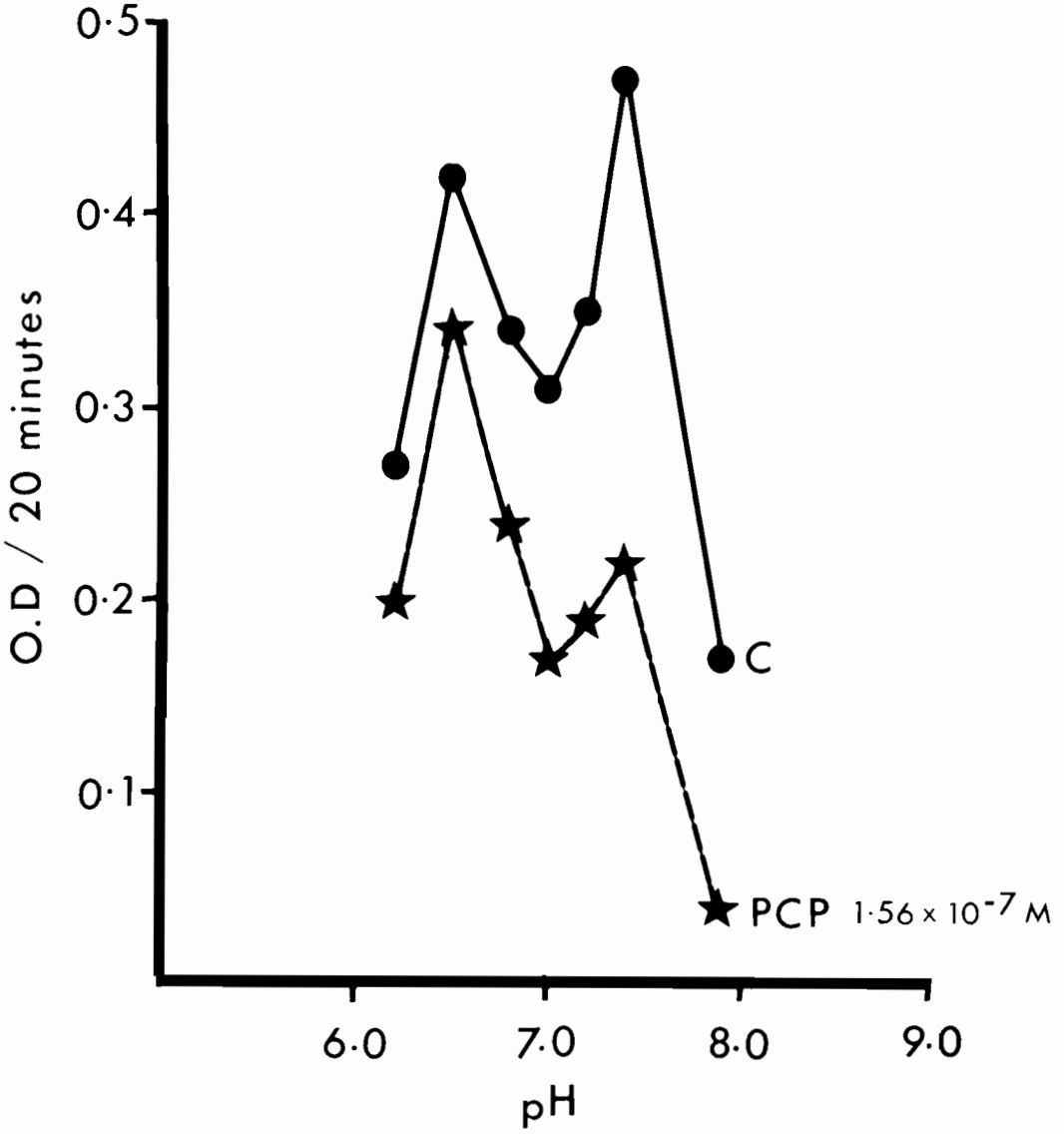


Fig. 16. The effect of phenycyclo propylamine (PCP) on the activity-pH curve of partially heat-inactivated (50 C, 30 mins.) enzyme. (C) is the control. The substrate is kynuramine (1.6 X 10^{-4} M).

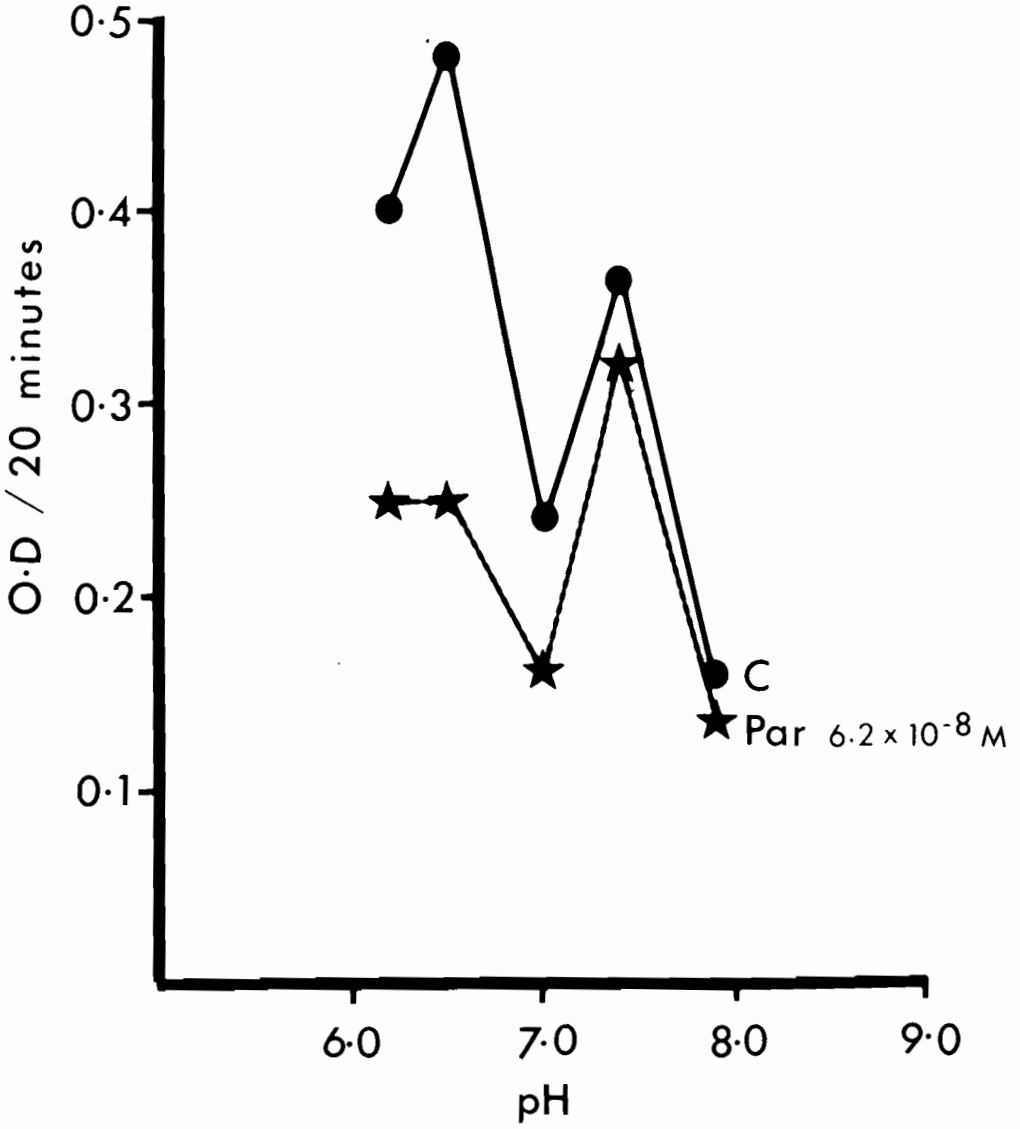


Fig. 17. The influence of pargyline (Par) on the activity-pH curve of partially heat-inactivated (50 C, 30 mins.) enzyme. (C) is the control. The substrate is kynuramine (1.6×10^{-4} M).

