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Ph.D. BIOCHEMISTRY

Short Title:

Iron-Dependency of Monoamine Metabolism in the Rat in Vivo.

Ph.D. Biochemistry

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EFFECTS OF DEFICIENCIES OF IRON, COPPER AND RIBOFLAVIN IN THE RAT ON MONOAMINE OXIDASE ACTIVITY

ABSTRACT

The mitochondrial monoamine oxidase (MAO) activity of livers from rats deficient in iron, copper or both those elements was compared with the activity in livers from normal rats. The activities towards isoamylamine, tyramine and benzylamine were studied by means of oxygen uptake using an oxygen electrode.

MAO activity of the livers decreased significantly ($P < 0.01$) when the levels of liver iron decreased. Copper-deficiency had no effect on the enzyme.

Expired radioactivity collected from rats injected with [1-^{14}C]n-pentylamine (5 μCi/kg) was measured as $^{14}CO_2$. The rate of catabolism of the amine decreased progressively in iron-deficient rats, reaching about 60% of control values in 3 weeks. Feeding with iron yielded normal rates within 6 days. Catabolism of amyl alcohol, which shares a common pathway with n-pentylamine via valeraldehyde, was unaffected by iron-deficiency.

These results are consistent with a function of iron in the maintenance of normal MAO activity in vivo.

Some iron chelators and hemolytic agents also inhibited MAO.

Polyacrylamide gel electrophoresis of the partly purified enzyme showed that MAO exists in multiple forms.

EFFECTS OF DEFICIENCIES OF IRON, COPPER AND RIBOFLAVIN IN THE RAT ON MONOAMINE OXIDASE ACTIVITY

By

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Submitted to the Faculty of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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March, 1972

THIS WORK IS DEDICATED WITH LOVE AND APPRECIATION TO MY DEVOTED WIFE, VALDA. DURING THE COURSE OF THE INVESTIGATIONS RECORDED IN THIS THESIS, SHE WAS FATHER AS WELL AS MOTHER TO OUR CHILDREN. HER INTEREST, ENCOURAGEMENT AND REASSURANCES HELPED ME TO PERSEVERE THROUGH MANY DIFFICULTIES IN MY FEEBLE ATTEMPTS TO FURTHER THE CAUSE OF SCIENCE.

ACKNOWLEDGMENTS

The author thanks Professor Theodore L. Sourkes sincerely for his stimulating guidance and encouragement throughout the course of these investigations.

I would like to express my gratitude to Mrs. Brenda Ley for her invaluable assistance in the preparation of diets, caring for the experimental animals and performing some of the assays for copper and iron in tissues.

Special thanks are due to Miss Elizabeth Wagmann for the care, skill and enthusiasm that she put into the typing of this Thesis. Also deserving of special mention are my colleagues, the Misses Linda Bloomer and Maureen Kiely, for their help in preparing and assembling copies of the finished manuscript.

Financial support was provided by a Research Grant from the Medical Research Council of Canada. This assistance is hereby gratefully acknowledged.

PREFACE

1. Monoamine oxidase (monoamine:oxygen oxidoreductase (deaminating) E.C. 1.4.3.4.) (MAO) catalyzes the oxidative deamination of biogenic monoamines such as tyramine, tryptamine, serotonin, the catecholamines dopamine, norepinephrine and epinephrine, and aliphatic monoamines such as butylamine, pentylamine and hexylamine. This enzyme, was first described by Hare in 1928, and is active *in vitro* as well as *in vivo*.

2. The typical mitochondrial enzyme is widely distributed in the animal body, occurring in vertebrate tissues such as liver, glandular organs, smooth muscle, cardiac muscle and in the nervous system. MAO is concerned mainly with the metabolism of a variety of biologically active amines. It is believed to play an important intracellular role in the regulation of the levels of biogenic monoamines in the central nervous system. This role of MAO is demonstrated by the pronounced increases in the levels of serotonin and catecholamines in the brain, induced by inhibition of MAO. Clinical findings have demonstrated a protective function of MAO for the enzymic deamination of highly active biogenic amines *in vivo*. Dramatic accounts of the markedly toxic (occasionally fatal) effects arising from the ingestion of tyramine-rich or amino-acid-rich foods, or sympathomimetic drugs following MAO-inhibitor treatment have been recorded in the literature.

3. Indirect evidence for the existence of multiple forms of MAO has long been accumulating. The physical separation of

the enzyme prepared from certain organs into multiple forms having distinct physicochemical and biochemical properties has been achieved recently.

4. Samples of partially purified enzyme were prepared by Youdim's method for use in some of the experiments reported in this Thesis.

5. The results of the investigations which were undertaken during the course of these studies are recorded in this Thesis, including:

(a) Studies on the nutritional cofactor requirements of MAO. The effects of deficiencies in copper, iron, both copper and iron, riboflavin and inositol on the activity of the enzyme were studied in the rat. Methods were developed to pursue these studies in vitro with the liver enzyme and in vivo in the whole animal.

(b) The requirement of sulfhydryl groups for MAO activity was studied. Qualitative and quantitative determinations were made on the content of sulfhydryl groups in the partly purified enzyme.

(c) Polyacrylamide gel electrophoresis was used to detect multiple forms of the enzyme.

(d) The MAO-inhibitory properties of the hemolytic agents phenylhydrazine and acetylphenylhydrazine were studied in vivo and in vitro.

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ABBREVIATIONS

MAO	Monoamine oxidase
MAOI	Monoamine oxidase inhibitor
DAO	Diamine oxidase
PLP	Pyridoxal phosphate
5-HIAA	5-Hydroxyindoleacetic acid
.SH	Sulfhydryl
AIA	Allylisopropylacetamide
APHZ	Acetylphenylhydrazine
PHZ	Phenylhydrazine
V_m, V_{max}	Maximal reaction velocity
Km	Michaelis Constant
4.HOQ	4-Hydroxyquinoline
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide, riboflavin-5'-phosphate
TCA	Trichloroacetic acid
mA	milliamperes
SDS	Sodium dodecyl sulfate
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
s.c.	subcutaneous, subcutaneously
i.p.	intraperitoneal, intraperitoneally
m.p.k.	milligrams per kilogram
XO	Xanthine oxidase
NHI	non-heme iron

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I. INTRODUCTION

1. MAO - Historical

Ewins and Laidlaw (1) were the first workers to report that tyramine could be converted in the body to p-hydroxyphenylacetic acid. They demonstrated this by adding tyramine to the perfusion fluid of surviving rabbit liver and isolating the acid from the perfusate. Guggenheim and Löffler (2) performed similar experiments and found that phenylethylamine was transformed quantitatively to phenylacetic acid. In 1928, Hare (3) described an enzyme in mammalian livers which catalyzed the oxidative deamination of tyramine, which she called tyramine oxidase. This is the earliest report in the literature of the enzyme now known as monoamine oxidase (MAO).

In 1937, Blaschko, Richter and Schlossmann (4) described an adrenaline oxidase of liver, and Pugh and Quastel (5) reported the presence in brain of an enzyme (amine oxidase) that oxidized aliphatic amines. Pugh and Quastel (6), Blaschko et al. (7) and Kohn (8) showed independently that the amine oxidase of brain, adrenaline oxidase and tyramine oxidase of liver had similar properties. They came to the conclusion that the three enzyme systems are identical and comprise a single enzyme that attacks adrenaline, tyramine and several aliphatic amines.

It was Zeller (9) who proposed the name "monoamine oxidase" for this enzyme to distinguish it from diamine oxidase (E.C.1.4.3.6) (DAO). MAO, unlike DAO, does not act on histamine or short chain

aliphatic diamines, nor is its action sensitive to cyanide or semicarbazide. The names MAO and DAO seem inappropriate nowadays since both types of enzyme have been characterized not only with regard to their substrate preferences, but also with regard to their histological location and their cofactors, which may be either metallic or organic.

Many of the particulate amine oxidases (MAO-type) have a flavin prosthetic group (10-18) while the soluble enzymes of plasma (19-21), pig kidney (22, 23) and some microorganisms, e.g. Aspergillus niger (24) (DAO-type) require copper and pyridoxal phosphate (PLP) as their prosthetic groups. Attempts to identify PLP in the amine oxidase of pea seedlings have been largely unsuccessful (25). Thus copper remains the only well established cofactor of this enzyme (26, 27). McEwen (28) recognized the likelihood that human plasma amine oxidase might be a metalloprotein, however, he was unable to identify its metallic cofactor. McEwen, Cullen and Sober (29) reported indirect evidence for the presence of PLP in the rabbit serum enzyme. The finding by Kapeller-Adler and Macfarlane (30) that pig kidney DAO contained riboflavin in addition to copper and PLP was a controversial one and could not be substantiated by other workers (31). The above-mentioned details are summarized in Table I.

Table I

A classification of amine oxidizing enzymes based upon their prosthetic groups

Enzyme	Source	Known Cofactors	References
Spermine oxidase (spermine: oxygen oxidoreductase (donor cleaving)) E.C. 1.5.3.3.	Beef plasma	Cu^{2+}, PLP	19, 20
Benzylamine oxidase (Histaminase) E.C. 1.4.3.6.	Pig plasma	Cu^{2+}, PLP	21
Diamine oxidase (Histaminase) E.C. 1.4.3.6.	Pig kidney	Cu^{2+}, PLP	22, 23
Plant amine oxidase (DAO) E.C. 1.4.3.6.	Pea seedling	Cu^{2+}	26, 27
Fungal MAO	Aspergillus niger	Cu^{2+}, PLP	24
E.C. 1.4.3.4.	Trichosporon sp.	Cu^{2+}	32

Table I continued

Bacterial MAO (Tyramine oxidase) E.C. 1.4.3.4.	Sarcina lutea	Riboflavin	33
Bacterial polyamine oxidase (Spermidine oxidase)	Serratia marcescens	Riboflavin	34
Mammalian tissue MAO E.C. 1.4.3.4.	Mitochondria of brain, liver and kidney	Riboflavin	10-18

2. Occurrence and Distribution

MAO has been found in all classes of vertebrates that have been examined up to now (7). It occurs in many different tissues, especially in glands, smooth muscle, cardiac muscle and nervous tissue (4, 35, 36). Highest activity of the enzyme is shown in the liver and kidneys of most animals. Rat kidneys are a relatively poor source of MAO, however. Other visceral organs usually display less activity than either the liver or the kidney (5-7). In the human and animal brain, MAO activity is to be found in the central gray matter (basal ganglia) and hypothalamus, nerve cells and capillary walls but not in glial cells or the nerve fibers (37). In man, the parotid and submaxillary glands contain the highest concentrations of the enzyme (38). MAO is also found in the male and female reproductive organs. Its activity in the uterus of the dog and sheep (36) and the endometrium of the human (39, 40), the rat and the guinea pig (39) appears to be related to the phases of the estrus (menstrual) cycle. The mammalian placenta (41), human blood platelets (42, 43) and the skin and sweat glands of the horse (44) also contain MAO. The enzyme appears to be absent from skeletal muscle (45) and the skins of other species examined (44).

The kidneys of the human neonate show approximately one-half the level of MAO activity found in the adult; the increase to adult concentrations takes place shortly after birth (46). The levels of activity in the liver and intestinal mucosa of the newborn and adult human are approximately the same (35).

Pre- and post-natal development of MAO activity has also been studied in various organs of the rat. Some differences in the rates of development and increase of the enzyme have been observed. Concentrations of MAO in the heart continue to increase after birth until long after the attainment of maturity (47, 48). The kidney showed a rapid increase in enzymic activity during the first ten days after birth and then the activity remained constant (49); the liver enzyme continued to increase in activity steadily for about three to four months after birth (47, 49). The pattern of development in rat brain MAO was biphasic (49, 50). It has been suggested that the rapid increase in enzyme activity which occurs immediately after a temporary cessation of development during the first week of postnatal life may coincide with myelination of the rat brain (49).

Levels of MAO activity have been compared in the adult and foetal blood vessels of the sheep (51). Maternal blood vessels contained greater amounts of enzyme activity than did those of either the foetus or umbilicus (51).

Eiduson (52) found that development of MAO activity in chicken brain regions commences in the embryo at about thirteen days and the levels of activity increased gradually from the embryonic stage through posthatching life. Shih and Eiduson have observed that the electrophoretic patterns of MAO isozymes in the brain of the chick (53) and different tissues of the rat (54) are age-dependent. Findings similar to these have been reported by Baker (55) for the South African clawed toad, Xenopus laevis.

Blaschko and his coworkers (56-61) have been diligent in examining the tissues of representative specimens from several phyla of invertebrates for MAO activity. There is a wide distribution of the enzyme in Eusepia officinalis (56). The digestive organs contain a high concentration of enzyme activity; nervous tissue and other organs contain lesser amounts. The enzyme has been found in several other marine molluscs and in some species of echinoderms (57). Boadle (62) has reported the occurrence of a MAO with unique substrate specificity in the marine cephalopod Eledone cirrhosa.

The enzyme has been studied relatively little in insects. The cockroach, Periplaneta americana L., contained significantly greater levels of MAO activity in the Malpighian tubules than was found in other tissues (58). Boadle and Blaschko (59) found that the enzyme in the Malpighian tubules of Blaberus discoidalis Serville displays a different substrate specificity from that of Periplaneta americana L. Up to the present time, the mealworm, Tribolium confusum Duval (63), is the only insect in which MAO has been studied during the life cycle.

MAO has also been found in the gut of the earthworm (60), the kidney of the snail, Helix aspersa (64), and in the trematode worm Schistosoma mansoni (65). The presence and nature of an oxidative deamination system in the ciliated protozoan, Tetrahymena pyriformis W. have been reported on by Iwata et al. (66). The suggestion that the flagellate, Crithidia fasciculata (67) may also

contain MAO has not yet been confirmed.

The properties that distinguish the extracellular, soluble amine oxidases of animal plasma (DAO, histaminase) have been mentioned earlier (p. 1 and Table I). Several thorough reviews of the chemistry of DAO have been published (9, 68-70, 70a).

Amine oxidases also occur in plants (71-75). The best known of these is the enzyme from pea seedlings (71). It has been purified and found to differ from MAO in its cofactor requirement (see Table I) and inhibitor sensitivity (26, 27). Smith (72) and Smith and Stevens (73) have established that the amine oxidase found in grain seedlings is also different from MAO in its substrate specificity. Werle and Roewer (74, 75) discovered an enzyme in extracts of plant tissues with properties similar to those of animal MAO.

Several microorganisms contain amine oxidases (76-89) only some of which behave like the typical intracellular MAO of animal origin (78, 79, 84-86, 89). So far, the tyramine oxidase of Sarcina lutea is the only one of these enzymes which has been purified and characterized (33, 84-86, 89).

An amine oxidase present in skin (90), bone (91) and connective tissue (92-95) specifically deaminates the ϵ-carbon atom of polypeptide-bound lysine. The corresponding aldehyde produced undergoes condensation and thereby initiates the cross-linking in collagen and elastin molecules (91-93). This enzyme is distinct from mitochondrial MAO in its substrate and inhibitor

specificities and its cofactor requirements, i.e. the presence of copper is absolutely necessary for its action (91-93).

3. Intracellular Distribution of MAO

The intracellular distribution of MAO was studied first by Cotzias and Dole (96) in 1951 and later by Hawkins (97). It was evident from their results that the activity of the enzyme resided predominantly in the mitochondrial fraction of the cell. The microsomal fraction of rat liver also contained MAO activity (97). These observations have been confirmed by several groups of workers, not only for the rat liver but also for various organs in the rat and different species of animals tested (98-107). A greater proportion of the MAO activity of the rat heart (104) and rat vas deferens (104, 105) is located in the microsomal fraction of those tissues than has been found in the microsomes of the liver. Blaschko et al. (98) have reported that the activity of the bovine adrenal medulla enzyme is found exclusively in the mitochondria. Most of the MAO activity in the pupa of the mealworm is bound to the mitochondria and the remaining activity is found only in the nuclear fraction (63).

The enzyme is bound firmly to the outer membrane of the mitochondria. Ultrastructural studies by several groups of workers, made in conjunction with assays for its activity, have now confirmed the validity of its use as a marker for locating the outer mitochondrial membrane (108-121). Not long ago, there was some disagreement as to which submitochondrial structure the enzyme was attached to (122). Green et al. (123) and Gorkin (124) had expressed

doubt as to whether or not MAO activity ought to be considered as a characteristic property of mitochondrial membranes. Green and his coworkers (125) have now concluded from recent work that MAO activity is enriched in purified outer membrane fractions of beef heart mitochondria. Racker and Proctor (126) were successful in reconstituting resolved outer membrane preparations of beef kidney and beef heart mitochondria with partly purified MAO. Olivecrona and Oreland (127) and Oreland and Olivecrona (128) also recombined soluble MAO from pig liver mitochondria with lipid-depleted mitochondria from the same source in the presence of added phospholipids. The enzyme also bound firmly to delipidated red cell membranes and delipidated milk fat globule membranes (127). The binding of MAO to mitochondria was shown to be dependent on the presence of anionic (acidic) phospholipids. Oreland and Olivecrona (128) have suggested that such phospholipids may take part in similar enzyme-mitochondria interactions *in vivo*. Heidrich *et al.* (129) found that the washed outer membrane fractions of rat liver mitochondria contained MAO activity and displayed the same electrophoretic mobility as did intact mitochondria. The other fractions of the disrupted mitochondria differed from the outer membrane preparations in their electrophoretic mobilities as well as their enzymic properties (129).

4. Chemical Properties and Action of the Enzyme

MAO is characterized by its oxidative action on monoamine substrates, its inability to deaminate histamine and short chain

diamines, sensitivity to octanol and certain heavy metals, and its resistance to cyanide ion, hydrazine, semicarbazide and carbon monoxide (130). The enzyme acts on secondary as well as primary amines but it attacks tertiary amines slowly or not at all (7).