Table XI.A. The Effect of Riboflavin-Deficiency on the Activity-pH Curve of MAO

Set I

Days on diet	Supplemented			Deficient		
pH	6.5	7.0	8.1	6.5	7.0	8.1
5	0.32*	0.53	0.76	0.28(88)**	0.38(72)	0.60(80)
12	0.39	0.56	0.93	0.26(78)	0.35(68)	0.58(62)
19	0.29	0.44	0.68	0.19(64)	0.21(48)	0.35(52)
26	0.44	0.63	1.06	0.26(60)	0.29(46)	0.53(50)
36	0.44	0.56	0.90	0.27(59)	0.19(33)	0.34(38)
46	0.43	0.56	0.95	0.21(48)	0.14(24)	0.32(33)

* Increase in optical density for the first 20 mins. of incubation with the substrate.

** Figures in the brackets represent per cent activity.

Rats on a deficient diet showed a decrease in MAO activity even after five days. The rate of fall in activity was first order and took place at all pH's. The loss in activity was greater at higher pH's (Fig.18). At about 30 days the activity at pH's 7.0 to 9.0 dropped rapidly so that the shoulder obtained in the activity-pH curve with the native enzyme became more prominent. If the animal is kept longer on the deficient diet most of the activity at the pH 7.0 to 8.1 is lost, but, at pH 6.5, 50% of the activity is retained. These results have been confirmed by experiments on a second set of animals (Table XI.B.).

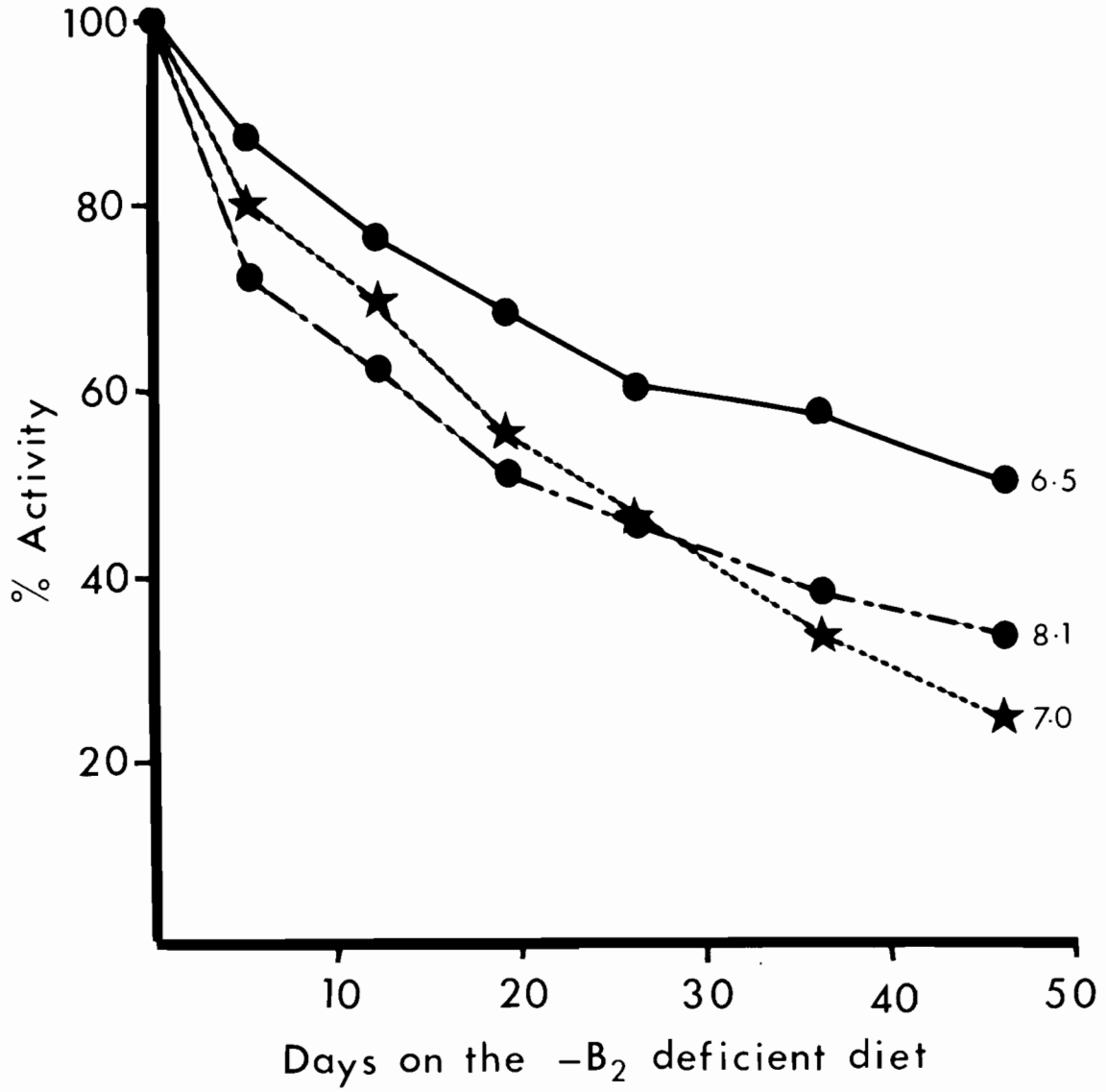


Fig. 18. The fall in the activity of MAO at variuos pHs with days on the riboflavin-deficient diet. Kynuramine (6.2×10^{-5} M) is the substrate.

Table XI.B. The Effect of Riboflavin-Deficiency on the Activity-pH Curve of MAO

Set II

Days on diet	Supplemented			Deficient		
pH	6.5	7.0	8.1	6.5	7.0	8.1
21	0.40*	0.57	1.19	0.31(78)**	0.40(70)	0.66(55)
36	0.16	0.28	0.44	0.11(68)	0.10(35)	0.15(35)
68	0.40	0.57	1.19	0.18(45)	0.07(12)	0.31(25)

* Activity in terms of optical density for the first 20 mins. incubation with the substrate.

** Figures in the brackets represent per cent activity of control.

Atebrin, an antimetabolite of riboflavin, was used in order to observe whether inhibition would be uniform across the activity-pH curve. Inhibition by this compound is presumptive evidence that flavin plays a role as a cofactor. Table XII shows the effect of atebrin on riboflavin-deficient rats and rats supplemented with the vitamin. Atebrin did not seem to affect the MAO activity of the riboflavin-deficient enzyme at pH's lower than 7.0 but activities at pH's above 7.0 were greatly reduced. Nearly 50% inhibition was produced at pH's 7.4 and 8.1 at a concentration of 1.6×10^{-5}M. At the same concentration, atebrin produced an inhibition in the enzyme prepared from the supplemented rats which closely resembled that obtained with the riboflavin-deficient enzyme (Table XII).