Several authors (3, 6, 7, 131) who isolated and identified the reaction products of liver extracts with different amines described the enzymic reaction as taking place according to the equation:

$$R.CH_2NHR' + O_2 \longrightarrow R.CHO + NHR' + H_2O_2$$

In the presence of cyanide, only one atom of oxygen is taken up per molecule of substrate. One molecule of ammonia is released per atom of oxygen consumed, or in the presence of a secondary amine substrate, one molecule of an alkylated amine (5, 131).

5. Substrate Specificity of the Enzyme

Primary and secondary amines are readily oxidized by MAO as was mentioned above. Blaschko (132) has suggested that an attack on methylated amines may be construed as a criterion for the presence of a true MAO. Although tertiary amines are poor substrates of MAO, they are oxidized more rapidly in some species, e.g., the cat, than in others, e.g., the rabbit (133). Rabbit liver is the only organ known which deaminates mescaline rapidly (134). McEwen et al. (29) found the purified amine oxidase of rabbit plasma to resemble the particulate enzyme in its substrate specificity. This enzyme also oxidizes mescaline readily (29).

Several authors have published reports on the oxidation of aliphatic amines by MAO since the phenomenon was first studied by Pugh and Quastel (135). The enzyme does not usually act on the lowest members in the series of aliphatic amines, but in some species, e.g., the cat and the bovine, it oxidizes ethylamine (136). In the homologous series $CH_3(CH_2)_nNH_2$, rates of oxidation increase at first with increasing chain length and decrease thereafter. In a systematic study of the substrate specificity of MAO, Alles and Heegaard (136) observed a species differentiation in the optimal chain length for hepatic MAO. These (136) and other workers (7, 134) showed that the short chain diamines were not deaminated by the enzyme. However, diamines having from 14 to 18 carbon atoms undergo MAO-catalyzed oxidation (137). Blaschko and Duthie (137) suggested that the second amine group of the short chain diamines interferes with the binding of the base to the enzyme and the interference tends to become less effective as the molecular distance between the amino groups increases. Similar effects of other polar groups, e.g., the carboxyl group of ω-amino acids and ω-amino-n-alkyl-trimethylammonium, on the enzyme-substrate interaction have been observed (45). Blaschko et al. (138) reported that some derivatives of ethylenediamine containing a substituted amino group serve as substrates for MAO.

The most important naturally occurring substrates of MAO are derivatives of β-phenylethylamine and tryptamine. Hydroxyl substitution in the aromatic ring of β-phenylethylamine at position 4

(p-tyramine) leads to faster rates of degradation than does substitution at either the 2-, or 3-position. The opposite is true for the corresponding methoxyl derivatives (133, 136). Of the tryptamine derivatives, the most important substrate of MAO is serotonin (5-hydroxytryptamine). The N,N-dimethyl derivative (bufotenine) is not attacked very rapidly by the enzyme (139).

The introduction of a methyl group in the α-position, the replacement of one β-hydrogen atom by a hydroxyl group or the replacement of both β-hydrogen atoms of the substrate by other groups causes changes in behaviour of the substrate towards MAO (9). For instance, α-methyl-substituted amines are not attacked by the enzyme; in fact, they inhibit amine oxidation competitively (6, 7, 136, 140). Mann and Quastel (140) suggested that the neurological effects of the amphetamines may stem from their blockade of cerebral MAO. In most instances, β-hydroxylation of the substrate leads to a considerable reduction in the rate of oxidative deamination (7, 133, 136). Substitution of both β-hydrogen atoms of an amine produces substances which are not substrates of MAO (136, 141, 142).

Structural formulae of some substrates of MAO are illustrated in Figure 1.

Blaschko and his coworkers (4, 143) had reported the preferential oxidation by MAO of l-isomers of certain substrates. Leeper et al. (144) reported contrary findings. However, Giachetti and Shore (145) confirmed the earlier reports of the enzyme's

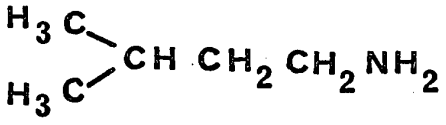
optical specificity (4, 143) when they found that soluble MAO preparations from various tissues of the rabbit and rat consumed the l-isomers of octopamine, norepinephrine and epinephrine more rapidly than the d-isomers. Rabbit heart slices also accumulated d-octopamine in preference to the l-isomer. Giachetti and Shore (145) regarded the preferential uptake of the d-form of this base as a reflection of its being a poorer substrate than the l-isomer.

Several reports of induced changes in the substrate and inhibitor specificity of MAO have appeared in the literature (89, 146-154). The enzyme when incubated with peroxides of higher unsaturated fatty acids, or with cupric ions, acquires the ability to deaminate substrates of DAO, lysine and spermine and loses the power to catalyze deamination of monoamines. MAO concomitantly becomes resistant to the action of MAO inhibitors (MAOIs) and becomes sensitive to the action of isoniazid, hydroxylamine and cyanide ion. Gorkin and his colleagues (89, 146-154) have referred to the alteration of MAO activity as "transformation". MAO activity can be restored by treatment of the "transformed" enzyme with reducing agents such as arsenite, borohydride and glutathione (153). "Transformation" can be prevented by preincubation of the enzyme preparations with MAOIs or glutathione (152, 153, 155).

Gorkin *et al.* (153) have reported that the opposite "transformation", that of DAO to an enzyme having MAO-like properties, has been observed.

Figure 1

Structural formulae of some common substrates of MAO. The monoamines shown are: (a) iso-amylamine; (b) 2-phenylethylamine; (c) benzylamine; (d) tyramine; (e) dopamine; (f) norepinephrine; (g) 5-hydroxytryptamine (serotonin); (h) kynuramine.



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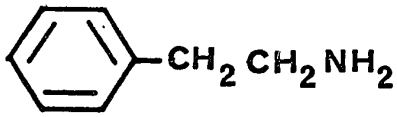

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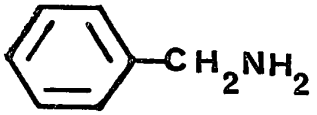

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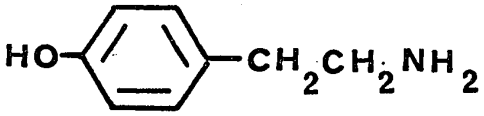

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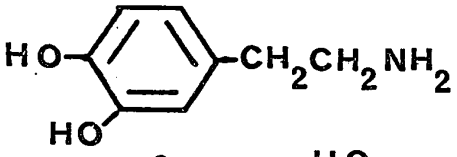

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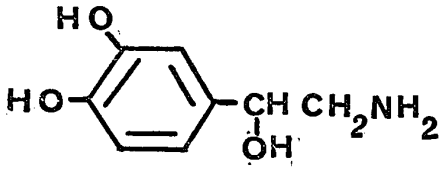

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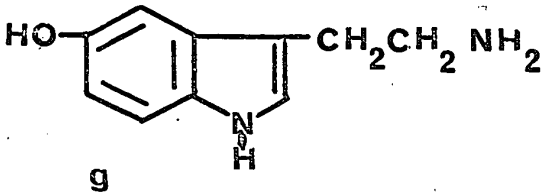

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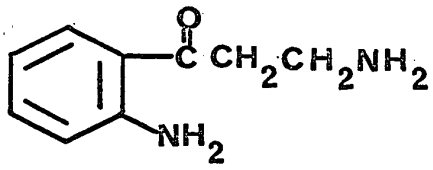

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6. Measurement of MAO Activity in Vitro

MAO activity can be measured by determining the rates of oxygen consumption, disappearance of substrate, appearance of organic product (the aldehyde or the acid produced by secondary oxidation of the aldehyde), production of ammonia or production of hydrogen peroxide.

A. Oxygen Consumption

Oxygen consumption may be measured either manometrically or polarographically.

i) Manometric Methods

The manometric determination of rates of oxygen consumption, using tyramine or tryptamine as substrate has found widespread application in the assay of MAO activity in tissue preparations. Reaction velocity is much greater at high than at low oxygen concentration therefore the reaction vessels are ordinarily filled with pure oxygen. Cyanide and semicarbazide are often added to the reaction mixture to minimize competing oxidative reactions thus preventing non-stoichiometric oxygen uptake (3, 7, 156).

The technique is now being supplanted by the polarographic technique using a Clark oxygen electrode (157) for the measurement of MAO activity as well as that of other oxidative enzyme systems (103, 158-161).

ii) Polarographic Methods

The theory and application of the oxygen electrode in biochemical systems have been reviewed by several authors (162, 163).

The oxygen electrode measures the concentration (tension) of oxygen dissolved in the medium in which its tip is immersed. The Clark type of electrode consists of a platinum cathode and silver anode. A minimal volume of electrolyte covers the electrode surface and the assembly is protected by an oxygen-permeable membrane.

The electrolytic reduction of dissolved oxygen at the weakly negative cathode causes more oxygen to diffuse towards it, thereby establishing an oxygen tension gradient between the sample and the electrode. When a low potential difference (- 0.4 to - 0.8 V) is applied across the electrode, immersed in an aqueous medium, the magnitude of the current produced is proportional to the oxygen tension of the solution (P_{O_2} in mm of mercury).

Calibration curves relating current at the electrode P_{O_2} can provide information on the percentage of oxygen in solution. However, if significant amounts of oxygen are bound by dissolved substances such as buffer salts or proteins, the values recorded may not indicate the true oxygen content of the solution. The solubility coefficients of oxygen in air for distilled water and some buffering media have been published (165, 166). Other methods exist for determining accurately the true oxygen content of more complex solutions (167, 168).

The advantages of the oxygen electrode are as follows:

(a) it provides continuous information at instantaneous rates; (b) it requires relatively short times for equilibration and monitoring reactions; (c) it can operate well in solutions having variable composition as regards substrate, gas content and pH. Thus, in vitro measurements with this instrument can simulate the physiological conditions of metabolizing systems much more nearly than do manometric methods.

The electrode itself consumes oxygen from the solution at a constant rate, dependent on the amount of oxygen dissolved in the solution. The electrode error may be reduced by using solutions equilibrated with air instead of 100% oxygen and also by using increased concentrations of biologically active material to reduce the time required for measurement.

B. Disappearance of Substrate

MAO activity may be determined by measuring the rate of disappearance of substrate, e.g., tyramine (169) and serotonin (170) or kynuramine (171) by optical methods. A new spectrophotometric method has been described by Obata et al. (172). It is based on the measurement of residual amounts of a strongly coloured 2,4,6-trinitrobenzene-1-sulfonate derivative of any amine, following its reaction with MAO.

Manukhin (173) used a bioassay technique to measure unreacted epinephrine. The pressor reaction of spinal rabbits and cats was measured following injection with an aliquot of the incubation

mixture. The responses elicited in the test animals were compared with those observed when they received injections of the amine.

C. Appearance of Product

The rate of appearance of aldehyde following the incubation of amine substrate with a tissue preparation containing MAO may be measured either by optical methods or by radiometric methods. Green and Haughton (173) developed an assay for MAO activity based on the formation of the dinitrophenylhydrazone of the aldehyde produced from enzymic deamination of tyramine. The method of Tabor et al. (174) is dependent on the enzyme-catalyzed formation of benzaldehyde from benzylamine. The rate of reaction can be followed spectrophotometrically at 250 nm. Similar assays have been developed using as substrates m-nitro-p-hydroxybenzylamine (175), m-iodobenzylamine (176) and p-dimethylaminobenzylamine (177). Their aldehyde products absorb strongly at wavelengths of 315, 253 and 355 nm, respectively.

Kraml (178) adapted the method of Weissbach et al. (171) to monitor fluorometrically the rate of appearance of 4-hydroxyquinoline. This product arises from the spontaneous cyclization of the aldehyde intermediate formed during the enzymic oxidative deamination of kynuramine. The method (178) is rapid, convenient and very sensitive, and is now being used widely in several laboratories. An assay procedure similar to that of Kraml (178) has been developed by Takahashi and Takahara (179) using 5-hydroxykynurenamine as substrate. The MAO deamination product cyclizes

to 4,6-quinolinediol which fluoresces maximally at 460 nm upon activation at 360 nm (179). Zeller et al. (180) have used the non-enzymic cyclization of o-aminophenylacetaldehyde to indole as a means of assaying for MAO activity in human blood platelets with o-aminophenylethylamine as substrate.

Several assays of MAO activity in vitro depend on the further oxidation of aldehyde to acid. One such method measures the rate of formation of indoleacetic acid from tryptamine in the presence of aldehyde dehydrogenase and nicotinamide adenine dinucleotide (NAD) (181). The acid product is detected by its fluorescence. A similar procedure using serotonin as substrate was developed by Hidaka and others (182).

There are several reports in the literature dealing with the use of radioactive amine substrates for the measurement of MAO activity in vitro. The methods depend upon the rate of production of deaminated ^{14}C- or ^{3}H-metabolites (65, 183-190).

D. Production of Ammonia

The rate of production of ammonia (or volatile organic amines) has been used as an index of MAO activity in tissue extracts (191-193). The analysis may be performed either in Conway diffusion chambers (191, 193) or in Warburg manometric flasks (192). The concentration of the trapped ammonia may be estimated by nesslerization. The procedure of Braganca et al. (192) has been used by Guha and Krishna Murti (194) to monitor the purification of rat liver MAO. It has been suggested that the rate of MAO-mediated release of ammonia from

a substrate may be measured by coupling the reaction to oxidation of reduced NAD (NADH) in the presence of glutamate dehydrogenase (159).

E. Hydrogen Peroxide Production

It is only comparatively recently that this method of determining MAO activity has been used. To date, all the procedures in use involve the coupling of the MAO reaction with peroxidase activity in the presence of o-dianisidine (195, 196) or homovanillic acid (HVA) (197-199) with the formation of a stable chromophore (195) or highly fluorescent substance (197-199), respectively. Guilbault et al. (200, 201) had modified their earlier method (198) by substituting p-hydroxyphenylacetic acid for HVA. The initial rates of formation of the secondary products, as measured by suitable optical techniques, are related linearly to MAO activity.

F. Histochemical Determination

Early histochemical studies of MAO made use of the fact that the enzyme produces a dark pigment in the presence of tryptamine or serotonin. The location of the pigment indicated the site of MAO in the tissues (202, 203). Pigment formation was blocked by inhibitors of MAO (203).

Tetrazolium dye reduction was first applied to the histochemical localization of MAO by other workers (204-206) at about the same time that Blaschko and Hellman (202) were conducting their

investigations. Tetrazolium is reduced by the aldehyde produced on oxidation of the amine and a blue formazan precipitate results. Glenner et al. (207) modified the method of Francis (205, 206) by using tryptamine as substrate instead of tyramine. The modified method is now widely used to examine different tissues from different species of animals for MAO activity in normal (208-212) as well as in pathological (213) conditions. It has also been used to study the post-partum development of the enzyme in rat brain (214, 215). The chemistry of the reaction as it applies to MAO has been studied extensively in different laboratories (216-222) and it is still being studied (223).

Koelle and Valk (224) developed another method for the direct localization of MAO in nervous tissue. Tryptamine is used as substrate and the indoleacetaldehyde-hydrazone is precipitated. The method has been criticized and has not been used much because of its labouriousness (130, 208).

7. Inhibitors of MAO (MAOIs)

Since Zeller and his colleagues (225, 226) reported the strong in vitro and in vivo inhibition of MAO activity by the antitubercular drug iproniazid, the list of compounds synthesized and tested as potential MAO inhibitors (MAOIs) has lengthened considerably. Numerous substances belonging to various classes of chemicals interfere with the action of MAO in vivo as well as in vitro. Several reviews and other studies have been published on the properties of these substances and the results of their

interaction with the enzyme (227-236). In addition to that, conferences have been held which dealt with problems in this field exclusively (237, 238).

There is no single substance which can be called a representative MAOI. Each compound (or group of compounds) appears to have its own individual properties with regard to degree of inhibition, rapidity of onset of action, organ specificity, toxicity and pharmacological action (231, 234). Marked species differences in responsiveness to MAOIs have also been observed (231). Although several groups of workers have studied the effects on MAO inhibition of alterations of structure of inhibitors (239-242) it is still difficult to make any generalizations as to the structural requirements for MAO-inhibitory action. Pscheidt (242) had suggested that many of the effects of MAOIs may arise either from their binding to the amines in the tissues or from direct action of the drugs themselves and may not be the result of MAO inhibition per se.

The principal types of MAOIs are based upon hydrazine, simple and α-alkyl-substituted amines, N-disubstituted amines and some heterocyclic, nitrogen-containing compounds. A heterogeneous group of substances which inhibit MAO also exists.

A. Hydrazine Derivatives

Hydrazine-derived MAOIs, the prototype of which is iproniazid, are classified as long-acting, irreversible inhibitors of MAO in vivo and in vitro. They may be subdivided into the acid hydrazide

and alkylhydrazine groups. The former seem to exert their anti-MAO activity as a result of biotransformation to the more potent corresponding alkylhydrazine derivative (243-247).

B. α-Alkyl-substituted Amines

Arylalkylamines in which a hydrogen atom at carbon-1 is replaced by a methyl group, e.g., the amphetamines and ephedrine, inhibit MAO competitively and reversibly (6, 7, 45, 136, 140). α-Alkyl-substituted derivatives of the indolylalkylamines are also inhibitory (45, 136).

Tranylcypromine (trans-2-phenylcyclopropylamine) is one of the most potent non-hydrazine MAOIs studied. A variety of its analogues have been synthesized and compared with it to determine its structural requirements (248, 249). The duration of action of tranylcypromine is not as long as has been observed for hydrazine derivatives administered in equiactive doses. Its onset is fairly rapid but its effects are not readily reversible, at least *in vitro*.

C. N-Disubstituted Amines

Pargyline (N-benzyl-N-methyl-2-propynylamine) is another powerful inhibitor of MAO. It is a long-acting, irreversible inhibitor with a slow onset of action. The structure-activity relationships of some of its analogues have been studied (250).

The inhibitor, clorgyline, (N-methyl-N-propargyl-3(-2,4-dichlorophenoxy)propylamine) is an extremely potent one *in vitro*. Johnston (251) interpreted the kinetics of its action on rat brain MAO as evidence for the existence of more than one form of the enzyme.

D. Heterocyclic Nitrogenous Compounds

Heterocyclic nitrogenous MAOIs include such diverse compounds as the β-carbolines, e.g., harmine and harmaline, and modaline (2-methyl-3-piperidinopyrazine). Harmaline is the most potent inhibitor of the β-carboline derivatives studied (252). In vivo it is a fast-acting drug of short duration and its effect in vitro is reversible. Pletscher and Besendorf (253) found that it could protect experimental animals against the actions of some long-acting MAOIs. This drug is poorly absorbed from the gastrointestinal tract in man and it can produce markedly toxic effects (254). Ozaki et al. (228) reported that slight changes in structure from the natural harmala alkaloids resulted in marked loss of inhibitory activity.

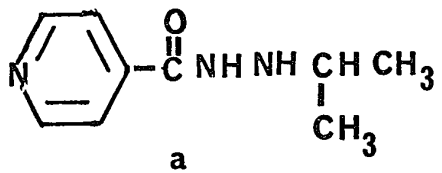
Modaline is a rapidly acting MAOI whose interactions with other drugs (255) and the structure-activity relationships of its analogues have been studied (256). Its effects are of long duration and it inhibits MAO irreversibly. Gylys and others (256) have suggested that biotransformation of modaline in vitro may occur before the drug exerts its effect.

E. Miscellaneous MAOIs

The activity of MAO has been reported to be sensitive to the actions of such varying types of chemical substances as local anaesthetics (257), amidines (258, 259), S-n-alkyl-iso-thioureas (260), aliphatic alcohols, e.g., octanol (135), methylene blue (261, 262), choline-p-tolyl ether (263-266), urea (36), derivatives

Figure 2

Structural formulae of some inhibitors of MAO. The formulae shown are those of: (a) iproniazid; (b) tranylcypromine; (c) phenelzine; (d) β-phenylisopropylamine (amphetamine); (e) pargyline; (f) clorgyline; (g) choline-p-tolyl ether; (h) modaline; (i) harmaline.



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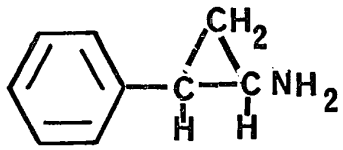

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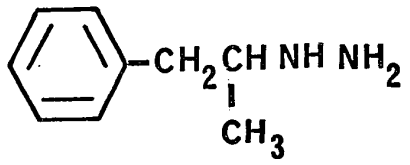

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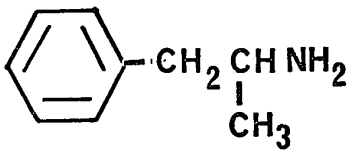

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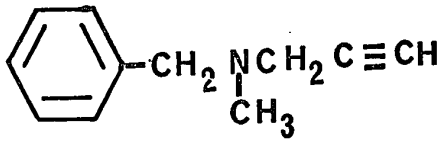

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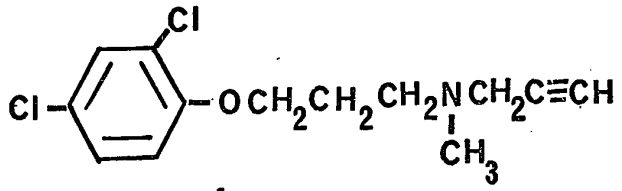

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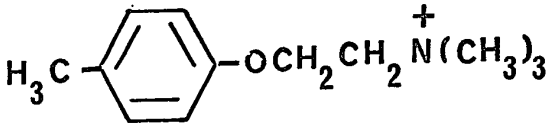

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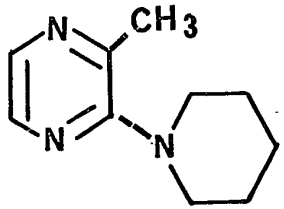

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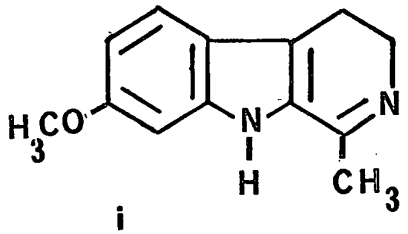

i

of thyroxine (267), thiol reagents (268) and sodium pentobarbital (269). Interpretations of the actions of some of these substances have been reported in several reviews (45, 130, 231, 270, 271).

The structural formulae of some representative MAOIs are shown in Figure 2.

8. The Estimation of MAO Activity and the Assessment of the Potency of MAO Inhibition in Vivo

Noticeable decreases in MAO activity have been induced in clinical practice by the administration of MAOIs. Attempts have been made to assess the losses of enzymic activity quantitatively by *in vivo* assays. None have been completely satisfactory.

Many of the tests used to determine the activity of MAO *in vivo* and to judge the efficacy of MAOIs *in vivo* are indirect procedures based on the assumption that inhibition of MAO will result either in interference with endogenous and exogenous amine metabolism or in some alteration of the pharmacological actions of other substances.

A. Interference with Amine Metabolism

This phenomenon manifests itself by decreased enzymic activity in isolated tissues or decreased rates of metabolism of endogenous and exogenous amines.

(i) Decreased Enzyme Activity of Tissues

Decreased MAO activity is found *in vitro* in the homogenates of tissues prepared from MAOI-treated animals. MAO activity in

normal or inhibitor-treated human patients may be measured directly in jejunal mucosa obtained by biopsy (272) or from buccal scrapings (183). It has been suggested that the MAO activity in human blood platelets may be used as an index of the status of the enzyme in other tissues of the patients (180, 186). However, Collins and Sandler (273) urged that caution ought to be exercized before extrapolating the status of human platelet MAO activity to other tissues.

ii) Decreased Metabolism of Endogenous and Exogenous Amines

(a) Sjoerdsma and his colleagues (254, 274, 275) found that the rate of conversion of exogenous serotonin to 5-hydroxyindole-acetic acid (5-HIAA) decreased noticeably in patients who received a MAOI before getting serotonin. The tests were conducted by comparing the urinary levels of 5-HIAA in the patients, following an oral loading dose of serotonin.

(b) MAO inhibition in man and in laboratory animals has been found to result in an increase in the urinary excretion of endogenous and exogenous amines (276-279). Increases in the concentrations of biogenic amines (usually serotonin) in organs, e.g., brain, of MAOI-treated animals have also been reported (280). Measurements were made by paper chromatography or fluorimetry in the above-mentioned instances.

Radioisotopic tracer methodology has also been applied to the in vivo study of MAO activity and its inhibition. For example, Breese et al. (281) administered labeled amines to animals and

estimated the total amounts of the radioactivity that were excreted in the urine as metabolites or stored in the tissues. Resnick (282) and Resnick and Elmadjian (283) found that the injection of rats with iproniazid followed by the injection of N-methyl-labeled epinephrine resulted in an increased rate of excretion of radioactivity, via the urine, which persisted for two weeks after the iproniazid treatment had ceased. Alivisatos et al. (284) observed that the prior treatment of mice with MAOIs blocked the incorporation into the acid-soluble components of the brain of (^{14}C)-labeled aldehyde from intraventricularly injected (^{14}C)-labeled serotonin.

B. Pharmacological Tests for MAO Inhibition

Pharmacological tests for the assessment of MAO inhibition in vivo include behavioral bioassays with whole animals, and the potentiation of amine effects either in the animal or in preparations of excised tissue.

i) Prevention of Reserpine Effects

The principal in vivo test used to measure the relative effectiveness of drugs as MAOIs is the prevention, or the reversal, of the pharmacological actions of reserpine or tetrabenazine in rodents (256, 285-288). Excitation and increased motor activity rather than depression and sedation has been observed in animals given MAOIs before reserpine (285, 287, 288). Injection of mice with a MAOI before reserpine blocked the generation of reserpine-induced hypothermia in the animals (256, 286). The intensity and

duration of action of the MAOI could be estimated quantitatively from the observed effects (256, 288).

ii) Potentiation of Amine Effects

The potentiation of amine effects following pretreatment of animals with MAO-inhibiting drugs has also been used as a tool to study MAO activity *in vivo* and also to compare the relative MAO-inhibitory potencies of different drugs (256, 279, 289-294).

Tryptamine and serotonin or amino acid precursors can elicit convulsive reactions when given to rats intravenously. When challenging doses of these amines (or their precursors) are injected after a MAOI has been given, potentiation of convulsion will result (289, 290).

Potentiation of the pressor activity of tyramine also occurs in MAOI-pretreated rats (292, 293). MAOIs will also potentiate the ability of tryptamine (291) and serotonin (292, 293) to contract isolated strips of smooth muscle. Use has been made of these phenomena to screen some new compounds as potential MAOIs (292, 293).

9. Interactions of MAOIs with Other Substances

MAOIs presently in clinical use have an irreversible action on the enzyme which persists long after the drug has been metabolized until the enzyme has been resynthesized. Their biochemical and pharmacological effects have led to the appearance in patients receiving MAOI treatment of potentiation or alterations in the actions of other drugs which may be in use concomitantly. The

consumption of foods rich in amines and their amino acid precursors evoke in MAOI-treated patients prolonged augmented effects of the drug. Reports of several such incidents (sometimes fatal) arising from MAOI interactions with various drugs and amine-containing foodstuffs, especially cheeses, have appeared in recent reviews (295-300).

10. Solubilization and Purification of the Enzyme

MAO is tightly bound to the insoluble structure of mitochondrial membranes. This makes the enzyme very resistant to solubilization and thus, difficult to purify. A soluble form of the enzyme which has been reported to occur in the liver of the guinea pig (301, 302) may be of microsomal origin. Several groups of workers were able to prepare soluble, partially purified MAO from mitochondria disrupted with detergents (303-306), or sonication (307-309) or a combination of both these treatments (310-312).

The combined use of these techniques with gel filtration and ion exchange chromatography has enabled other workers to achieve greater purification of MAO from beef liver (313), kidney (12), thyroid (314) and brain (13, 14), rat liver (315), human liver (316), placenta (317, 318), brain (18) and blood platelets (273), rabbit liver (319), monkey intestine (320) and pig brain (160). Hollunger and Oreland (321) described the preparation of soluble MAO from lysed pig liver mitochondria by extraction with 2-butanone (ethylmethylketone). Kapeller-Adler (322) has published a detailed survey of several of the above-mentioned purification procedures.

Attempts to study the cofactor requirements and the apparent inhomogeneity (multiplicity) of MAO in vitro, by direct means, have been facilitated by its solubilization and purification. This is also true for investigations made into the reaction pathway of the enzyme found in pig brain (323), beef thyroid (314) and beef liver (324, 325). The results of those experiments are consistent with the operation of a ping-pong mechanism (326) in the reaction of MAO with tyramine (314, 323) and benzylamine (324, 325). The mechanism of the reaction of Sarcina lutea tyramine oxidase has also been studied (327). Evidence has also been presented for ping-pong catalytic mechanisms of soluble amine oxidases purified from pig kidney (328), pig plasma (329), beef plasma (330) and pea seedlings (331).

11. Cofactor Requirements of MAO

Prior to the advent of methods for purifying mitochondrial MAO, indications of possible cofactors of the enzyme had been suggested by indirect evidence. The involvement of riboflavin, metals and sulfhydryl groups in the action of MAO are considered here in this connection.

A. Riboflavin

In 1937, Richter (131) compared the reactions catalyzed by D-amino-acid oxidase and MAO and suggested that the latter might also be a flavoprotein. Hawkins (332) found later that MAO activity in the livers of rats made nutritionally deficient in riboflavin

decreased appreciably. This report was substantiated by Sourkes and his colleagues (333-337). Distler and Sourkes (335) found that MAO was more effectively inhibited in riboflavin-deficient rats and that recovery of enzymic activity *in vivo* was considerably retarded under these conditions. All these findings and the observations that riboflavin antagonists could inhibit the enzyme (334, 336, 338, 339) suggested that MAO may have a flavin cofactor.

Riboflavin has been identified recently in a prosthetic group that is bound covalently to mitochondrial MAO purified from beef liver (10, 11), kidney (12) and brain (13, 14), pig brain (15), pig liver (358), rat liver (16, 17) and human brain (18).

B. Possible Metal Cofactor of MAO

Reports from several laboratories have indicated the possible requirement of MAO for a metal cofactor (315, 340-344). The soluble amine oxidases found in pea seedlings (26, 27), plasma (19, 21, 345), hog kidney (23, 346), *Aspergillus niger* (347) and *Trichosporon sp.* (32) have been purified and found to contain copper as a prosthetic group. It has been shown that the purified mitochondrial enzyme does not contain copper in sufficient quantities to serve as a cofactor (12, 13, 160, 315, 337, 348). Youdim and Sourkes (315) showed that there was relatively more iron (6 atoms per mole of enzyme) than copper (less than 1 atom per mole of enzyme) in purified rat liver MAO.

No functional metal component has been found in MAO up to now. However, it has been shown that the maintenance of normal enzymic

activity in the rat depends upon an adequate supply of dietary iron (161, 349, 350 and this Thesis).

C. Essential Sulfhydryl Groups

The involvement of sulfhydryl (.SH) groups in the action of MAO was first described by Friedenwald and Herrmann (351) in 1942. These workers reported that the inhibition of MAO by organic mercurials could be reversed on incubation with glutathione or cysteine. Singer and Barron (352) and Lagnado and Sourkes (340) showed that MAO was inhibited by organic arsenicals and a variety of heavy metal ions, respectively. Both these groups of investigators also found that thiol compounds afforded the enzyme some protection against the action of sulfhydryl reagents (340, 352). These findings have led to the classification of MAO as a .SH enzyme (340). Direct evidence for the involvement of .SH groups in the action of MAO purified from different sources has come from the results of quantitative studies with .SH group reagents (12, 89, 353-359).

12. Multiplicity of MAO

Many groups of workers have recorded observations on MAO which pointed to the possible existence of more than one form of the enzyme (343). The phenomena which suggested the occurrence of MAO isozymes in certain organs include different inhibitor sensitivities (251, 360-365), substrate specificities (366-368), heat stabilities (302, 336, 369), and competitive substrate effects (370).

Several reports of MAO isozymes have now appeared in the literature (371). Some authors have described a differential substrate specificity for MAO isozymes separated physically (305, 372, 373). Other investigators have achieved separations of MAO by electrophoresis into several bands of activity that display distinct substrate and inhibitor specificities, as well as different thermal stabilities (53-55, 317, 318, 371, 374-381). The extent to which these in vitro findings may reflect the role of MAO in vivo is not clear, however.

II. EXPERIMENTAL

1. MATERIALS AND METHODS

A. Animals, Diets and Injections

Male, albino Sprague-Dawley rats were used in all the experiments . Those animals that were used in experiments with inhibitors and in purification studies weighed between 100 and 200 g. They were fed on Purina rat pellets and had tap water to drink.

The rats used in the nutritional deficiency experiments weighed 40-50 g initially. They were fed solid semisynthetic diets designated as control, iron-, copper-, riboflavin-, inositol- and doubly-deficient (deficient in both iron and copper, and jointly deficient in riboflavin and inositol). The deficient diets had the same composition as the control diets except for the omission of the indicated nutrients. The diets were prepared from the following ingredients (grams per 3 kg batch): vitamin-free casein 660, corn starch 870, sucrose 900, partially hydrogenated fat (Crisco) 180, corn oil (Mazola) 60, powdered cellulose (MN 300, Macherey, Nagel and Co., Germany) 60, and Rogers-Harper salt mixture 150 (382). The composition of the salt mixture (percentage by weight) was as follows: ammonium molybdate 0.0025, calcium carbonate 29.29, calcium phosphate 0.43, cupric sulfate 0.156, ferric citrate 0.623, magnesium sulfate 9.98, manganous sulfate 0.121, potassium iodide 0.0005, potassium phosphate 34.31, sodium chloride 25.06, sodium selenite 0.0015, zinc chloride 0.02.

Each batch of feed contained the following vitamins (milligrams): biotin 6, folic acid 12, riboflavin, thiamine hydrochloride and pyridoxine hydrochloride 75 each, vitamin B_{12} and menadione 150 each, niacin 300, inositol and p-aminobenzoic acid 330 each, and choline chloride 5010. Glass-distilled water for drinking was supplied in glass bottles fitted with glass spouts. Each rat was given, in addition, 1 drop of cod-liver oil daily, by mouth.

MAOIs were prepared for injection as buffered solutions in water or in 0.15M NaCl. They were given to rats starved overnight by intraperitoneal injection at the dosage levels and indicated times as follows: iproniazid and tranylcypromine 100 and 5 mg per kg body weight respectively, 16 hours before testing for MAO activity; harmaline HCl 5 and 20 mg per kg at 30 and 15 minutes respectively, before testing; SKF 525-A 100 and 75 mg per kg at 60 and 90 minutes respectively, before testing. Control rats were injected with water or 0.15M NaCl solution.

Allylisopropylacetamide (AIA) a porphyria-producing drug, was dissolved in absolute alcohol. Sufficient sodium chloride dissolved in water, was added to the AIA solution to make it 40% (v/v) in alcohol and 0.15M in NaCl. The test animals were given 200 mpk, subcutaneously, once daily for each of 5 consecutive days. Control rats received the alcoholic saline injection only. The rats were sacrificed on day 6. The livers were perfused *in situ* with ice-cold 0.25M sucrose solution, removed and weighed, then homogenized in sucrose. The homogenates were assayed for MAO activity and protein.

Acetylphenylhydrazine (APHZ)* was prepared for injection as described for AIA, except that the concentration of alcohol required to keep it soluble in the saline medium was 20% (v/v).

(i) Test animals were given single (s.c.) injections of the drug daily, at various dosage levels, for 6 days. Hemoglobin levels of tail blood were measured on day 8. The dosage level causing a significant decrease in hemoglobin concentration concomitant with survival at day 6 was selected as the minimum effective dose of APHZ to be used in more extensive experiments described below.