Table XII. The Effect of Atebrin on the MAO Prepared from Riboflavin-Deficient Rats

The conditions for these experiments were similar to those outlined in Table IV. The supplemented animals received a daily dose of 30 mg/kg diet of riboflavin 5-phosphate in 5% sucrose solution. The deficient group received an equivalent amount of 5% sucrose solution.

		Supplemented			Deficient		
	pH	6.5	7.0	8.1	6.5	7.0	8.1
No atebrin							
12+		0.39*	0.56	0.93	0.30(77)**	0.35(63)	0.58(62)
36		0.16	0.24	0.89	0.11(69)	0.14(58)	0.40(45)
Atebrin, 1.6×10^{-5}M							
12		-	-	-	0.27	0.29	0.43
36		0.14	0.21	0.44	0.10(71)	0.12(57)	0.21(48)

* Optical density for the first 20 mins. incubation with the substrate.

** Figures in the brackets represent per cent of control activity.

\+ Days on riboflavin-deficient diet.

10. Sucrose Gradient Studies

The particulate fraction, freshly prepared as described under "Methods", was resuspended above the gradient as shown in Fig. 19a.

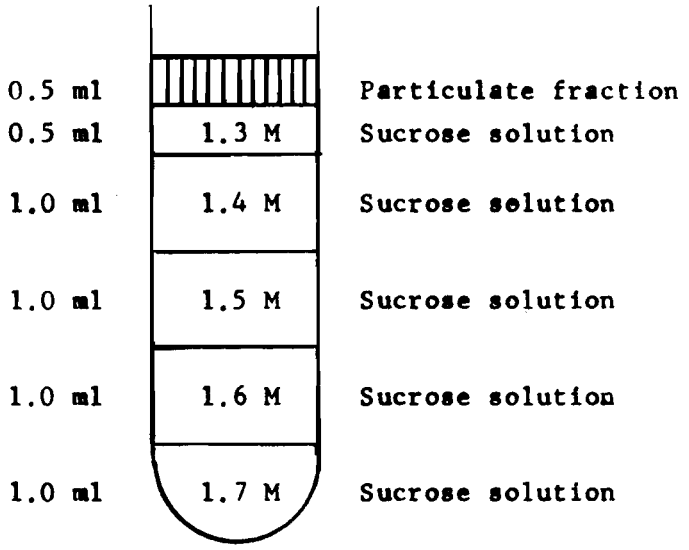


Fig. 19a

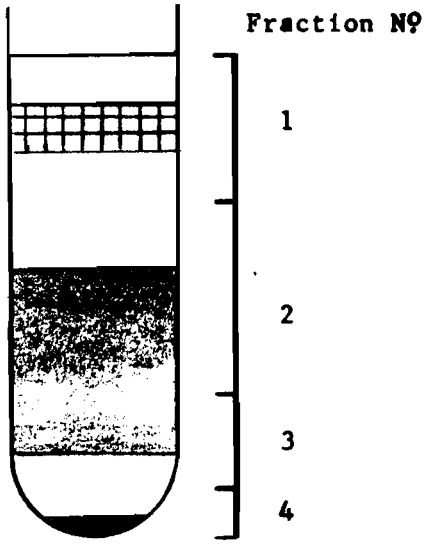


Fig. 19b

The typical appearance of the tube at the end of the 2 1/2 hour centrifugation at 40,000 RMP. (156,000xg) in a swinging bucket rotor is shown in Fig. 19b. In all experiments, a brown sediment was seen but occasionally a very small amount of whitish material had also sedimented. Several layers had formed at the boundaries of the sucrose solution. One well-defined band was always seen at the junction of the 0.3M and 1.3M. Between 1.5M and 1.6M, a rather thick band usually appeared. Sometimes however, instead of one band, there were two, but these were not too distinct.

Fig. 19b also shows the positions at which the fractions were collected. This was done with the use of 0.5 ml. micro-pipette.

The fractions were numbered as shown in Fig. 19b. Fig. 20 gives the results obtained in these experiments with the riboflavin-deficient enzyme and with the enzyme prepared from the rats receiving a supplement of riboflavin. In these experiments, the activity was calculated per mg. protein. Fig. 20 shows the result of an experiment in which monoamine oxidase activity were determined.

Monoamine oxidase activity at all pH's had a maximum in fraction 2; this fraction contains 44% of the activity recovered in all fractions. Similar results were obtained with the riboflavin-deficient enzyme, except that the activities were much lower at all pH's (Table XIII).

Table XIII. The Distribution of MAO Activity on a Sucrose Density Gradient

pH	Supplemented			Riboflavin-deficient (45 days)		
Fractions	1	2-3	4	1	2-3	4
6.5	0.170*	0.270	0.11	0.07(41)**	0.09(33)	0.05(45)
7.0	0.240	0.351	0.17	0.10(42)	0.12(34)	0.06(35)
8.1	0.351	0.431	0.20	0.16(46)	0.21(49)	0.07(35)

* Activity per mg. protein (optical density for the first 20 mins. incubation with the substrate).

** Per cent activity of the supplemented.

Recently Gorkin has been able to obtain from mitochondrial fraction two fractions capable of oxidizing two different amines. It is not known whether the two fractions obtained by Gorkin are as a result of two different particulate (mitochondrial) fractions or that more than one enzyme exist in the same particulate fraction. Attempts to obtain fractions in which monoamine oxidase would show different activity-pH curves gave negative results. Though the activities (per mg. protein) for the fraction were different, the type of curves obtained were very similar (Table XIII) (Fig.20).

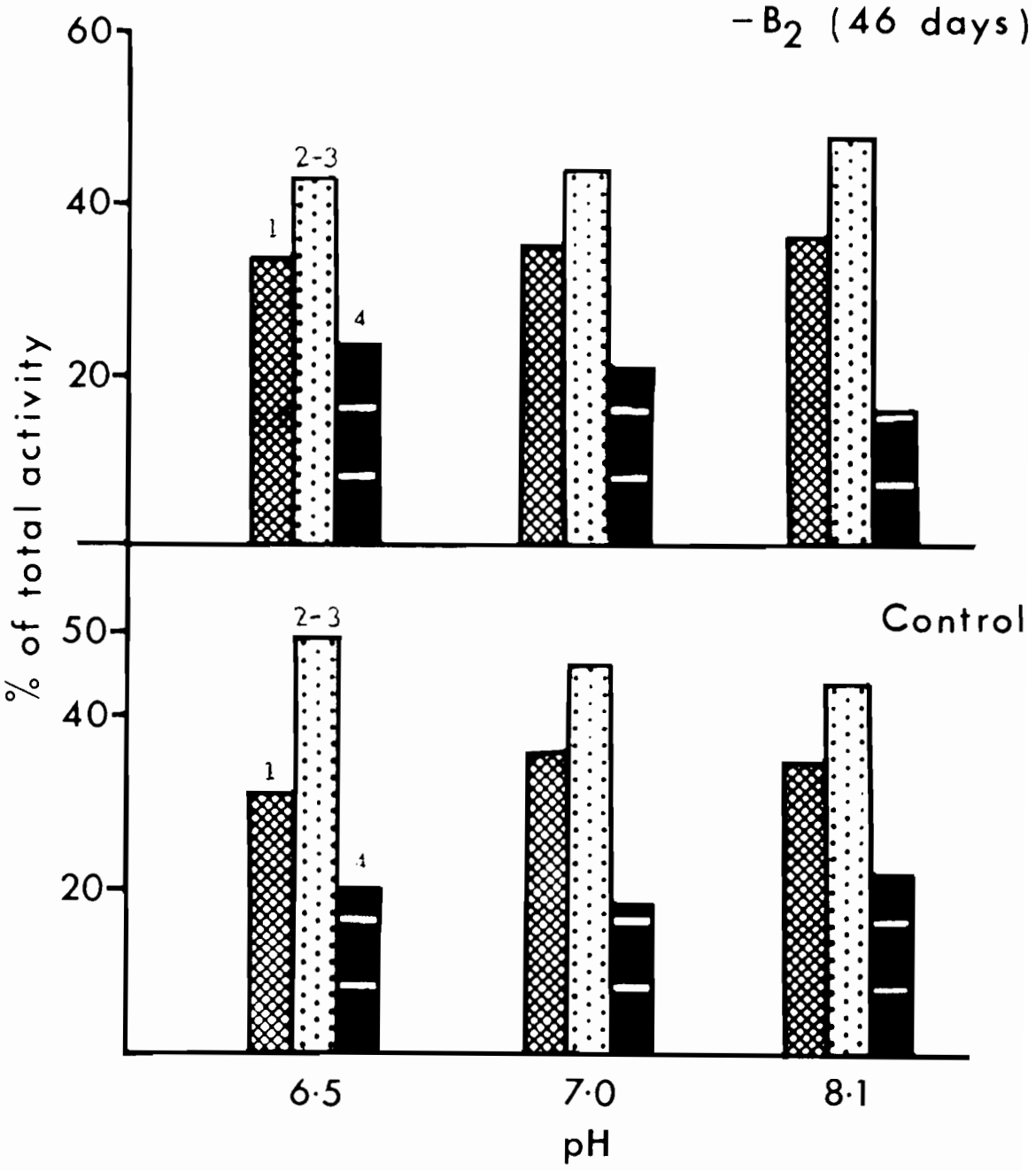


Fig. 20. Distribution of Monoamine oxidase in a specific density gradient. The fractions are numbered as shown in Fig. 19b. Ordinate: Recovery as percentage of recovery in all fractions.

11. The Effect of Pyridoxine-Deficiency

Rats fed a pyridoxine-deficient diet showed more or less the same monoamine oxidase activity as that of the control, Table XIV. Even after 54 days on the B_6 deficient diet, there was virtually no change in the activity. In some cases, the activity even increased. These results are in contrast to those obtained by using serotonin as a substrate. From unpublished works in this laboratory, it has been shown that the *in vivo* metabolism of serotonin, measured as 5-hydroxyindoleacetic acid, is reduced in pyridoxine-deficient animals, while riboflavin-deficiency did not impair the *in vivo* metabolism of serotonin.

Table XIV. The Effect of B_6 Deficiency on MAO Activity

All animals were fed pyridoxine-deficient diet. The supplemented animals received a daily supplement of pyridoxine 30 mg/kg diet in a 5% sucrose solution. The deficient animals received an equivalent amount of 5% sucrose solution.

Day on the diet	Supplemented			Deficient		
pH	6.5	7.0	7.4	6.5	7.0	7.4
20	540*	560	780	490(90)**	480(86)	720(92)
23	420	550	670	600(143)	470(85)	600(89)
36	365	401	612	210(56)	368(90)	542(89)
43	280	400	550	280(100)	430(108)	590(107)
54	425	565	1000	390(92)	560(100)	740(74)

* Increase in optical density for the first 20 mins. incubation with the substrate.

** Figures in the brackets represent per cent activity of control.

12. The Effect of Carbonyl Reagents on MAO Activity

Since no success was achieved with B_6 deficiency experiments, attention was turned to the carbonyl reagents. Inhibition by these reagents is regarded as evidence for the presence of pyridoxal phosphate. Of the carbonyl reagents studied, semicarbazide and hydrazine sulfate were the most effective (Table XV). These reagents apparently inhibit the enzyme at pH's lower than 7.0 and did not affect the activity at 7.4. Semicarbazide, at concentrations of 1.4×10^{-4}M and 8.6×10^{-4}M, inhibited the activity at pH 6.5 by 34% and 46% respectively, while at the same concentrations, virtually no inhibition was produced at pH 7.4 (Fig.21). The *in vivo* effects

Table XV. The In Vitro Effect of Carbonyl Reagents

The conditions for these experiments were similar to those outlined in Table IV.

Carbonyl reagents	pH	Control 6.5	Control 7.0	Control 7.4	Experimental 6.5	Experimental 7.0	Experimental 7.4
Hydrazine, 6.2×10^{-4}M		0.40*	0.52	0.70	0.28(30)**	0.42(20)	0.56(20)
Semicarbazide, 1.4×10^{-4}M		0.40	0.56	0.72	0.26(34)	0.48(14)	0.63(10)
8.6×10^{-4}M		0.35	0.43	0.57	0.19(46)	0.34(21)	0.53(7)

* Increase in optical density for the first 20 mins. of incubation with the substrate.

** Figures in the brackets represent per cent inhibition.

of isoniazid and desoxypyridoxine on the MAO of pyridoxine-deficient rats were also studied. Two groups of rats, having been on a B_6