(ii) Experimental animals were given repeated injections of APHZ (80 mpk s.c. per day) for 3 days. The first group of rats was sacrificed 48 hours after the last injection. Blood was collected for determination of its hemoglobin concentration and tissues were removed for assaying their MAO activity and iron content. The remaining animals were killed on the 4th, 11th and 18th days after the last injection to follow the recovery of the rats from the effects of the drug. The same procedure described above was followed in another series of experiments except that the rats were given a single injection of APHZ. They were sacrificed at 3, 6, 12, 24, 48 hours and at 4, 8, 12 and 22 days following the injections. The time course of inhibition and recovery of MAO activity was followed by the polarographic method and by the kynuramine fluorescence assay. The effects of the treatment on brain serotonin content were also assessed (383).

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*The chemical used in the earlier experiments was a gift from Dr. David Rubinstein, Department of Biochemistry, McGill University.

(iii) The interaction of the drug and riboflavin-deficiency (4 weeks' duration) on liver and brain MAO activity was studied. Groups of rats were killed at 24 and 48 hours after they had been injected with 0, 50 and 80 mpk of APHZ. Hemoglobin was determined in their blood and tissues were removed for estimation of enzymic activity.

(iv) An investigation was conducted to assess the ability of the APHZ-treated iron-deficient rat to metabolize radioactive [1-^{14}C]-pentylamine *in vivo* (349, 350 and this Thesis). Rats that had been fed the iron-deficient diet for 5 weeks and 3 weeks were injected subcutaneously with 80 mpk and 35 mpk, respectively, of APHZ 16 hours prior to injection with the radioactive amine. Food was withdrawn at the same time but the animals were allowed free access to water.

Phenylhydrazine hydrochloride (PHZ). This study of the *in vivo* effects of this agent on MAO activity in the rat was less extensive than was undertaken for APHZ treatment. A single injection of PHZ (80 mpk) in 0.15M NaCl solution was administered subcutaneously to the experimental animals. The control animals received injections of saline only. Groups of animals were killed at 24 and 48 hours after they were injected. Blood was collected for estimations of hemoglobin, and the livers and brains were taken for assaying their MAO activity. The serotonin levels of the brains were also estimated (383).

B. Enzyme Assays

MAO activity was estimated from the rates of oxygen consumption of mitochondrial suspensions prepared from liver homogenates made in 0.25M sucrose (97). Oxygen uptake measurements were made by means of a Y.S.I. Model 5331 Biological Oxygen Monitor (Yellow Springs Instrument Co., Ohio) equipped with a Clark electrode (157) and connected to a 100 mv Heath EUW 20 Servo-recorder (Heath Company, Benton Harbor, Michigan). The reaction mixtures (156) contained: semicarbazide hydrochloride 20 μmoles, sodium cyanide 2 μmoles, 50 mM phosphate buffer (pH 7.4), 0.5 ml enzyme preparation, equivalent to 50-100 mg fresh weight of liver and water to 3.0 ml. The reaction was started by addition of the substrate: 40 μmoles iso-amylamine, 20 μmoles tyramine or 5 μmoles benzylamine. With tyramine, the buffer used was at pH 7.0. In experiments where benzylamine was substrate, cyanide and semicarbazide were omitted from the reaction mixtures. All incubations were carried out at 37.5° under air-saturating conditions.

In the studies employing metal-chelating agents, the enzyme was preincubated for 5 minutes with various concentrations of the respective chemicals prepared from neutralized stock solutions. Preincubation for longer periods of time did not increase the inhibitory effects of any of the chelators tested.

Protein content of the enzyme was estimated by the method of Lowry et al. (384).

MAO activity was also determined in vitro by a microfluoro-

metric method with kynuramine as substrate (178) (Aminco-Bowman Spectrophotofluorometer, American Instrument Company, Silver Spring, Maryland).

Ceruloplasmin oxidase activity (385) was measured in the serum obtained from tail blood of the copper-deficient and copper-supplemented rats.

C. Assay of Cofactors

The nutritional status of the experimental animals was monitored by recording their body weights.

In studies on the effects of iron-deficiency, hemoglobin estimations were made on tail blood (386) at weekly intervals to follow the progress of the deficiency. When the rats fed the iron-deficient diets became definitely anemic, as indicated by their severely lowered hemoglobin levels (after about 4 to 5 weeks), some of the animals from each dietary group were sacrificed immediately. The remainder were killed thereafter at weekly intervals for the duration of the experiments.

The livers of the decapitated animals were perfused *in situ* with freshly prepared ice-cold 0.25M sucrose, removed quickly, and then washed and chilled thoroughly before being dried and weighed. Portions of each liver were removed for assaying concentrations of iron and copper, and monoamine oxidase activity.

When the concentrations of iron and copper were to be determined, the samples of tissue (100-300 mg and 0.5-1.0 g fresh weight, respectively) were dried and then incinerated as described by

Thiers (387). The ash was extracted with hydrochloric acid solution and portions of the extracts were taken for the determination of copper with zinc dibenzyldithiocarbamate (388) and of iron with o-phenanthroline (389).

D. In Vitro Experiments

(i) In the initial sets of experiments, buffered aqueous solutions of PHZ and APHZ were added to the incubation media containing mitochondrial preparations of the enzyme. The mixtures were preincubated for 15 minutes at 37° and pH 7.0. The reaction was initiated by the addition of kynuramine and allowed to proceed for 20 minutes. The activity of the inhibited enzyme was compared with that of untreated enzyme for different concentrations of the inhibitors.

(ii) The ability of the substrate to protect the enzyme against inhibition was studied. Kynuramine was added to the reaction mixture and the incubation started before either inhibitor was added. The concentrations of PHZ and APHZ that were used were expected to produce about 50% inhibition of enzymic activity if they were preincubated for 15 minutes with the enzyme before the substrate was added.

(iii) When MAO activity is assayed by means of oxygen uptake measurements, the addition of cyanide ion and semicarbazide to the incubation mixtures is useful for suppressing competing oxidations (7, 156). The effects of cyanide and semicarbazide on the inhibition of MAO in vitro by PHZ and APHZ were studied. Quantities of either

2 μmoles of sodium cyanide or 20 μmoles of semicarbazide hydrochloride were added to the incubation mixtures containing enzyme, with or without inhibitors. The media were incubated for 20 minutes at 37° and pH 7.4 before the reaction was initiated.

(iv) A study was made in separate experiments of the influence of pH on the extent to which MAO was inhibited by both substances. Reaction mixtures (3 ml total volume) contained 33.3 mM concentrations of the following buffers: pH values 5.0-6.0, $Na_2HPO_4.7H_2O$ and citric acid; 6.2-8.0, $Na_2HPO_4.7H_2O$ and $KH_2PO_4.H_2O$; pH values 8.6-10.0, glycine and sodium hydroxide. Recipes for the buffering reagents were obtained from tables in Dawson's Methods (390). The enzyme and inhibitors, in suitably buffered mixtures were preincubated for 20 minutes at 37° before the reaction was initiated.

(v) It was observed in several of the previous experiments that inhibition of MAO activity increased when the period of preincubation of the enzyme preparation with the inhibitors was changed from 15 to 20 minutes. Such a time-dependence of inhibitory action is indicative of irreversible inhibition (or, rather, irreversible binding of an inhibitor to an enzyme) (243). Indeed, many hydrazine derivatives are classified as irreversible inhibitors of MAO (391). Irreversibility was tested by the following two methods:

(a) Extensive dilution of an enzyme-inhibitor mixture that has been allowed to stand for some time should restore enzymic activity if enzyme and inhibitor form a reversible complex. Conversely, little or no change in enzymic activity should result

from dilution of the inhibited enzyme if enzyme and inhibitor bind each other irreversibly (392-394). Concentrations of PHZ and APHZ, capable of producing 70-80% inhibition of MAO were incubated with suspensions of washed mitochondria from rat liver for 20 minutes at 37° and pH 7.4. At the end of that time, two sets of aliquots were withdrawn from those media and transferred to buffer containing kynuramine held at 37° (pH 7.4). The incubation with substrate was continued for 20 minutes. In one set of samples, the buffer contained sufficient inhibitor to maintain its original concentration; the other set contained no added inhibitor.

(b) Time-dependence of inhibition was also studied. Buffered mixtures of enzyme preparations and the inhibitors were incubated at 37° for different intervals of time and the enzymic reaction was started by addition of kynuramine at the end of the respective preincubation period. Comparisons were made with untreated enzyme.

(vi) The type of inhibition of MAO was ascertained by assaying activity of the enzyme preparations in the presence and absence of different concentrations of inhibitors and varying amounts of substrate.

E. Enzyme Assay in Vivo

Principle: n-Pentylamine, a good substrate for MAO (5) is readily absorbed and rapidly oxidized by the rat, to valeraldehyde. This metabolite is further oxidized to valeric acid and the degradation products of the latter are expired as carbon dioxide

after entering intermediary metabolism via the citric acid cycle. Pentylamine-1-[^{14}C] should be degraded to $^{14}CO_2$ and the rate of excretion of the latter provides an estimate of MAO activity in the intact animal.

Procedure: Rats fasted overnight were injected intraperitoneally with 5 μCi of n-pentylamine-1-[^{14}C] per kg of body weight. The isotope was diluted with buffered, unlabeled carrier amine and administered at a dosage level of 100 mg per kg of body weight. The rats were placed immediately into separate glass metabolic cages. The expired air containing $^{14}CO_2$ was removed from each cage by a current of dry air, and passed first, by means of a manifold, through a bath of aqueous ethyleneglycol (1 + 1, v/v) maintained at -17°. It was then trapped in 50 ml of a mixture of ethanolamine and ethyleneglycol monomethyl ether (1 + 2, v/v).

Aliquots (3 ml) of the trapping agent were removed at intervals, transferred to scintillation vials, and then mixed with 15 ml of a scintillation "cocktail" that consisted of PPO (5.5 g/L) and DM POPOP (100 mg/L) in a mixture of toluene and ethyleneglycol monomethyl ether (2 + 1, v/v). The radioactivity of the samples was measured in a Beckman LS-250 liquid scintillation spectrometer.

The amount of radioactivity expired at any time was expressed as a percentage of the total amount of radioactivity injected initially. The data were usually expressed as a percentage of the administered radioactivity recovered as $^{14}CO_2$ during the first

hour after injection, because the rates were linear over that period. Buffered aqueous solutions of the various MAOIs were injected (i.p.) in advance of the radioactive amine. The times of pretreatment are mentioned in the tabulated results.

The rates of catabolism of valeric acid-1-[^{14}C] and n-pentanol-1-[^{14}C] were measured as described above and compared in iron-deficient and iron-supplemented rats. These drugs were administered as follows: pentanoic acid-1-[^{14}C], 7.5 μCi per kg of body weight diluted in neutralized carrier valeric acid; pentanol-1-[^{14}C], 5 μCi per kg of body weight in carrier amyl alcohol. Each preparation was injected (i.p.) at a final dosage level of 100 mg/kg. The amyl alcohol solution contained propylene-glycol to 35% (v/v) to ensure that it remained monophasic. Its pH was about 6.0.

F. Enzyme Purification

(i) Washed mitochondrial fractions or low speed (600 x g for 10 minutes) supernatant fractions from homogenates of normal rat livers prepared in 0.25M sucrose were used as the starting material. The mitochondria were either processed immediately or stored in the frozen state (-17° C) until sufficiently large batches had been accumulated. MAO was purified by the method of Youdim and Sourkes (315) up to the ion exchanger step which followed the first salt fractionation. The following steps were used:

(a) Mitochondrial preparations were resuspended in 0.01M phosphate or tris-HCl buffer, pH 7.4, and were sonicated for 70-80

minutes at the maximum intensity of a Branson Model S-75 Sonifier (Heat Systems Inc., Norwalk, Conn.) in the presence of benzylamine (3 mM final concentration) and a detergent (either 1% sodium deoxycholate or 0.5% Triton X-100). The temperature of the suspension was maintained at 2-5° C by means of a salt-ice-water bath.

(b) The clarified, soluble enzyme solution (supernatant fraction from the 105,000 x g for 90 minutes ultracentrifugation in a Spinco Model L ultracentrifuge Type 50 Rotor in earlier experiments; a Type 30 Rotor was used in subsequent preparations) was fractionated with solid ammonium sulfate. NH_4OH solution (3N) was added in small amounts to keep the pH of the mixture at pH 7.4. The precipitate obtained at 30-55% saturation was collected by centrifuging at 15,000 x g for 20 minutes in the SS-34 Rotor of a Sorvall 2B centrifuge, and redissolved in buffer. After re-centrifugation the resulting solution was desalted by gel filtration on a column of Sephadex G-25 (Pharmacia (Canada) Ltd., Montreal) swollen in 0.05M phosphate or tris buffer, pH 7.4.

(c) The enzyme solution was then subjected to ion exchange column chromatography on either DEAE cellulose ("Cellex D", Bio Rad) or DEAE Sephadex A-25. The ion exchangers were equilibrated with either 0.05M phosphate or tris-HCl, pH 7.4, and the column washed with 2 volumes of the same buffer. MAO activity was usually eluted from the column in the starting buffer (0.05M phosphate or tris-HCl, pH 7.4). It was found that the presence of sodium chloride in the eluant (up to 0.01M) could improve the stability of the enzyme.

(ii) Assays

(a) Enzymic activity was followed at each step of purification by measurements of oxygen uptake as previously described (161 and this Thesis) using isoamylamine as substrate.

(b) The protein concentrations of the enzyme preparations were determined by the procedure of Lowry et al. (384). Human plasma albumin (Cutter Laboratories, Berkeley, Calif.) served as a reference standard.

(c) The concentration of iron was measured in some preparations of the enzyme at successive stages of purification using a previously described method (161 and this Thesis).

(d) Estimates of the molecular weight of the partially purified enzyme were obtained by comparison with standard proteins, using Andrews' technique of gel filtration chromatography (395, 396). Columns (1.5 x 90 cm) of Sephadex G-150 and G-200 were employed in these experiments. The adsorbents were equilibrated with 0.05M tris-HCl buffer, pH 7.4, and 0.05M phosphate buffer, pH 7.4, containing 0.10M NaCl, respectively.

(iii) Detection of Riboflavin in MAO

(a) The absorbance spectra of the partly purified enzyme were scanned on the Zeiss PMQ II spectrophotometer. The enzyme was reduced by the addition of sodium tetrathionate (dithionite).

(b) The fluorescence emission spectra of the partially purified enzyme and a flavopeptide obtained from it were scanned on an Aminco-Bowman Spectrophotofluorometer. The spectra were compared with those of riboflavin phosphate (FMN) using an activation wave-

length of 450 nm. The MAO flavopeptide was prepared by incubating a sample of the enzyme (4.25 mg protein) with 4 mg Pronase for 4 hours at 37° and pH 7.4 (0.05M phosphate buffer) in a final volume of 3 ml. Proteolysis was terminated by adding an equal volume of 20% (w/v) trichloroacetic acid (TCA) to the digest, and removing the precipitated protein by centrifugation. The clarified digest was extracted three times with peroxide-free diethyl ether to remove the excess of TCA. The extraction and subsequent procedures were carried out in a darkened room to minimize photodecomposition of the liberated flavin. The emission (oxidized) spectrum of the flavopeptide preparation was scanned at pH 3.2 (0.05M glycine-HCl buffer) as well as at pH 7.4.

(iv) Acrylamide Gel Electrophoresis

(a) Disc gel electrophoresis was carried out in a Buchler "Polyanalyst" unit on samples of enzyme representing MAO at different stages of purification. The method of Davis (397) and Ornstein (398) was used. Six-cm long acrylamide columns (7% gels), polymerized with ammonium persulfate and buffered with 0.4M tris-HCl to pH 8.9, were used. The cathode chamber contained 0.005M tris-glycine buffer, pH 8.3, and the anode chamber 0.4M tris-HCl, pH 8.9. A current of 1.25 mA per gel was used to stack the proteins in the spacer gels (3% acrylamide in 0.06M tris-HCl buffer, pH 6.7). Experiments were performed at a constant current of 2.5 mA per gel after the tracking dye (bromophenol blue) had just penetrated into the running gels. Running tap water was used as a coolant to maintain the anode

chamber at approximately 20°. Electrophoresis was discontinued after the dye front had travelled about 5 cm into the gels (by about 1 1/2 hours). Protein was localized by staining the gels with a 2% solution of amido black (Buffalo blue-black NBR, Allied Chemical) in 7% (v/v) acetic acid. The gels were destained electrophoretically in a 7% aqueous acetic acid solution using a current of 8 mA per gel. Enzymic activity was located by incubating the gels with a dye-substrate mixture (207) for 1 hour at 37°, in the dark. The incubation mixture had the following composition: Nitroblue tetrazolium, 1 mg/ml; phenazine methosulfate, 0.1 mg/ml; sodium cyanide, 1 mg/ml; semicarbazide hydrochloride, 1 mg/ml; tryptamine hydrochloride or tyramine hydrochloride, 2 mg/ml; sodium sulfate, 0.5 mg/ml. All the ingredients were dissolved in 0.25M phosphate buffer, pH 7.4. After the incubations were discontinued, the gels were rinsed thoroughly with glass-distilled water to remove the yellow colour of the dyes.

Samples of MAO at different stages of purification were also subjected to gel electrophoresis in continuous systems, i.e., only running gels were used (10% acrylamide buffered to pH 7.0 with 0.05M phosphate and 5% gels buffered to pH 7.4 with 0.05M tris-HCl) and both electrode compartments of the apparatus contained the same buffer (0.1M phosphate, pH 7.0 or tris-HCl, pH 7.4).

(b) SDS-acrylamide gel electrophoresis was performed according to the procedure described by Weber and Osborn (399). Samples of partially purified MAO, found to be homogeneous by

Sephadex gel filtration, were incubated, with shaking, for 2 hours at 37° in a medium that was 1% (w/v) in sodium dodecyl sulfate (SDS) and 1% (v/v) in 2-mercaptoethanol, all in 0.01M sodium phosphate buffer, pH 7.0. The protein concentration of the incubation mixture was approximately 1 mg/ml. About 0.05-0.10 mg of the denatured protein was mixed with bromophenol blue and incubation mixture and then applied to 10% acrylamide gels, 7 cm long, prepared in 0.2M sodium phosphate buffer, pH 7.0, that contained 0.2% SDS. Both buffer chambers of the apparatus contained 0.1M sodium phosphate buffer pH 7.0 that was 0.1% in SDS. Electrophoresis was performed at a constant current of 8 mA per gel. Protein was visualized by the staining procedure already described. Proteins consisting of subunits whose polypeptide chain molecular weights have been determined were treated similarly and run at the same time as MAO. They served as comparison standards for the estimation of the molecular weights of the dissociated oligomers of MAO.

(v) Sulfhydryl Groups of MAO

(a) Inhibition Studies

Experiments were performed to assess the ability of .SH-reacting compounds, thiol-containing reducing agents and other substances to inhibit partially purified rat liver MAO. Aliquots of 0.10 ml of a solution of the enzyme (about 180 units of activity to isoamylamine) were incubated with various concentrations of sodium arsenite, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), cysteine, dithiothreitol (Cleland's reagent), 2-mercaptoethanol

sodium nitroprusside and potassium thiocyanate for 15 minutes at pH 7.0 and 37° prior to the addition of kynuramine (1 mM final concentration). The final concentration of enzyme was 0.95 μM assuming a molecular weight of 290,000. In some experiments, the period of preincubation of enzyme with inhibitors was extended to 25 minutes without causing any additional inhibition of MAO.

(b) Determination of Number of .SH Groups

The number of sulfhydryl groups per molecule of the enzyme was estimated by the method of Ellmann (400) using DTNB reagent. Aliquots of the reagent dissolved in 0.1M phosphate buffer, pH 8.0, and containing 0.01M EDTA (disodium salt) were added to a solution of the protein in tne same buffer to give a final DTNB concentration of 1×10^{-4}M after mixing. The reaction was carried out at room temperature and followed by measuring the extinction of the yellow thionitrobenzoate anion produced in a Zeiss PMQ II spectrophotometer set at 412 nm. The reaction was usually complete by 20 minutes as no change in absorbance was detectable after that time. Corrections were made for the absorbance values of protein (enzyme blank) and unreacted DTNB (reagent blank) at 412 nm. Progress curves of the reaction (E 412 nm vs time) were plotted and the corrected final extinction values extrapolated to the ordinate at zero time. The number of reactive .SH groups in the enzyme was calculated from the molar absorptivity index of the thionitrobenzoate ion ($E=1.36 \times 10^4$/M/cm) and the concentration of enzyme used. Total .SH content of the enzyme was estimated by carrying out the reaction in buffers that contained

8M urea. A preliminary assessment of the method was made using cysteine, dithiothreitol and methionine as test standards.

G. Chemicals

The inorganic salts used in the dietary supplements were of the highest purity that was commercially available.

Compounds used were obtained from the following suppliers: ammonium persulfate, glacial acetic acid, EDTA disodium salt, semicarbazide hydrochloride, sodium desoxycholate, sodium dodecyl sulfate, sodium phosphate, dibasic, potassium phosphate, monobasic, sodium arsenite, sodium sulfate, anhydrous, sodium tetrathionate (dithionite), sucrose, α,α'-dipyridyl, 8-hydroxyquinoline, o-phenanthroline, ethylene glycol monomethyl ether, monoethanolamine, propyleneglycol, toluene, phenylhydrazine hydrochloride (PHZ), semicarbazide hydrochloride, (ninhydrin) triketohydrindene hydrate, n-heptane, and n-butanol, Fisher Scientific Co., Montreal; ammonium hydroxide, ammonium sulfate, trichloroacetic acid (TCA), tris, valeric acid and n-amyl alcohol, J.T. Baker Chemical Co.; Triton X-100, Hartmann-Leddon Co.; kynuramine dihydrobromide, Regis Chemical Co.; benzylamine, pentylamine, isopentylamine, N,N,N',N'-tetramethylene diamine, 2-mercaptoethanol, Eastman Organic Chemicals; Cyanogum 41, bromophenol blue, BDH (Canada) Ltd.; tryptamine hydrochloride, tyramine hydrochloride, γ-globulin, bovine serum albumin, ferritin, ovalbumin, thyroglobulin, Pronase B, serotonin creatinine sulfate complex, Calbiochem; catalase, Worthington Biochemicals; rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, Boehringer

and Son; cytochrome c, cysteine hydrochloride, methionine hydrochloride, 5,5'dithio-bis-(2-nitrobenzoic acid) (DTNB), Sigma Chemical Co.; Blue dextran, Pharmacia (Canada) Ltd.; nitroblue tetrazolium, phenazine methosulfate, Aldrich Chemicals; dithiothreitol, Pabst Biochemicals, Milwaukee, Wisc.; glycine, potassium thiocyanate, sodium cyanide, sodium nitroprusside and urea, Merck Chemical Co.; riboflavin-5'-phosphate dihydrate, sodium salt, Nutritional Biochemicals Corp., Cleveland; bis-cyclohexanone-oxaldihydrazone (Cuprizone), G. Frederick Smith Chemical Co., Columbus, Ohio; 4-hydroxyquinoline (4-HOQ), K and K Fine Chemicals, N.Y.; 1-acetyl-2-phenylhydrazine (APHZ), sodium diethyldithiocarbamate, Matheson, Coleman and Bell, East Rutherford, N.J.; absolute alcohol, Regie des Alcools de Quebec, Montreal; zinc dibenzyldithiocarbamate (Arazate), Naugatuck Chemicals, Elmira, Ontario; pentylamine HCl (1-^{14}C) specific activity 1.0 mCi/mmole, pentanoic acid sodium salt (1-^{14}C), specific activity 2.01 mCi/mmole, and pentanol (1-^{14}C), specific activity, 1.76 mCi/mmole, Mallinckrodt Nuclear, St. Louis, Mo.; 2,5-diphenyloxazole (PPO), New England Nuclear, Boston, Mass.; (1,4,bis-2-(4-methyl-5-phenyloxazolyl)benzene) (DMPOPOP), Picker Nuclear, White Plans, N.Y. Harmaline hydrochloride was supplied by Fluka AG, Buchs, Switzerland. Other monoamine oxidase inhibitors were gifts from the following companies: iproniazid phosphate, "Marsilid", Hoffmann-LaRoche, St. Laurent, Quebec; tranylcypromine hydrochloride, "Parnate", β-diethylaminoethyl-2,2-diphenylpentanoate hydrochloride, "SKF 525-A", Smith Kline and French, Senneville, Quebec.

Allylisopropylacetamide (AIA) was a gift from Hoffmann-LaRoche, Inc., Nutley, N.J.

2. MAO ACTIVITY IN THE LIVER OF THE IRON- AND COPPER-DEFICIENT RAT

Introduction

Information about the role of metals in the action of the amine oxidases has been accumulating (315, 340-344). Previous work in this laboratory (401, 402) and elsewhere (343, 344) has suggested that rat liver mitochondrial MAO contains a metal cofactor. Youdim and Sourkes (315) showed that the purified enzyme contains insufficient copper (less than 1 atom per molecule of enzyme) to serve as a cofactor of MAO, but relatively more iron (approximately 6 atoms per molecule).

Since it appeared from this that iron, and not copper, might be the possible metallic cofactor of MAO, nutritional experiments, using iron-deficient rats, as well as experiments with metal-chelating agents (in vitro) were conducted to investigate this question further.

Although copper does not participate in the action of rat liver MAO (337, 402), it is known to be involved in the utilization of hepatic iron in the biosynthesis of hemoglobin (403-405). Therefore, the effects of copper deficiency were studied to see whether or not the interaction of joint iron- and copper-deficiencies could influence hepatic MAO activity in the rat.

It was observed that the MAO activity of the livers of the iron-deficient and doubly-deficient rats decreased significantly.

In contrast to this, the activity of the enzyme remained unaltered in rats fed copper-deficient diets for up to 10 weeks. The double-deficiency had no effect beyond that of iron-deficiency alone.

Metal-chelating agents considered to be chiefly iron-complexing inhibited the oxidation of the substrates studied when they were incubated with mitochondrial preparations of MAO. Those substances which chelate copper preferentially were not inhibitory towards MAO.

RESULTS

MAO Activity of Copper- and Iron-deficient Rats

The feeding of diets deficient in copper led to a marked decrease in the concentration of copper in the liver and of plasma ceruloplasmin oxidase activity (385) as shown in Table II. However, the MAO activity of the liver ranged from 102-114% of the control activity in various stages of the deficiency (Table III). Similar results had been obtained by Youdim (402) with kynuramine as substrate.

In contrast to the copper-deficiency which did not have any significant effect on MAO activity, iron-deficiency led to a moderate but significant lowering of enzymic activity (Table IV). The decrease ranged from 18-24% and 25-31% in iron-deficiency, in Series 1 and 2, respectively. In Series 1, the enzymic activity of the liver mitochondria from the doubly-deficient rats was compared with that of rats in the other dietary groups. There was no effect of the combined iron- and copper-deficiency on MAO beyond that observed in

Table II

The effects of diets deficient in iron and copper on the hepatic concentrations of those elements. Means ± SE are shown.

Diet	Duration of Expt (Days)	n	Hepatic Concentration of Iron (μg/g fresh wt)	P	Copper (μg/g fresh wt)	P
Series 1						
Control (1,2)	36-69	8	73.0±6.3		4.45±0.46	
Copper-deficient	36-69	12	95.8±5.2	< 0.05	3.19±0.26	< 0.05
Iron-deficient	36-69	12	24.1±5.2	< 0.001	11.77±2.25	< 0.01
Doubly-deficient	36-69	12	24.9±1.1	< 0.001	3.38±0.22	< 0.05
Series 2						
Control	35-80	8	64.2±4.1		4.42±0.34	
Iron-deficient		8	23.3±0.8	< 0.001	7.93±1.36	< 0.05

1. For control rats, mean hemoglobin concentration ± SE was 14.7%±0.4%; for iron-deficient rats, the value was 4.8%±0.3% (14 rats/group), and for doubly-deficient rats, 4.8%±0.1% (12 rats).

2. Ceruloplasmin oxidase activity was determined in the plasma of individual rats. For 12 control rats the mean value ± SE was 0.07±0.0 absorbance units/0.1 ml/15 min incubation. No readings were detectable for 8 copper-deficient rats tested.

Table III

Monoamine oxidase activity of livers of rats deficient in copper. Means ± SE are shown.

Diet	Duration of Expt (Days)	n	MAO Activity[a]		
			isoAmylamine	Tyramine	Benzylamine
Control	36-69	20	5.86±0.25	2.55±0.10	4.46±0.28
Copper-deficient		23	6.24±0.24	2.90±0.10	4.59±0.26

(a) Enzyme activity is expressed as μl of oxygen consumed per hour per mg protein.

Measurements made at 37.5° under air-saturating conditions.

Table IV

Monoamine oxidase activity of livers of rats deficient in iron. Means ± SE are shown.

Diet	Duration of Expt (Days)	n	MAO Activity[a]		
			isoAmylamine	Tyramine	Benzylamine
Series 1					
Control	36-69	20	6.69±0.25	3.14±0.10	5.08±0.28
Iron-deficient		23	5.53±0.24	2.39±0.10	4.08±0.26
Probability			< 0.01	< 0.001	< 0.05
Series 2					
Control	35-80	14	6.78±0.39	5.74±0.27	5.99±0.38
Iron-deficient		14	5.07±0.44	4.19±0.36	4.13±0.39
Probability			< 0.01	< 0.01	< 0.01

(a) Enzyme activity is expressed as μl of oxygen consumed per hour per mg protein. Measurements were made at 37.5° under air-saturating conditions.

Table V

Monoamine oxidase activity of livers of rats doubly deficient in iron and copper. Means ± SE are shown.

Diet	Duration of Expt (Days)	n	MAO Activity[a]		
			isoAmylamine	Tyramine	Benzylamine
Control	36-69	20	6.69±0.25	3.14±0.10	5.08±0.28
Doubly-deficient	36-69	24	5.71±0.42	2.50±0.17	4.10±0.31
Probability			< 0.05	< 0.001	< 0.05

(a) Enzyme activity is expressed as the rate of consumption of oxygen (μl/hr) per mg protein. Measurements were made at 37.5° under conditions of air saturation.

iron-deficiency alone (Table V). The second series of experiments in which the effect of simple iron-deficiency only was studied was continued for a longer period of time than was Series 1.

Effect of Copper Loading

The possibility was considered that the observed effects of iron-deficiency on MAO, shown in Table IV, could be due to excessive amounts of copper in the hepatocytes rather than a deficit of iron. Experiments in which rats were loaded with copper (by means of repeated intraperitoneal injections of solutions of copper sulfate) were conducted to test this possibility. The results obtained are shown in Table VI and demonstrate that the highly elevated concentrations of hepatic copper attained by this treatment did not change MAO activity. These results confirmed that the reduction in enzymic activity observed in iron-deficient rats was due to depressed levels of liver iron.

Effect of Chelating Agents

Chemicals known to chelate iron and copper were examined for their effects on MAO preparations from normal rat livers. The results of those experiments are shown in Tables VII and VIII. Sodium diethyldithiocarbamate and cuprizone, regarded as specific chelators of copper did not inhibit the oxidation of either iso-amylamine or tyramine at the concentrations tested (Table VII). In contrast to that, those chemicals considered to be chiefly iron-complexing inhibited the oxidation of those substrates (Table VIII).

Table VI

The effects of repeated injections with copper on the level of hepatic copper and MAO activity of the liver. Rats were injected once daily for 7 consecutive days with aqueous copper sulfate 0.15M in NaCl. Control animals received saline only. The animals were killed 24 hr after they received the last injection. The livers were perfused in situ with ice-cold 0.25M sucrose solution, removed, chilled and portions taken for analysis of copper and estimation of MAO. Enzyme assays were performed either on the 600 x g supernatant fractions or mitochondrial fractions of homogenates prepared in 0.25M sucrose. (Means ± SE).

Cu^{2+} injected		Liver [Cu]	MAO Specific Activity*		
(mg/kg)	n	(μg/g fresh wt)	isoAmylamine	Tyramine	Benzylamine
0.0	6	3.95±0.10	4.19±0.26	1.90±0.24	2.10±0.10
0.5	5	11.14±1.86**	4.76±0.54	2.16±0.43	2.31±0.28
1.0	6	43.05±5.79***	4.83±0.51	2.71±0.34	2.23±0.22
2.0	3	90.13±21.58**	4.89±0.37	2.55±0.45	1.82±0.10
0.0	6	5.99±1.14	7.62±0.00	--	6.39±0.36
2.0	8	97.96±11.20***	6.59±0.44	--	5.31±0.76

*Specific activity is expressed as the rate of consumption of oxygen (μl/hr) per mg protein measured at 37.5° under air saturating conditions. **$p < 0.01$; *** < 0.001 (student t-test).

Table VII

Effects of copper-chelating agents on MAO activity of rat liver in vitro

Chelator	Concentration mM	Monoamine oxidase activity*			
		isoAmylamine		Tyramine	
		n	Mean ± SE	n	Mean ± SE
Sodium diethyldithiocarbamate	0.167	3	99.0±1.1	3	102.0±2.9
	1.67	4	93.5±2.8	4	103.0±3.7
Cuprizone	0.167	4	91.1±2.1	4	95.1±3.7

*Activity measured in the presence of the chelating agent as a percentage of the control value. Chelator was incubated with the enzyme preparation at 37.5° for 5 min before substrate was added. The mean specific activity (± SE) was 6.01±0.81 µl of oxygen consumed per hour per mg protein using isoamylamine as substrate in 7 experiments; it was 5.21±0.13 in 11 experiments with tyramine.

Table VIII

Effect of iron-chelating agents on monoamine oxidase activity of rat liver in vitro

Chelator	Concentration mM	Monoamine oxidase activity* Isoamylamine n	Isoamylamine Mean ± SE	Tyramine n	Tyramine Mean ± SE
α,α'-Dipyridyl	0.167	3	108±6.1	3	93±3.6
	1.67			6	34±2.1
	6.67	6	50±1.6		
8-Hydroxyquinoline	0.167	3	115±3.8	3	64±3.7
o-Phenanthroline	0.167	5	103±5.8	6	88±2.6
	0.833			2	14, 48
	1.67			5	14±4.3
	13.33	6	29±1.6		

*Activity measured in the presence of the chelating agent, as a percentage of the control value. Chelator was incubated with the enzyme preparation at 37° for 5 min before substrate was added. Enzyme activity was measured in units of μl of oxygen consumed/hr/mg of protein. The mean specific activity (± SE) was 6.8±0.81 units in 20 experiments with isoamylamine as substrate; it was 4.1±0.19 units in 21 experiments with tyramine.

α,α'-Dipyridyl, 8-hydroxyquinoline and o-phenanthroline all inhibited the oxidation of tyramine to some extent. When iso-amylamine was the substrate, inhibition was observed only at high concentrations in the cases of α,α'-dipyridyl and o-phenanthroline.

DISCUSSION

The experiments described above show that prolonged deficiency of dietary copper results in (a) reduced levels of this element in the liver, (b) decreased activity of plasma ceruloplasmin oxidase activity, but (c) essentially unchanged MAO activity of the hepatocytic mitochondria. This last observation is in agreement with the low concentrations of copper found in purified preparations of mitochondrial MAO prepared from rat liver (315, 337), beef liver (348), beef kidney (12), and pig brain (160). Youdim (402) had found earlier that rats maintained for as long as 13 weeks on a copper-deficient diet had the same levels of MAO activity in their liver mitochondria as animals that were fed the control diet. In those experiments, enzymic activity was measured by the kynuramine oxidase (171) and benzylamine oxidase methods (174).

Chronic iron-deficiency resulted in (a) a mean reduction of the hepatic iron concentration of about 65% during the period studied, (b) a reduction in blood hemoglobin level of the same order, and (c) a decrease in MAO activity that ranged from 18-31% for both series of experiments. Thus, the effect of iron-deficiency on MAO is in strong contrast to that observed in the copper-deficient rat.