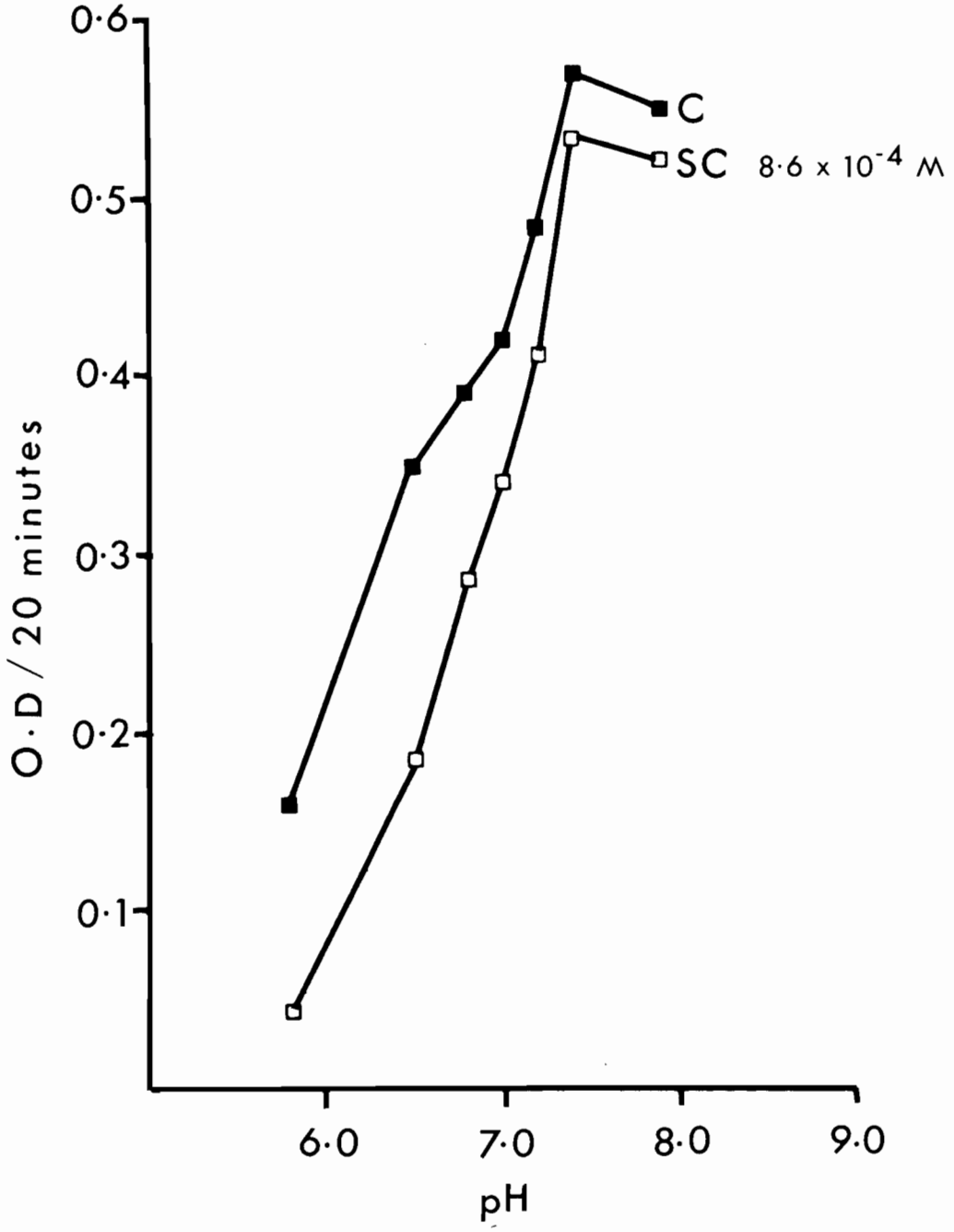


Fig. 21. Activity-pH curve of the native enzyme with (SC) and without (C) semicarbazide. Kynuramine (6.2 X 10^{-5} M) is the substrate.

deficient diet for 26 days, were injected intraperitoneally daily with 50 mg/kg isoniazid and 25 mg/kg body weight desoxypyridoxine respectively for seven days. A control group was also kept. On the 33rd day of deficiency, they were killed, their livers removed, and activity was determined.

Table XVI. The *In Vivo* Effect of Carbonyl Reagents on the MAO of Pyridoxine-Deficient (33 days) Rat Liver

The control is the 33rd day pyridoxine-deficient animals. The experimental is also 33rd day pyridoxine-deficient animals which had received the daily injection of carbonyl reagent.

Carbonyl reagents	Control (33 days deficient) pH 6.5	7.0	8.1	Experimental 6.5	7.0	8.1
Desoxy-pyridoxine,	0.56*	0.77	1.19	0.31(45)**	0.48(37)	0.83(30)
Isoniazid,	0.56	0.77	1.19	0.23(55)	0.43(44)	0.56(50)

* Increase in optical density for the first 20 mins. incubation with the substrate.
** Figures in the brackets represent per cent inhibition.

In both cases, rats receiving isoniazid and desoxypyridoxine showed reduced activity at all pH's but more at pH's lower than 7.0 (Table XVI). Where isoniazid had no inhibitory effect *in vitro*, it exerted its inhibitory property *in vivo*. The *in vivo* effect of carbonyl reagents on the MAO of B_6 supplemented rat liver has not been studied due to the lack of supplemented animals.

13. The Stabilization of Monoamine Oxidase by Substrates, Inhibitors and Possible Prosthetic Groups

O'Sullivan and Tomson (140) in 1890 were the first to describe the effect of the stabilization of an enzyme by its substrate.

Delory and King (141) concluded that stabilization of alkaline phosphatase by different substrates increases with the rate of hydrolysis. Thorough studies of the stabilization of D-amino-acid oxidase by various compounds such as substrate, competitive inhibitors and flavin-adenine dinucleotide were done by Burton (146).

The procedure for studying the stabilization is similar to that described for pH and thermal inactivation (page 40). The compound whose effect was under study was added to the enzyme-buffer preparation prior to heating. Heating was carried out at 50°C for 15 mins. After the heating the mixture was cooled and dialyzed for three hours against distilled water. The activity was then measured per mg. protein.

Various compounds were chosen to observe whether they would act to protect the enzyme from loss in activity or not. Substances which were substrates for MAO, such as benzylamine, epinine and isoamylamine stabilized the enzyme against heat, whereas non-substrates such as ethanolamine and diamines like histamine and diaminobutane did not (Table XVII). Each result is the result of one experiment.

Reversible inhibitors like harmaline and 8-hydroxyquinoline also protected the enzyme but their stabilization property was not as effective as the substrates of MAO. Also studied were the effect of pyridoxal phosphate and FAD (flavin-aenine dinucleotide). Neither of these two had any effect on the rate of inactivation of the enzyme (Table XVII).

The stabilization of MAO resembles that of D-amino-acid oxidase, also in other respects both enzymes act very similarly, but this will be discussed later.

Table XVII. Stabilization of Monoamine Oxidase by Substrates and Inhibitors

The enzyme-buffer (pH 7.4, 0.066 M concentration) preparation was heated (50°C, 15 mins.) in the presence of the substance whose protective property was to be studied. All compounds were present at 1.6×10^{-4}M (final concentration) except the last two.

Compounds	Control (100%)	Control heated	Experimental (heated in presence of indicated compound)
Benzylamine	0.60*	0.38(63)**	0.55(91)
Dopamine	0.53	0.15(28)	0.27(51)
Epinine	0.55	0.15(28)	0.26(49)
Isobutylamine	0.47	0.12(25)	0.19(40)
Isoamylamine	0.47	0.12(25)	0.16(34)
Ethanolamine	0.60	0.38(63)	0.38(63)
Histamine	0.60	0.19(32)	0.20(33)
Diaminobutane	0.60	0.38(63)	0.42(70)
Harmaline	0.53	0.16(30)	0.24(45)
8-Hydroxy-quinoline	0.53	0.16(30)	0.22(40)
FAD, 1.6×10^{-5}M	0.40	0.15(38)	0.15(38)
Pyridoxal phosphate, 1.6×10^{-5}M	0.40	0.15(38)	0.13(32)

* Increase in optical density, per mg. protein, for the first 20 mins. incubation with the substrate.

** Figures in the brackets represent per cent activity of one experiment.

IV. DISCUSSION

The greater susceptibility of MAO to thermal inactivation at extreme pH values is typical of most enzymes. It may be noted that the pH range of greatest thermostability is 6.0-8.0. Since the enzyme has not been purified and the isoelectric point identified, one cannot say exactly where in the pH range the enzyme exhibits its greatest thermostability. Johnson, Eyring and Polissar (142) have suggested that enzymes with an acid isoelectric point exhibit the greatest stability at more alkaline pH values and vice versa. As indicated in the explanation of the results, MAO tends to be more sensitive to heat at the alkaline pH. The greatest loss in activity occurs between pH's 7.0 and 8.1. This fall in activity gives rise to a second peak at pH 6.5. The fact that the inactivation of MAO does not follow first-order kinetics is illustrated by the time courses (Fig.1,2), and this is confirmed by the fact that a plot of log. activity against time is not linear. The order with respect to time is obviously not unity, and the falling off of the first-order constant may be explained in various ways:

1. The inactivation is reversible and an equilibrium is reached. This explanation is ruled out by the fact that MAO inactivation is irreversible.
2. Two MAO enzymes may exist, having different thermostabilities and they may differ in other respects.

The activity-pH curve showed a shoulder around pH 6.5-7.0 and a peak at pH 7.4 using phosphate buffer, or at pH 8.1 using borate buffer. It was thought that the borate buffer may affect the product

of kynuramine oxidation, 4-hydroxyquinoline, in that it increases the absorbancy at 315-330 mμ. This increase might be the result of the reaction of borate with 4-hydroxyquinoline causing it to enolize and therefore increase the absorbancy. The ultra-violet spectra of kynuramine and 4-hydroxyquinoline in borate buffer pH 8.1 were not different from that in phosphate buffer pH 7.4. The shoulder obtained at pH 6.5 has also been reported by Barbato and Abood (49). Also using kynuramine, they obtained an activity-pH curve which had a shoulder around pH 7.0. They have obtained a similar activity-pH curve with benzylamine as substrate. Horita (143), studying the influence of pH on the serotonin metabolism by rat heart homogenate, also obtained a shoulder around pH 7.4-8.8 and a peak at pH 9.5. In order to eliminate any doubt that the shoulder might be an artifact, the effect of pH on the absorbancy of kynuramine and 4-hydroxyquinoline were studied. No effect was observed. The effects of various concentrations of buffers on the activity of the native enzyme were also studied. There was no change in activity or in the position of the shoulder.

When the enzyme was heated in the presence of various phosphate buffers 0.031M final concentration for various times (Fig.3) two peaks, at pH 6.5 and at pH 7.4, were obtained. The fall in activity at these pH's was first order (Fig.4). The presence of a double pH optima can be explained in a number of ways: (1) the presence of 2 distinct isoenzymes with different pH optima, (2) formation of active enzyme-substrate complexes by ionic supplement, of the enzyme which differs by at least 2 protons, (3) the presence of an ampholyte

inhibitor and (4) the enzyme having more than one active site. Schwimmer (144) has been able to put the above postulate into mathematical form. His mathematical theories are appliable for a pure preparation of the enzyme only, and are too complicated. Because our studies have been done on the mitochondria, we do not know what really happens when inactivation takes place. In a pure state, the enzyme may act completely differently. But we have evidence that may rule out postulations 2-4 and increase the possibility for the presence of two isoenzymes. The fact that heat inactivation at various pH's is first order (Fig.4), and that when heating took place in buffer concentrations of various strengths, the activity-pH curve did not change, rule out the possible existence of two ionic sites or more than one enzyme-substrate complex formation. The possibility that the results are due to polymerization of monoamine oxidase is discounted because of the low enzyme concentrations used and because the initial rates of enzyme activity were proportional to enzyme concentration at both high and low substrate concentration/support for the presence of two isoenzymes comes from studies on heat inactivation at various pH's. The time course for the inactivation of MAO, obtained at pH 6.5, 7.0 and 7.4 are different. The loss of activity was greatest at pH 7.0, followed by the loss at pH 7.4 and then at 6.5. Kamaryt and Zdenek (145), working with isoenzymes of lactic acid dihydrogenase and their susceptibility to heat inactivation, have shown that the isoenzymes of lactic acid dehydrogenase differ in their time course for the inactivation. Indirect evidence also for the multiplicity of MAO comes from the fact that the enzyme lacks substrate specificity.

The effect of the concentration of coenzyme, substrates and inhibitors on the rate of enzyme-catalyzed reaction is often discussed in terms of a combination of the enzyme and the coenzyme, substrate or inhibitors. Burton (146), studying stabilization of D-amino acid oxidase by cofactors, substrates and competitive inhibitors, has come to the conclusion that if stabilization of the enzyme by cofactors, substrates of competitive inhibitors were also connected with a similar combination, at the same site on the enzyme the protection constants should be related to the Michaelis constants determined at the same temperature and pH. He is supported in this respect by Boyer, Lum, Ballos, Luck and Rice (147) and Boyer, Ballou and Luck (148).

The relation between the rate of inactivation and the concentration of the protector can be explained if the apo-enzyme P combines with the protector X to form a complex PX, which is less readily denatured than the apo-enzyme.

Let α_X be the dissociation constant of the PX comples, $\alpha_X = \frac{(P)\ (X)}{(PX)}$ if (P), (X) and (PX) are equilibrium concentration.

Let k_o be the rate constant of inactivation of the free apo-enzyme P and let k be the rate constant of inactivation of the complex PX.

The resultant rate constant of inactivation of the enzyme in an equilibrium mixture of P, X and PX is

$$K = \frac{(P)k_o + (PX)k}{(P) + (PX)}$$

$$= \frac{\alpha_x k_o + (X)k}{\alpha_x + (X)}$$

when $(X) = \alpha_x$, $K = \frac{k_o + k}{2}$

Therefore $\Pi_x = \alpha_x$

Π_x is the protection constant.

Burton in his studies supported by others (148) has found that the curve of stabilization of an enzyme by its substrate at various concentrations resembles the effect of substrate concentration on the rate of enzyme action. By analogy with the Michaelis constant, the protection constant II can be evaluated.

When benzylamine protects the MAO, Π_B should be equal to the expression $\frac{(P)\ (\text{benzylamine})}{(\text{P-benzylamine})}$. The protection constant of any compound can be represented by the above equation. Compounds which are substrates of the enzyme are found to protect the oxidase. With a better substrate, there is more protection. Several substances, not being substrates, do not protect the oxidase under the condition studied. Flavin-adenine dinucleotide (FAD), suggested to be the co-factor of MAO, does not protect the enzyme. In this respect MAO differs from that of D-amino acid oxidase. Pyridoxine (B_6) did not have protective properties either. Some of the inhibitors of MAO, harmaline and 8-hydroxyquinoline, protected MAO from inactivation, but they were not as good as the substrates. During the course of inactivation, the active site is involved. The substances which protect the enzyme from denaturation must react with the active site of the enzyme and protect it.

It has been reported that an -SH group is involved in the active site of the oxidase. Studies with sulfhydryl compounds have shown that these compounds exert inhibitory effects in vitro. This is in agreement with the view that sulfhydryl group functions in the oxidation of the substrate and that an excess of some -SH compound or of a disulfide could inhibit MAO at one phase of the catalytic process. If a sulfhydryl group is necessary for the activity of the oxidase, then the enzyme heated in the presence of oxygen might show less activity than the enzyme heated in presence of nitrogen. As shown on Table III, the results are in agreement with the above suggestion. Oxygen would tend to oxidise the -SH group which may give rise to -S-S-. Substrates and inhibitors tend to protect the active site from such oxidations. After heating the preparation in the presence of nitrogen, some MAO activity was lost. This might be due to the presence of oxygen in the nitrogen and dissolved oxygen in the enzyme mixture. To check against the presence of oxygen, nitrogen was passed through a solution of hydroquinone to remove any oxygen which might be present. The results of this experiment showed that the enzyme, in the presence of nitrogen which had been passed through a solution of hydroquinone, was not inactivated as much as in the previous preliminary experiments. The small amount of inactivation obtained with the nitrogen might be as a result of surface denaturation. The results of these experiments are in agreement with those of previous findings that an SH group is necessary for the activity of the enzyme.