The observed effect of the iron-deficiency on MAO indicates that the activity of the enzyme in the normal rat may depend on the presence of iron. From a consideration of the results recorded above, the following alternative roles may be postulated for the participation of iron in the action of MAO: (1) the effect of iron-deficiency could stem from the loss of a metallic cofactor, or (2) iron could be required for binding particles containing the enzyme to the mitochondrial structure so as to maintain it in some spatial orientation most suitable for its full activity, or (3) iron could function as part of a catalyst involved, at some stage, in the biosynthesis of MAO. If iron is a part of the MAO molecule in rat liver, then it may be held firmly by the apoenzyme, and be removed at a much slower rate than either hemoglobin or total hepatic iron during the development of a nutritional deficiency. The inhibition of the enzyme by iron-chelating agents is consistent with that possibility.

Iron deficiency was accompanied by an increased concentration of hepatic copper (Table II), thus confirming previous reports of this inverse relationship (406, 407). It is significant that copper-loading of rats failed to inhibit liver MAO activity towards any of three different substrates tested. This made the possibility unlikely that elevated hepatic copper rather than decreased iron concentrations *per se* could be responsible for the depressed enzymic activity observed in iron-deficiency. Furthermore, Lagnado and Sourkes (340) had reported earlier that copper inhibits MAO weakly *in vitro*.

Purified MAO from three sources (12, 315 and 348) has an absorption band in the Soret region of the spectrum. This is suggestive of the presence of an iron complex in these preparations. Youdim and Sourkes (315) found that the rat liver enzyme contained 0.12% iron while Nara et al. (313) found about 0.02% in their purified beef liver preparation. Such inconsistencies may be due to species differences.

Youdim (402) also observed inhibition of the rat liver MAO by the above-mentioned chelators. Murali and Radhakrishnan (320) found that these agents inhibited the partly purified mitochondrial MAO from the monkey small intestine. These workers found that sodium diethyldithiocarbamate and DL-penicillamine, another well-known chelator of copper, were also inhibitory. Severina and Gorkin (342) found that o-phenanthroline and 8-hydroxyquinoline were not inhibitory when benzylamine was the substrate. o-Phenanthroline and $\alpha,\alpha,'$-dipyridyl are weak inhibitors of the beef liver enzyme (313), however, the latter compound seemed more active in later experiments reported by Yasunobu and his coworkers (348). The beef brain (312) and pig brain (160) enzymes were also inhibited by iron chelators. Mitochondrial MAO from beef kidney (12) was also more susceptible to inhibition by 8-hydroxyquinoline and o-phenanthroline than to cuprizone and sodium diethyldithiocarbamate. Erwin and Hellerman (12) found that the inhibition by 8-hydroxyquinoline could be reversed by the addition of some divalent metallic ions to the reaction mixture. (Copper caused further inhibition and not reversal, however.) Gabay and Valcourt (319)

observed dual effects of metal-chelating agents on purified MAO from rabbit liver mitochondria: i.e., o-phenanthroline and neocuproine at low concentrations (23-330 μM) produced an enhancement of activity whereas higher concentrations (1.0-5.0 mM) were inhibitory towards the oxidation of kynuramine.

In addition to these observations, inhibition of MAO by agents considered to be strong iron-binding chelators in biological experiments calls attention to the iron content of purified rat liver MAO (315, 337). However, the evidence from the present studies with livers of iron-deficient rats and with chelating agents indicates that the presence of iron is important for the proper functioning of the normal enzyme.

SUMMARY

1. Chronic nutritional iron-deficiency produced a significant decline, amounting to 18-31%, in the MAO activity of rat liver mitochondria.

2. Prolonged feeding of a diet deficient in copper was without effect on the enzyme.

3. A decrease in enzymic activity of the same magnitude that arose from simple iron deficiency was observed in the livers of rats that were fed a diet low in both iron and copper.

4. Repeated intraperitoneal injections of copper sulfate produced elevated concentrations of hepatic copper, which were greater than the levels observed in iron-deficient livers. The MAO activity of the copper-loaded rat livers did not differ from

that found in normal livers.

5. The results of the nutritional experiments and the inhibitory action of iron-chelating agents on MAO together implicate iron in the activity of the enzyme.

6. Possible roles are postulated for iron in the action of MAO.

3. ON THE ESTIMATION OF MONOAMINE OXIDASE ACTIVITY IN VIVO IN THE ~~LIVING~~ WHOLE ANIMAL. THE SITE OF PARTICIPATION OF IRON IN THE METABOLISM OF AN ALIPHATIC MONOAMINE

Introduction

Many of the tests used to assess the activity of MAO and the efficacy of MAOIs *in vivo* either in human patients or experimental animals are based on the assumptions that inhibition of the enzyme will elicit the following responses: (a) decreased enzymic activity in homogenates of tissues obtained from inhibitor-treated animals; (b) decreased MAO activity *in vivo* which results in decreased metabolism of endogenous as well as exogenous biogenic amine substrates. Examples of such indirect methods have been mentioned in the introductory review of this Thesis.

The present work is concerned with the development of a more direct method of estimating MAO activity *in vivo*, by means of injecting animals with tracer amounts of radioactive [1-^{14}C]-labeled substrates. The method, adapted from that of Madras and Sourkes (408), was developed primarily to extend our previous work on the effects of iron deficiency on MAO activity in the rat (161 and this Thesis), and to assess the effects of iron deficiency on the *in vivo* rate of catabolism of monoamines.

By this means, it was confirmed that iron is an essential dietary factor, when given in sufficient amounts, for full activity of MAO in the rat (*in vivo* as well as *in vitro*). The requirement for iron was at the oxidative deamination (MAO-catalyzed) stage of amine catabolism and not at the sites of oxidation of the aldehyde

or acid metabolites. The method was also used to determine whether or not riboflavin deficiency and copper deficiency affect MAO activity *in vivo*.

A preliminary study of the effects of some MAOIs by means of this method, was also undertaken.

RESULTS AND DISCUSSION

Monoamine Oxidase Inhibitors

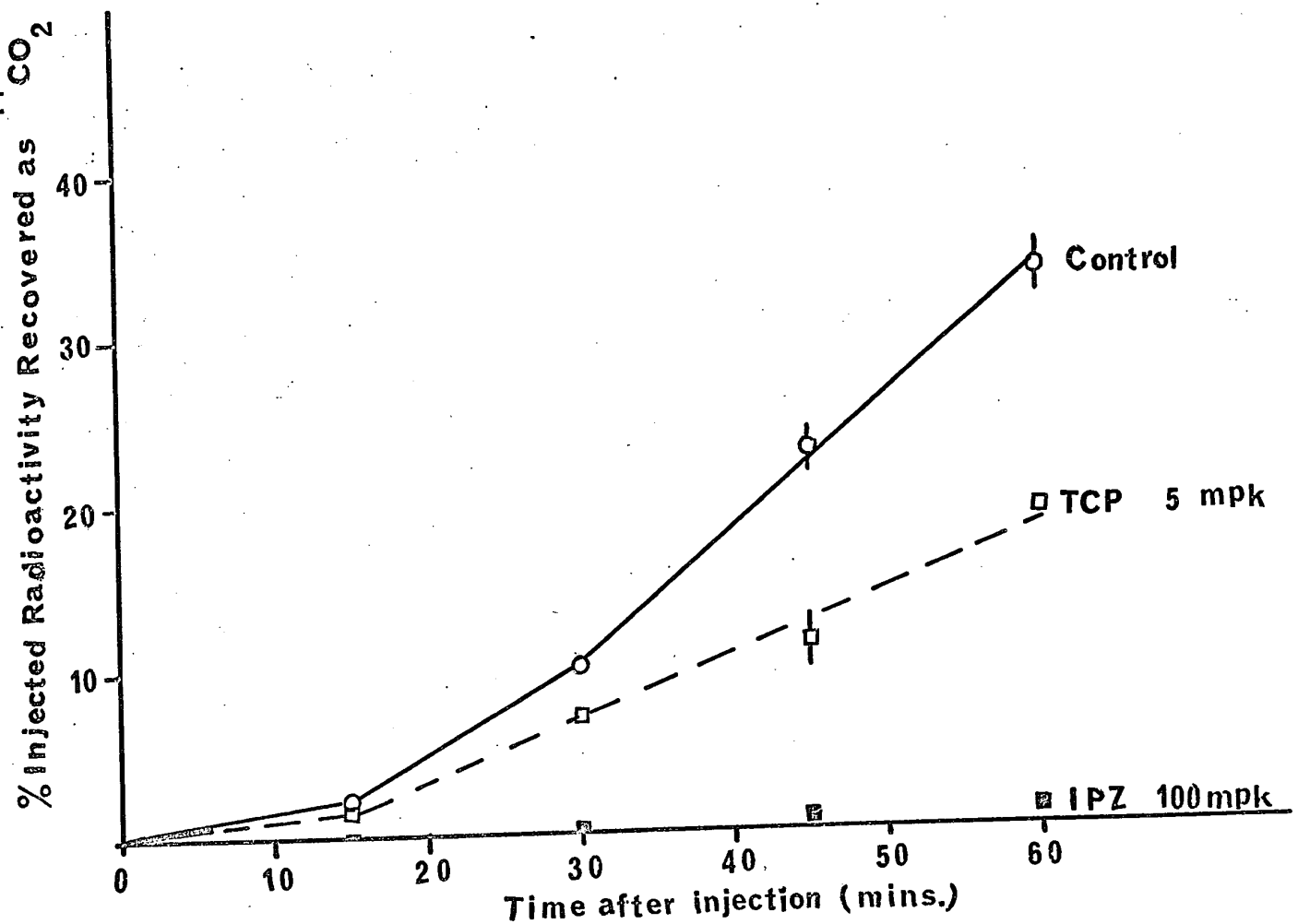
The data from some experiments with MAOI are shown graphically in Fig. 3. The effects of tranylcypromine (TCP) and iproniazid (IPZ) indicate that the method is specific for measuring MAO activity *in vivo*. TCP, at the dosage level employed, caused a reduction of MAO activity to about 55% of control values. IPZ blocked catabolism of the amine almost entirely; only 3-7% of the injected radioactivity was recovered as $^{14}CO_2$ during the first three hours after its administration.

Other MAOIs tested by this method were harmaline, a reversible, short-acting inhibitor (252) and SKF 525-A, an inhibitor of several microsomal drug-metabolizing enzymes (409), also found to have anti-monoamine oxidase action (410, 411). The data that were obtained are shown in Table IX. Acetylphenylhydrazine, an inhibitor of MAO *in vivo* and *in vitro* (412) was also tested by this method. The results of that experiment are reported in another section of this Thesis.

Harmaline, at the combinations of dosage levels and pre-treatment times employed in these studies, was apparently without

Figure 3

Effect of MAO inhibitors on the rate of catabolism of 1-^{14}C-labeled n-pentylamine by normal rats. Buffered saline solutions of the inhibitors were given to the experimental animals at the indicated dosage levels by intraperitoneal injection 16 hours prior to the administration of amine. Food was withdrawn simultaneously. The control rats were injected with saline. Each rat was injected i.p. with 5 μCi of labeled compound per kg body weight. The label was diluted with nonradioactive carrier amine and administered at a dosage level of 100 mg per kg. Each point represents the mean (± SE) of observations made on 6 control animals and on groups of 3 animals given each of the inhibitors.


% Injected Radioactivity Recovered as $^{14}CO_2$
40
30
20
10
0
10
20
30
40
50
60
Time after injection (mins.)
Control
TCP 5 mpk
IPZ 100 mpk

effect on the rate of catabolism of n-pentylamine. Perhaps its overall effect (mainly on the MAO of brain) was insufficient to block the activity of the MAO in liver.

SKF 525-A appeared to have dose-dependent effects on MAO. The enzyme was inhibited by this drug by about 20% at 100 mpk. Notwithstanding earlier reports on the inhibitory effects of SKF 525-A towards MAO activity of rat liver in vitro (410) and rat brain in vivo and in vitro (411), these results should be interpreted with caution, in view of the difference in the intervals of time that elapsed between pretreatment with the inhibitor and injection with the labeled substrate.

Effects of Iron-deficiency on Rate of Catabolism of Labeled [1-^{14}C] Pentylamine

First Series

The rats were used in this series of experiments after they had been maintained for 7 weeks on the iron-deficient diet. The hemoglobin concentrations of tail blood had not been measured because it had been observed in earlier experiments that they fell and remained consistently lower than control values after 4-5 weeks on the iron-deficient diet. It is evident from Table X that iron-deficient rats metabolize pentylamine more slowly than the control rats (about 60% of control rates). Previously, it was found that the average decrease in mitochondrial amine oxidase activity of rat liver due to chronic iron-deficiency ranged from 18-31% (161 and this Thesis) when measured in vitro. This method then appears

Table IX

Effects of some MAOIs on the rate of catabolism of pentylamine-[1-^{14}C] in the rat in vivo. Drugs were administered in buffered (pH 6.8-7) aqueous solutions of 0.15M NaCl by intraperitoneal injection at the dosage levels and intervals of treatment in advance of amine as indicated. Control animals received equivalent amounts of saline. The radioactive amine (5 μCi/kg) was diluted with unlabeled n-pentylamine and injected intraperitoneally in a dose of 100 mg/kg body weight.

Experiment Number	Treatment	% of injected radioactivity recovered as $^{14}CO_2$ (Means ± SE) 30 min	45 min	60 min
1	Saline	12.4±0.6 (3)[a]	26.0±1.5	40.8±1.6
	Harmaline HCl 5 mpk 30 min prior to receiving amine	12.9±0.4 (3)	27.6±0.8	41.7±0.6
2	Saline	8.7±0.4 (3)	18.3±0.5	27.5±1.2
	Harmaline HCl 20 mpk 15 min prior to receiving amine	8.0±1.7 (3)	18.5,14.0	24.0
3	Saline	9.4±0.3 (3)	20.2±0.4	30.0±0.6
	SKF 526-A 100 mpk 60 min prior to receiving amine	8.2±0.0 (2)	16.5±0.4	24.5±0.3
		$P < 0.05$	$P < 0.01$	$P < 0.01$
4	Saline	8.3±0.2 (3)	18.2±0.7	28.3±0.8
	SKF 525-A 75 mpk 90 min prior to receiving amine	9.0±0.8 (2)	17.7±2.8	27.7±3.6

[a] Numbers in parentheses indicate numbers of animals tested in each experiment.

to be more sensitive as a means of assessing the effects of iron deficiency on MAO activity in rats than the in vitro methods used earlier.

The effect of realimentation with iron is a complete restoration of the rate of amine oxidation to control levels by 6 days (Table X). These results indicate that iron is involved in the metabolism of pentylamine, although the hemoglobin and hepatic iron concentrations have not yet increased to the levels found in the control rats. Reference to Table XI will show that these parameters for the iron-supplemented deficient rats although within the normal range of values, are significantly different from control values for a long time after MAO activity is back to normal.

Figures 4 and 5 represent the effects of iron-deficiency and realimentation with iron, respectively, on rates of amine metabolism in the rat. The cumulative recovery of $^{14}CO_2$ was significantly lower at all times for the iron-deficient than for the control rats (Fig. 4). On the other hand, cumulative rate curves for the realimented and control rats are nearly identical up to 3 hours (Fig. 5).

The lag in $^{14}CO_2$ excretion rates observed for all the test animals during the 0-15 min recovery interval in these progress curves appears to be due to the period of time required for complete absorption of the drug (amine or analogue) from the peritoneal cavity. The rates of $^{14}CO_2$ production soon accelerate, become linear up to 60 min and gradually level off thereafter.

Table X

Effect of iron-deficiency and subsequent realimentation with iron on the rate of catabolism of pentylamine-[1-^{14}C] in the rat *in vivo*. For the experiments on repletion, the test animals received the iron-deficient diet for 56 days followed by the iron-supplemented diet for the succeeding number of days indicated. The dose of radioactive amine and route of injection were as described in Table IX.

Experiment Number	Diet	Days	% of injected radioactivity recovered as $^{14}CO_2$ during the first 60 min (Means ± SE)
1	Control	51	38.3±1.14 (6)*
	Iron-deficient	51	23.1±1.09 (6)
2	Control	62	32.1±3.04 (4)
	Iron-deficient then supplemented	6	33.9±2.63 (6)
3	Control	65	34.9±0.88 (3)
	Iron-deficient then supplemented	9	35.0±2.08 (3)
4	Control	68	34.3±1.78 (3)
	Iron-deficient then supplemented	12	34.2±1.12 (3)

*Numbers in parentheses refer to the numbers of animals tested in each experiment.

Table XI

Effect of iron-deficiency and subsequent realimentation on nutritional status of the experimental animals. Realimented rats had received the iron-deficient diet for 56 days, followed by the iron-supplemented diet for the succeeding number of days indicated. Means ± SE are shown.

Diet	Days	n	Body weight (g)	Liver weight (g)	Hemoglobin concentration (g/100 ml blood)	Hepatic iron concentration (μg/g fresh tissue)
Control	73	10	264.8±5.8	7.20±0.19	15.4±0.53	123.4±16.01
Iron-deficient	73	5	222.2±6.5***	6.40±0.10*	5.1±0.66***	24.94±0.66***
Iron-deficient then supplemented	17	5	284.4±5.4	8.72±0.40**	12.9±0.23**	62.64±16.26*

*Statistically significant $P < 0.05$ (Student t-test)

** $P < 0.01$

*** $P < 0.001$

Figure 4

Progress curves: Comparison of the time courses of catabolism of [1-^{14}C]-labeled n-pentylamine by normal and iron-deficient rats. The experimental rats were fed for 51 days on a semisynthetic diet lacking iron. They were fasted overnight before being injected i.p. with 5 μCi of labeled compound per kg body weight. The drug was diluted with nonradioactive n-pentylamine and injected at a dosage level of 100 mg per kg. Experimental points represent the means (± SE) of observations made on groups of 6 animals.

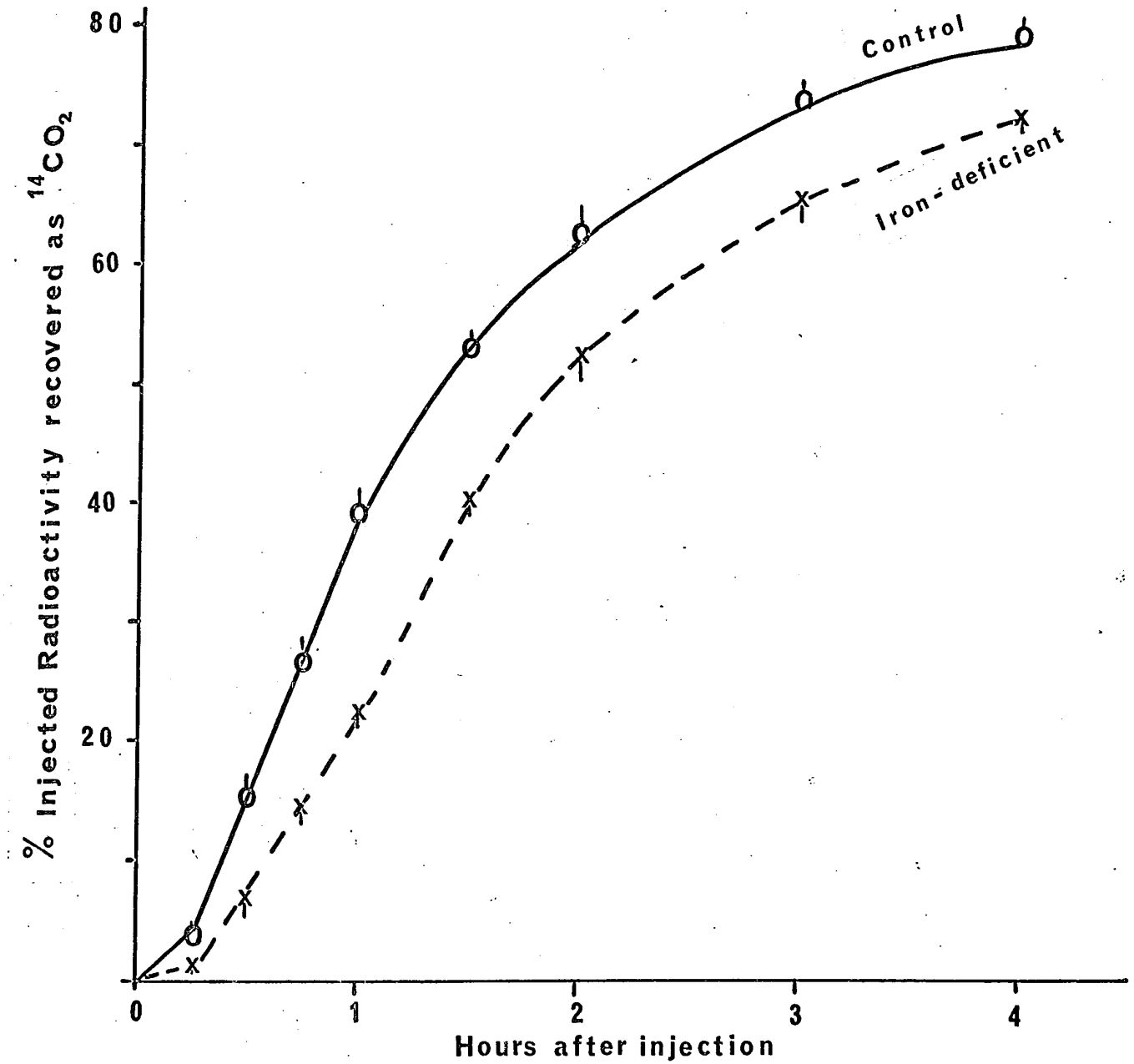
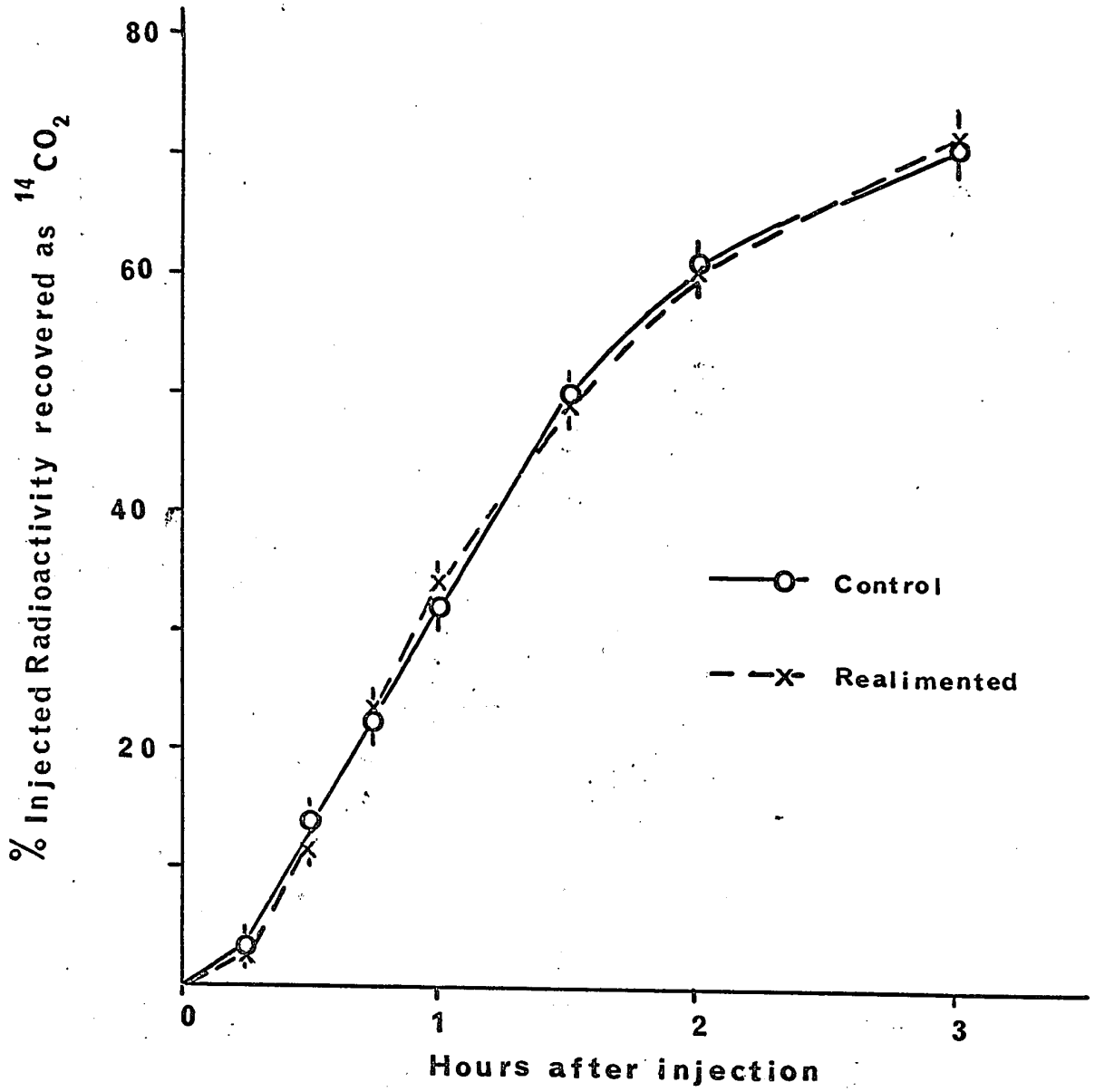

80
60
40
20
0
1
2
3
4
% Injected Radioactivity recovered as $^{14}CO_2$
Hours after injection
Control
Iron-deficient

Figure 5

Progress curves: Comparison of the time courses of catabolism of [1-^{14}C]-labeled n-pentylamine by normal rats and iron-deficient rats supplemented with iron. The experimental rats were fed for 51 days on a semisynthetic diet lacking iron. They were then placed on an iron-supplemented diet for 6 days, at which time they were tested for their ability to catabolize the radioactive amine. Details of the experimental procedure are described in the caption to Figure 4. Experimental points represent the means (± SE) of observations made on groups of 6 animals.


80
60
40
20
0
1
2
3
% Injected Radioactivity recovered as $^{14}CO_2$
Hours after injection
Control
Realimented

Second Series

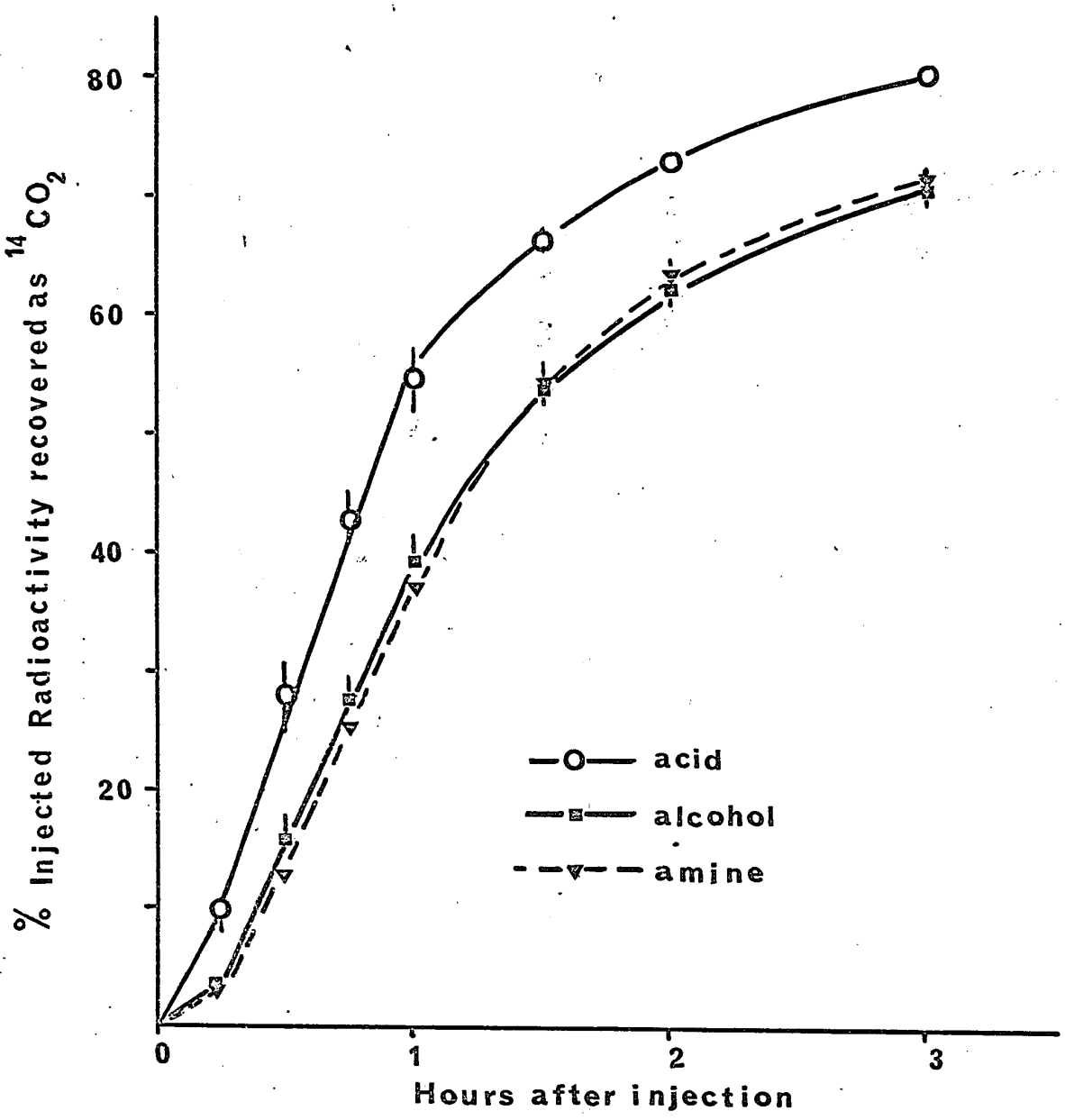
The results obtained in this series of experiments confirm those obtained in the first series, and are represented by the graphs shown in Fig. 7. The rate of oxidation of radioactive pentylamine was seen to decrease steadily as iron-deficiency progressed (Fig. 7A). This phenomenon and the fact that realimentation of the iron-deficient rats restored the rate of amine oxidation *in vivo* to normal indicated iron dependence of MAO activity in the rat.

The hemoglobin content of tail blood of the same animals used in these radioactive tracer studies of pentylamine catabolism was estimated. Nutritional iron-deficiency caused a decline in blood hemoglobin concentration to about 30% of the control level within 4 weeks from the time the rats were first fed the iron-deficient diet. A rapid return to the normal range of values was observed when some of the deficient rats were fed the iron-supplemented diet (Fig. 7B).

Examination of the growth curves (Fig. 7C) shows that iron-deficiency soon produced a significantly slower rate of weight gain in the experimental rats than in the control rats. The mean growth rate of the iron-realimented rats remained parallel with that of the iron-deficient rats. This may be explained by the fact that a much longer period of time than was necessary for realimentation to restore hemoglobin concentration and MAO activity to normal was required for iron-deficiency to exert its maximum effect on decreasing growth rate (4-5 weeks on the diet).

Figure 6

Progress curves: Comparison of the time courses of catabolism of some [1-^{14}C]-labeled substrates in intact normal rats. Rats were fed complete semisynthetic diets for 28, 30 and 32 days and then fasted and tested for their ability to catabolize radioactive n-pentylamine (5 μCi per kg), n-pentanol (5 μCi per kg) and n-pentanoic acid (7.5 μCi per kg), respectively, injected i.p. Details of the experimental procedure are described in the text. Experimental points represent the means (± SE) of observations made on groups of 3 animals.


% Injected Radioactivity recovered as $^{14}CO_2$
80
60
40
20
0
1
2
3
Hours after injection
acid
alcohol
amine

Effects of Iron-deficiency on Rates of Catabolism of Radioactively Labeled [1-^{14}C] Analogues of Pentylamine in Vivo

The results referred to above implicate iron in the enzyme oxidation of amines in vivo as well as in vitro. However, the site of the requirement for iron in the metabolic pathway of pentylamine was found by comparison of the rates of catabolism in vivo of pentanoic acid [1-^{14}C] and n-pentanol [1-^{14}C], estimated as described above, in the iron-deficient and iron-supplemented rat. Pentylamine and n-pentanol are both metabolized by the rat, after their enzymic oxidation to valeraldehyde, to carbon dioxide via the same pathway (5, 413). The [1-^{14}C]-labeled aldehyde was not available, and so the rates of catabolism, by normal and iron-deficient rats, of its succeeding metabolite, pentanoic acid-[1-^{14}C] were compared. Data from these experiments appear in Table XII.

It is evident that iron-deficiency did not impair the ability of the rat to metabolize these substrates. This is in contrast to the results that were obtained when pentylamine oxidation was studied.

Fig. 6 shows progress curves of cumulative rates of recovery of $^{14}CO_2$ from groups of control rats that were fed the semisynthetic diet (iron-supplemented) for approximately the same length of time, and had been injected with the various [^{14}C]-labeled substrates.

Neither valeraldehyde oxidation nor pentanoic acid oxidation can be rate-limiting for the oxidation pathway of pentylamine, since pentanol, which passes through the aldehyde stage, and pentanoic acid itself are both metabolized more rapidly than pentylamine, during the initial stages at least, in control rats. Hence, under

Table XII

Effect of dietary iron deficiency on metabolism of [1-^{14}C]-labeled substrates to $^{14}CO_2$ in the rat *in vivo*. Three animals per group were used in all the experiments. Means ± SE are shown.

Diet	Days	Substrate	% of injected radioactivity recovered as $^{14}CO_2$ in the first hr
Control	30	n-amyl alcohol	39.1±2.4
Iron-deficient			42.9±2.4
Control	70	"	39.2±1.4
Iron-deficient			42.6±2.0
Control	11	valeric acid	53.8±3.4
Iron-deficient			54.7±2.4
Control	32	"	54.6±2.0
Iron-deficient			51.9±0.9

these experimental conditions, MAO is rate-limiting in the conversion of carbon-1 of pentylamine to carbon dioxide. Furthermore, since iron-deficiency decreased the rate of oxidation of the amine, and not that of the acid or alcohol (nor aldehyde, by extension of the results for the alcohol), then it follows that iron is necessary at that stage (oxidative deamination) of catabolism of pentylamine. In other words, the rat requires adequate supplies of dietary iron to ensure the proper functioning of its MAO.

In an earlier publication (161) and elsewhere in this Thesis the following alternative possibilities of a function for iron in the activity of MAO had been considered: (1) iron could be attached to the enzyme as a prosthetic group; (2) it may be necessary to bind the enzyme to the mitochondrial membrane in some orientation suitable for its proper action; or (3) iron could be a requirement at some stage of the biosynthesis of MAO.