The question whether the enzyme has a metal or a cofactor

has not been fully answered. But there is much evidence to support that the enzyme has a metal and a cofactor. Other amine oxidases such as plasma-amine oxidase (Yamada and Yasunobu, 137), benzylamine oxidase (138) and diamine oxidase (pea seed plant amine oxidase) (Mann, 139) are known to have copper and a carbonyl as prosthetic groups. The group of amine oxidases inhibited by carbonyl reagents such as hydrazine and hydroxylamine includes the plant and animal diamine oxidases, histaminase, spermine oxidase (E.C.1.5.3.3.) and benzylamine oxidase (Bergeret, Blaschko and Hawes (149)). This is in contrast to the animal monoamine oxidase which is not inhibited by hydrazine or by the carbonyl reagents. Gorkin, studying the effect of chelating agents on the enzyme, came to the conclusion that the enzyme had a metal as a prosthetic group. He was able to antagonize the effect of inhibition produced by chelating agents with metal ions. He was unable to reach a definite conclusion as to which metal is involved in MAO. Lagnado and Sourkes have studied the effects of various metal ions on the activity of MAO. With some metals, at a high concentration, the enzyme was inhibited and while with other metals at lower concentration, MAO activity was manifested. They were not able to activate the enzyme by a specific metal. Green (64), with his studies on the mechanism of inhibition of monoamine oxidase with hydrazine inhibitors, has outlined a theory suggesting that MAO is a copper-containing enzyme. His theory is based upon the assumption that inhibition by hydrazine derivatives results from a copper-catalysed liberation of free radicals in the vicinity of the enzyme's active centre. There are a number of faults with this theory: (1)

rats made copper deficient did not show any decrease in MAO activity even though the copper store in the liver is decreased by 75%. It may be possible that the remaining 25% of copper would be sufficient for the activity of MAO. However, with riboflavin-deficiency there was some deterioration of enzymatic activity, (2) Diethyldithiocarbamate, a chelator used for the estimation of copper, showed significant inhibitory effects only when a final concentration of 10^{-3}M was used. In comparison to the other chelators, such as 8-hydroxyquinoline and thenoyltrifluoroacetone (an iron reagent), diethyldithiocarbamate was weak in inhibiting the enzyme. (3) No extensive studies have been carried out by Green to show whether or not other metal ions act in the same manner as copper on the hydrazine-inhibited active site of the enzyme.

Attempts to dialyze the metal with the use of chelating agents have failed. The metal, if present, must be tightly bound to the enzyme. The bond produced between the chelating agent and the metal ion is not strong enough to separate it from the enzyme.

Hitherto no prosthetic group has been resolved from amine oxidase, but on many occasions, the suggestion has been proposed that the enzyme possess a flavin nucleotide in its structure (55). No direct evidence has been found to support this hypothesis. On the one hand, it has been shown that the livers of riboflavin-deficient rats have less activity than those from control animals (57,58). Inhibition by atebrin further implicates a flavin, since this inhibitor exerts a specific action on flavoproteins (150) and also decreases

the oxidation of amines *in vivo* (59) and *in vitro* (58). Richter in 1937 drew attention to some of the similarities in the reaction catalyzed by this enzyme and amino acid oxidases. The parallel in reaction with the amino acid oxidase, which is known to be yellow enzymes is food for thought. Both these enzymes catalyze the conversion of $-CH-NH_2$ to $-C=NH$; both are efficiently carried out only under high partial pressures of oxygen, a characteristic of flavin enzymes, and both result in the formation of hydrogen peroxide.

Hawkins, in her studies with riboflavin-deficient rats, has suggested that the vitamin may be contained in enzyme protein. With inositol present in the diet, riboflavin was more effective in restoring amine oxidase activity. There was no rapid restoration of the enzymatic activity after the rats received riboflavin. Hawkins has further suggested that inositol is required either for the synthesis of a prosthetic group, or of a new enzyme protein. In the studies on riboflavin-deficiency using kynuramine as substrate, the protein content of the washed mitochondria was measured. There was no decrease in the protein content of mitochondria. This might rule out Hawkin's suggestion that the vitamin is necessary for the synthesis of the enzyme.

Considering the various functions of pyridoxal phosphate in the intermediary metabolism of amino acids and amines, a decrease in action by MAO resulting from rats deprived of this vitamin, has been anticipated. Rats put on a pyridoxine-deficient diet, showed more or less normal levels of MAO activity with a decrease on a few

occasions. Wiseman and Sourkes (151) have reported that riboflavin-deficient rats did not reveal any decrease in MAO activity, as measured in vivo by excretion of 5-hydroxyindoleacetic acid (5-HIAA) after administration of a serotonin (5-HT) load. In a recent paper (152) they have shown, as they had previously demonstrated with rats receiving 5-HT intragastrically, that riboflavin-deficiency does not alter the metabolism of intraperitoneally injected amine, when measured by the excretion of its acid metabolite 5HIAA. But from unpublished work of this laboratory, it was observed that the in vivo metabolism of serotonin was impaired with rats on pyridoxine-deficient diet. The results obtained with deficiency studies are conflicting and confusing. Some of these results may be explained if one assumes that there are more than one monoamine oxidase, and that they are responsible for the metabolism of various amines. A number of investigators (143) have pointed out that serotonin metabolism may involve more than one enzyme. This may answer the question raised by the above result that riboflavin-deficiency does not impair the activity of MAO when serotonin is used as substrate. When riboflavin-deficiency sets in, the normal route of serotonin metabolism may be upset and a new pathway involving the role of B_6 comes into being. There is another justification for this hypothesis; when a load of serotonin is given to a rat, only about 40% of it can be accounted for as 5HIAA. What happens to the rest of the serotonin is unknown. As mentioned earlier, there might be a different pathway of serotonin metabolism. Kapeller-Adler and Macfarlane (153) have reported that the prosthetic group of histaminase contains pyridoxal phosphate and FAD. In studying the absorption spectrum of the enzyme, two maxima

at 330 and 405 were obtained. These can be attributed to pyridoxal phosphate. From the absorption and fluorescence spectra reported by Kapeller-Adler and Macfarland (154), Mann (155) has calculated that 1 mole of pyridoxal phosphate is present in 40,000 - 50,000 g. of the preparation and 1 mole of FAD in 1,000,000 - 2,000,000 g. This suggests that further evidence is necessary to exclude the possibility that FAD is present as an impurity. The original suggestion that the enzyme might contain pyridoxal phosphate and FAD was made by Werle and Pechmann (156). When plant saps containing diamine oxidase were dialyzed for several days to remove the prosthetic group, the rates of the oxygen uptake were increased by adding pyridoxine hydrochloride to the dialysed saps. Goryachenkova (157), studying the same system, reported that both the uptake of oxygen and the liberation of ammonia were increased when pyridoxal phosphate and FAD were added. For the above reasons, and because rats put on a riboflavin-deficient diet did not show a complete deterioration of MAO activity, both FAD and pyridoxal phosphate may act as the prosthetic group of MAO. The presence of pyridoxal phosphate would explain why the enzyme is inhibited slightly <u>in vitro</u> and by 50% <u>in vivo</u> by carbonyl reagents such as hydrazine sulfate, and semicarbazide.

Horita (143), studying the influence of pH on serotonin metabolism in various tissue homogenates, has indicated the presence of at least two enzyme systems in the heart tissue of rat which are capable of metabolizing serotonin. In his studies with inhibitors, he found that KCN inhibited the heart enzyme at pH 9.5 while phenylisopropylhydrazine (a MAO inhibitor) inhibited the enzyme at the physiological pH 7.4. In my own studies, I have found similar results

with the rat liver mitochondrial MAO using kynuramine as substrate. Phenylisopropylhydrazine inhibits the enzyme at pH 7.4 and 8.1 but there is little inhibition at pH 6.5. In the control, activity-pH curve of the enzyme shows a shoulder at pH 6.5 and a peak at pH 8.1, while the enzyme inhibited with phenylisopropylhydrazine shows only a peak at pH 6.5. Semicarbazide (10^{-3}M), a carbonyl reagent, inhibited the native enzyme at the lower pH's. Whatever the explanation, it is clear from these experiments that at pH levels of 6.5 and 8.1, rat liver mitochondria contain two systems which can metabolize kynuramine.

Studies with chelating agents and other inhibitors of MAO have revealed very interesting results. Not all the chelating agents or MAO inhibitors had the same effect on activity-pH curve. With the exception of phenylisopropylhydrazine, the other hydrazine inhibitors and phenycyclopropylamine seem to act the same on the activity-pH curve. However pargyline, a non-hydrazine inhibitor, appeared to act differently for it inhibited the enzyme more at the lower pH's. In this respect, pargyline is acting very similarly to chelating agents. The possible explanation of this is that the acetylene group on the side chain of phenyl group may be reacting with a metal on the enzyme, thus forming an irreversible complex. (Attempts to dialyze the inhibitor at pH 6.5 have failed).

The oxidative deamination of various monoamines in the animal is usually attributed to the action of a single enzyme, monoamine oxidase/oxidoreductase (deaminating), EG 1.4.3.4. But in recent

years numerous data have been published to support the theory of the multiplicity of mitochondrial amine oxidases.

Most of these data have been obtained with inhibitors. Using different substrates of MAO, an inhibitor will give different degrees of inhibition. This has been regarded by some workers (2, 3,50) as evidence to support the theory of the multiplicity of MAO.

In my own studies, I have obtained similar results. The activity-pH curves show a shoulder around pH 6.5 and a peak at 7.4. The fact that it has been possible to inhibit selectively the shoulder at pH 6.5 with some chelating agents and inhibitor and the peak at pH 7.4 with some other inhibitors would suggest the presence of two systems both capable of oxidizing kynuramine, one system being more active than the other. The two systems differ from each other in many respects.

(a) Heat-inactivated studies have revealed that the rate of denaturations (the fall in activity) differ at pH's 6.5 and at 7.4, that is, the enzyme is more heat stable at pH 6.5. Gorkin (48), using tyramine and benzylamine as substrates, has shown that tyramine oxidation system is considerably more sensitive to controlled heating than that of benzylamine. After heating the enzyme preparation at 48°C for 45 minutes, the system that oxidizes benzylamine loses 54% of its activity while that of tyramine loses 100%. When the enzyme is heated at 50°C for 80 minutes, using kynuramine as substrate, it still has most of its activity (Fig.1). Here again the enzyme(s) is acting differently towards kynuramine. This would tend to support Gorkin's findings.

In a recent paper Gorkin (158), studying the *in vitro* effects of proflavine on MAO, found that proflavine acts as a competitive inhibitor in the case of serotonin oxidation by MAO prepared from rat liver. In similar experiments with tyramine as substrate, proflavine causes a non-competitive inhibition. The amount of inhibition by proflavine in the presence of each substrate was significantly different. The inhibition of oxidation of tyramine by proflavine could not be reversed whereas with serotonin as substrate, partial reversibility of the inhibitory effect of proflavine was achieved. This would support the suggestion of Hardegg and Heilbron (50) that the two enzymes oxidizing serotonin and tyramine are different. The fact inhibition by proflavine was partially reversible when serotonin was used as substrate may also lend support to the possibility of more than one enzyme, none reversibly inhibited (similar to the inhibition produced by proflavine when tyramine is used as substrate) and the other one reversibly, being involved in the deamination of serotonin. It would be interesting to determine the effect of proflavine on the activity-pH curves of serotonin and kynuramine and to see whether the inhibition is reversible at the different pH values.

(b) The mitochondrial MAO is also inhibited differently at the various pH's by inhibitors, chelating agents and carbonyl reagents. These have already been discussed.

(c) Riboflavin-deficiency studies have also contributed to the increased evidence that whatever systems are involved in oxidizing kynuramine at the different pH's are different. Carbonyl reagents inhibit the enzyme at pH's lower than 7.0. At these pH's the

loss in activity is less than at pH's above 7.0 in the MAO prepared from riboflavin-deficient livers of rats.

The data obtained can be explained by assuming (1) the existence of either two different structure-bound monoamine oxidases in the rat liver mitochondrial preparation or (2) of ionization sites on the single enzyme molecule.

All indications are that two ionization sites are not involved in the case of rat liver MAO. Gorkin (46, 159) has been able to separate partially rat liver mitochondrial amine oxidases. Using a non-ionic detergent OP-10 he has solubilized the mitochondria. Applying the solubilized material to a "Brushite" column and stepwise elution he has obtained two fractions capable of attacking two different amines, namely, m-nitro-p-hydroxybenzylamine (PHB) and p-nitrophenethylamine (PN). Ultracentrifugation of washed rat liver mitochondria, using kynuramine as substrate has not revealed different MAO activity, though using other substrates may. This would readily be understood if one assumes the existence in mitochondrial membranes of systems of multiple amine oxidases differing in substrate specificity or if one assumes that MAO may exist as a number of subunits, and each subunit acts differently with respect to heat-inactivation, oxidation of substrates, and its reaction towards inhibitors. Each subunit may also differ in cofactor requirement(s). Further investigations are necessary to find out whether the enzyme acts in this manner or not.

In summary evidence has been provided to support the theory that more than one monoamine oxidase exist, capable of oxidatively

deaminating a wide variety of amines. This evidence is also supported by the works of other investigators. By no means can this be counted as final proof since the enzyme has not been purified.

V. SUMMARY

In the recent years it has become popular to speak of not one monoamine oxidase but a series of them, each differing in its action towards substrates and inhibitors. Most of the work, done as evidence for the multiplicity of MAO, has been carried out on the impure enzyme prepared from the mitochondrial fraction. In these studies MAO was prepared very similar to that of Hawkins. These investigations have revealed that when kynuramine is used as substrate the activity-pH curve shows a shoulder around pH 6.5 and a peak at pH 7.4 (phosphate buffer) or 8.1 (borate buffer). Heat-inactivation studies revealed that the rate of inactivation at pH 6.5 and 7.4 are different, indicating that two systems may be involved in the oxidation of kynuramine. This point has been further strengthened with other studies such as the effect of inhibitor, chelating agents, carbonyl reagents and riboflavin-deficiency on the activity of MAO. While riboflavin-deficiency and most inhibitors had their greatest effect in reducing the oxidation of kynuramine at pH's higher than 7.0, the chelating agents and carbonyl reagent inhibited the enzyme much more at pH's lower than 7.0. The data from the riboflavin-deficiency experiments in vitro indicate that riboflavin may take part in the metabolism of kynuramine, though attempts to restore MAO activity by adding riboflavin has failed. Atebrin, an antimetabolite of riboflavin, inhibited the enzyme prepared from the supplemented and riboflavin-deficient rats. Inhibition by this reagent is regarded by many investigators as presumtive evidence for the role of riboflavin as a cofactor of MAO. Neither pyridoxine or copper-deficiency had any effect on the activity-pH curve of the enzyme. The possibility

that the enzyme has a metal has been increased by the fact that chelating agents inhibited strongly the activity of the enzyme. The activity of the enzyme could be restored by dialyzing the enzyme-chelating preparation against water. As yet no metal has been assigned to MAO.

Whatever the explanation, it is clear from the results that at pH levels of 6.5 and 7.4 or 8.1 the rat liver mitochondrial MAO contains two systems which can oxidize kynuramine. That both these systems are active is seen by the degree of inhibition in the presence of inhibitors of MAO and chelating agents. The results can be explained (a) assuming that there are two or more monoamine oxidases or (b) that the enzyme has more than one ionizable site capable of oxidation. Evidence obtained in these studies and by other workers in this field point strongly to the assumption that isoenzymes of monoamine oxidase exist in the mitochondria.

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