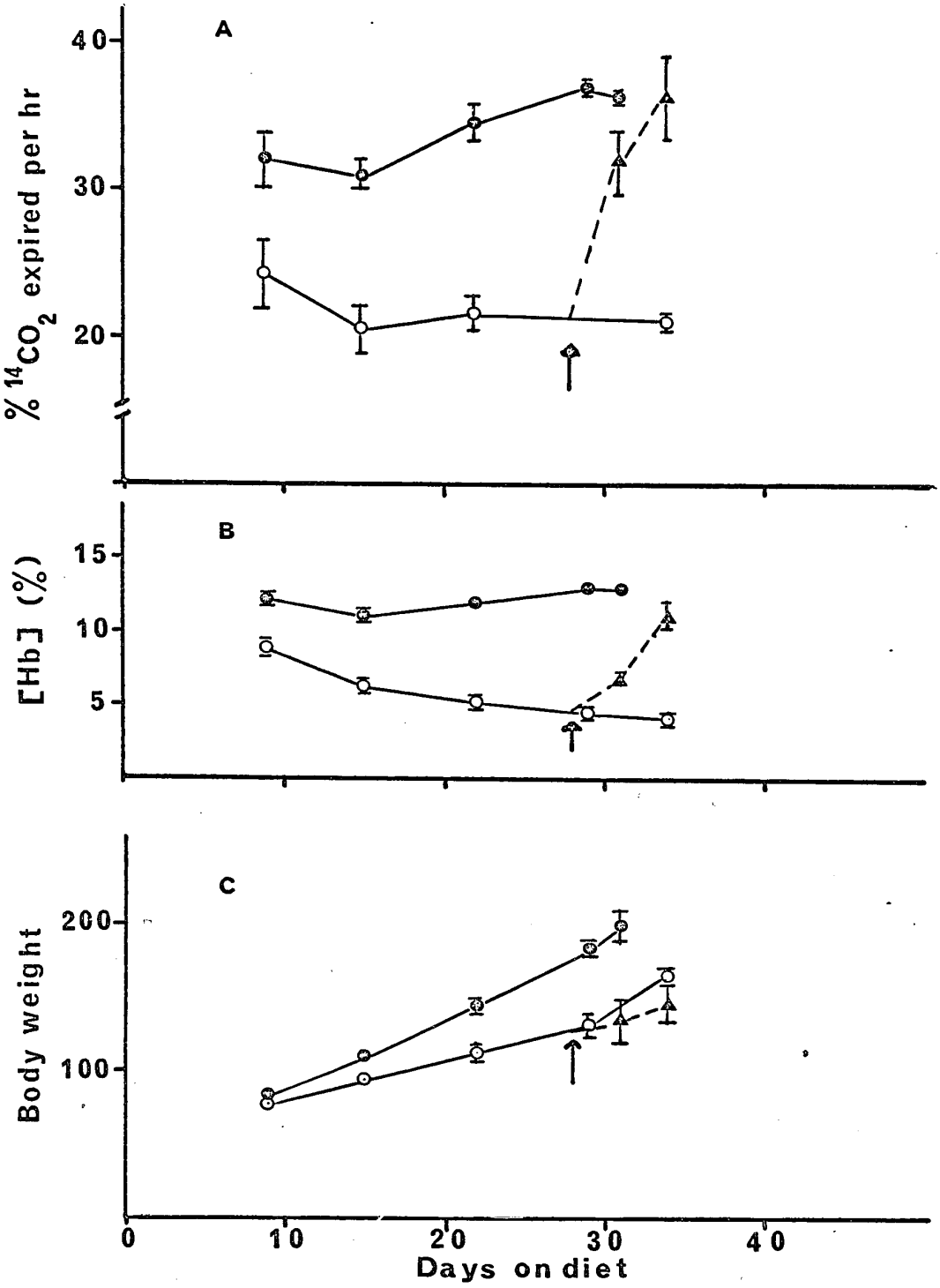
Concerning the first possibility, the purified rat liver preparation of Youdim and Sourkes (315) contained iron in significant amounts (6 atoms/300,000 Daltons), and iron chelators inhibit the enzyme (161). While these facts cannot be ignored, they are not yet sufficient evidence to confirm that iron is a prosthetic group of mitochondrial MAO.

Effects of Copper and Riboflavin Deficiencies on the Rate of Catabolism of n-Pentylamine-[1-^{14}C] in Vivo

The second alternative function for iron in the activity of MAO, mentioned above, was tested by indirect means. Dallman and

Figure 7

Effects of iron deficiency and realimentation with iron on: (A) the rate of catabolism of pentylamine-[1-^{14}C] *in vivo* in the rat; (B) concentration of hemoglobin in tail blood; (C) growth rate of rats fed on a semisynthetic solid diet. Details of the experimental procedures followed appear in the caption to Figure 4 and in the text. The experimental points represent means (± SE) of observations made on at least 3 animals in each group. The open circles represent iron-deficient rats. The closed circles represent control (iron-supplemented) rats, and the triangles, deficient rats repleted with the complete diet. The arrows indicate the day on which realimentation with iron commenced.


A
40
30
20
% $^{14}CO_2$ expired per hr
B
15
10
5
[Hb] (%)
C
200
100
Body weight
0
10
20
30
40
Days on diet

Goodman (414) described abnormal ultrastructural changes which occur in liver mitochondria of iron-deficient rats. Those changes were readily reversible when the rats were realimented with iron. If such structural abnormalities can decrease MAO activity, then a similar decrease could be expected to occur, however the abnormalities should arise. Dallmann and Goodman (415) and other workers (416, 417) have also reported that similar abnormal changes occurred in the hepatocytic mitochondria of rats and mice that were made deficient in copper or riboflavin.

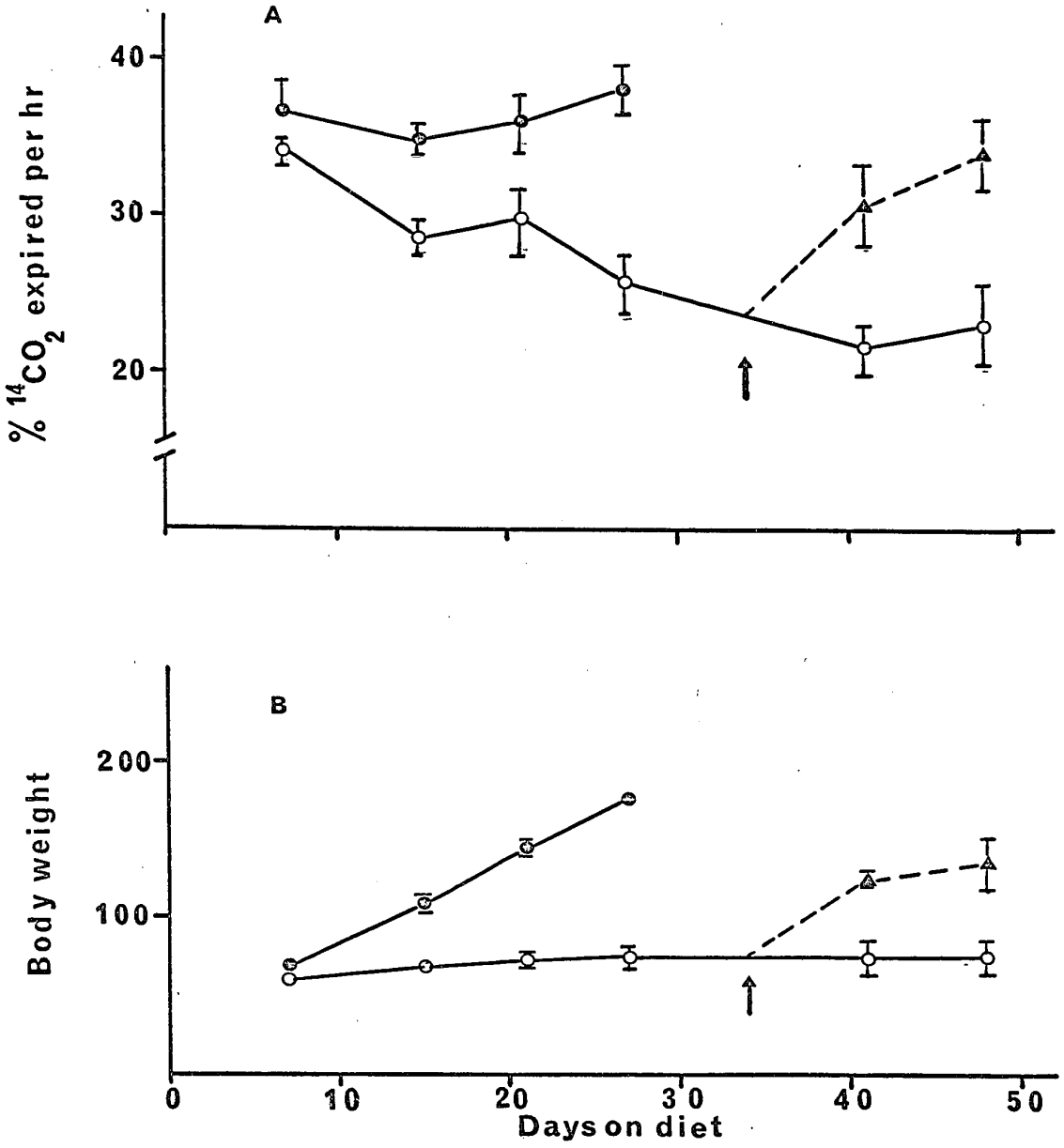
Two series of studies were therefore undertaken to assess the relative effects of copper-deficiency and riboflavin-deficiency on the rate of catabolism of radioactive pentylamine by the rat. The purpose of these experiments was to ascertain whether or not the ultrastructural integrity of mitochondria plays any part in the action of MAO.

The results of these experiments are shown in Figures 8 and 9. MAO activity in vivo decreased steadily as riboflavin-deficiency progressed with time, and was rapidly restored in deficient rats that were fed riboflavin (Fig. 8A). This is understandable, since the presence of riboflavin has now been detected in the prosthetic group of several mammalian MAOs (10-18) including that of the rat liver (16, 17) and a considerable reduction in MAO activity has long been associated with riboflavin deficiency (332-336; 418).

Examination of the growth curves in part B of Fig. 8 shows that there was almost complete cessation of growth as a result of the deficiency. There was a marked increase in growth rates of

Figure 8

Effects of riboflavin deficiency and realimentation with riboflavin on: (A) the rate of catabolism of pentylamine-[1-^{14}C] *in vivo* in the rat; (B) growth rate of rats fed on a semisynthetic solid diet. Details of the experimental procedures followed appear in the caption to Figure 4 and in the text. The experimental points represent means (± SE) of observations made on at least 3 animals in each group. The open circles represent riboflavin-deficient rats. The closed circles represent control (riboflavin-supplemented) rats, and the triangles, deficient rats repleted with the complete diet. The arrows indicate the day on which realimentation with riboflavin commenced.


A
40
30
20
% $^{14}CO_2$ expired per hr
B
200
100
Body weight
0
10
20
30
40
50
Days on diet

those deficient rats that were fed the riboflavin-supplemented diet.

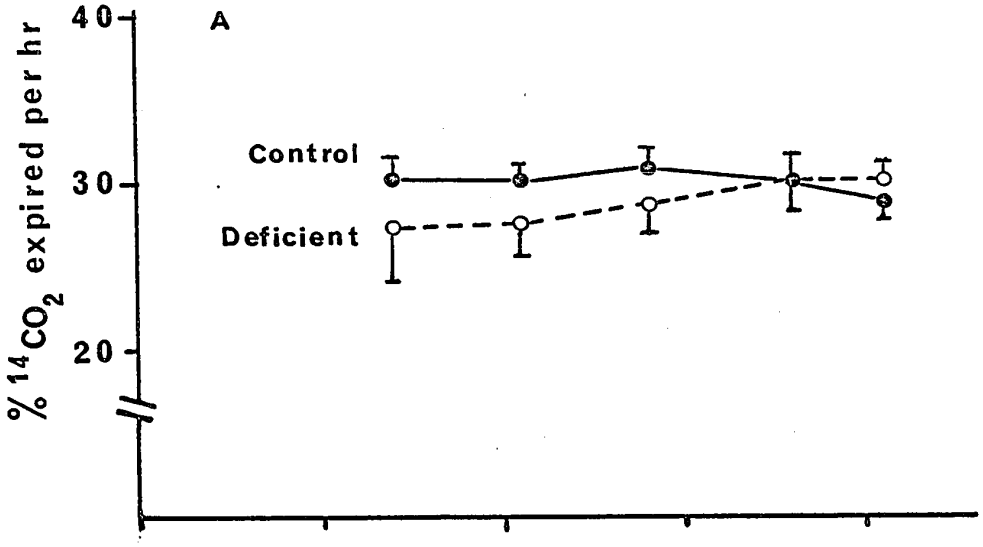
On the other hand, copper-deficiency of up to six weeks's duration did not interfere with the ability of the rats to catabolize radioactive pentylamine (Fig. 9A). This observation agrees with previous results obtained from in vitro assays of MAO (161 and this Thesis).

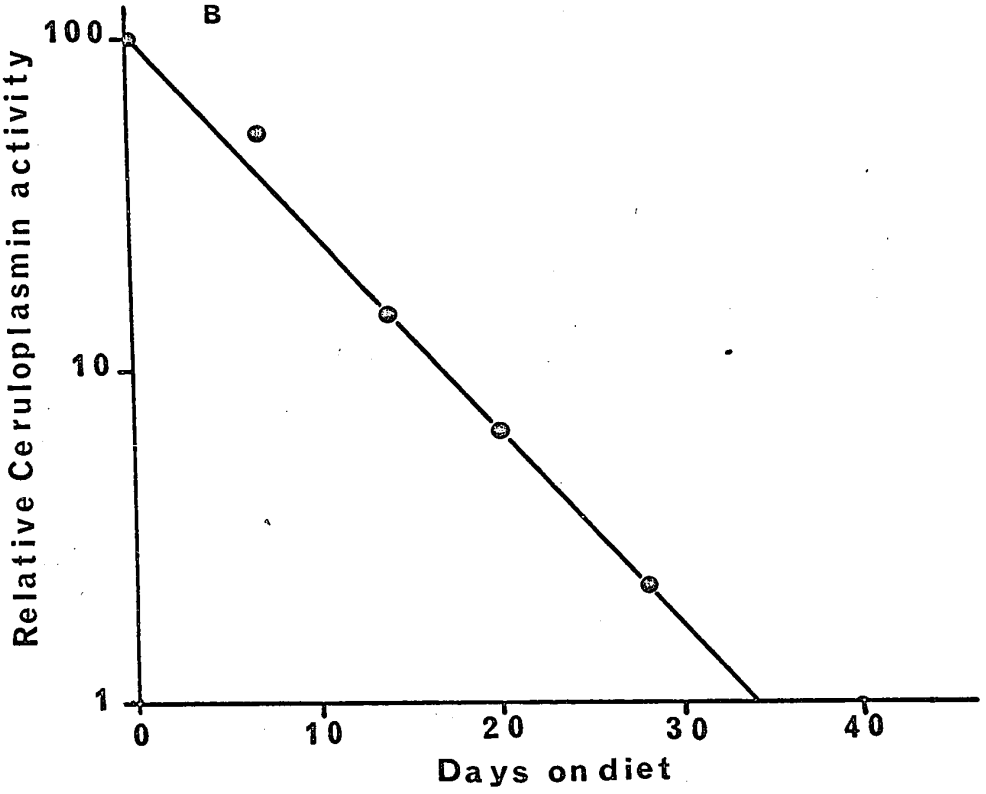
Copper deficiency did not affect either the growth rate or organ weight of the animals at the time of sacrifice (Table XIII). It did produce a considerable, progressive decline in serum ceruloplasmin oxidase activity in the experimental rats which differed significantly from levels found in control rat serum at the time of sacrifice (Fig. 9B and Table XIII, respectively). Table XIII also shows that the differences between the copper concentration of various tissues examined in experimental and control animals were also statistically significant. The order of decrease in tissue copper levels which resulted from chronic deprivation of dietary copper was: kidney > heart > liver.

Because of the observed discrepancy (no effect of copper-deficiency on MAO activity), the possibility that iron- and riboflavin-deficiencies act on MAO by provoking ultrastructural changes in the mitochondria is unlikely. It is also unlikely that the rate of absorption of pentylamine from the peritoneal cavity may be reduced as a result of iron- and riboflavin-deficiencies since the effects of ~~all three~~ both deficiencies on MAO observed originally in vitro (161, 332, 333), and now in vivo are parallel. In addition to that, no accounts have appeared in the

Figure 9

Effects of duration of copper deficiency on: (A) the rate of catabolism of pentylamine-[1-^{14}C] *in vivo* in the rat. Details of the experimental procedures followed appear in the caption to Figure 4 and in the text. The experimental points represents means (± SE) of observations made on at least 4 animals in each group. The open circles represent copper-deficient rats, and the closed circles, control (copper-supplemented) rats. (B) Serum ceruloplasmin oxidase activity. The ability of 0.1 ml of serum to oxidize p-phenylene diamine hydrochloride at 37^{o} and pH 5.2 during a 15 min incubation was expressed as absorbance at 540 nm. This value served as an index of ceruloplasmin activity. The experimental points represent the means of observations made on at least 4 animals and are expressed as a percentage of mean values found for control serum.


A
40
30
20
% $^{14}CO_2$ expired per hr
Control
Deficient


B
100
10
1
Relative Ceruloplasmin activity
0
10
20
30
40
Days on diet

literature as yet which report any diminution in the rates of absorption of amines from the peritoneal cavity due to either iron-deficiency or riboflavin-deficiency.

The remaining alternative role of iron in MAO seems to be an attractive one although it has not been tested up to the present time. Iron could be involved somehow in the biosynthesis of MAO, e.g., it might be part of a catalyst which mediates the incorporation of the riboflavin coenzyme into the apoprotein.

MAO Activity in the Liver of the Riboflavin- and Inositol-deficient Rat

Hawkins (332) had observed that a restoration of MAO activity in the livers of rats made nutritionally deficient in riboflavin occurred more rapidly when the animals were refed with inositol- and riboflavin-containing diets than when the diets did not contain extra inositol. This led to the suggestion that inositol could play a role as an additional cofactor in the action of MAO, or alternatively, it could have a function in the biosynthesis of the riboflavin-containing holoenzyme. The results of experiments conducted to test that hypothesis indicated no function of inositol in the action of the enzyme.

MAO activities of livers of rats that were kept for 28 days on diets made deficient in riboflavin, inositol and both riboflavin and inositol were compared with those of rats that were fed on a normal semisynthetic diet. There were 6 animals in each group that was tested. The differences between enzymic activities of the

Table XIII

Comparison of the nutritional status of copper-supplemented and copper-deficient rats. Duration of the experiment was 47 days. Means ± SE are shown.

		Diet				
		Control	n	Copper-deficient	n	
Body weight (g)		173.1±3.61	12	172.2±6.14	11	
Organ weight (g) - liver		6.08±0.14	12	6.75±0.22	11	$P > 0.01$
	kidney	1.65±0.00	12	1.55±0.00	11	
	heart	0.57±0.014	12	0.563±0.014	11	
Serum ceruloplasmin oxidase activity		0.070±0.00	12	n.d.*	8	
Tissue concentration of copper - liver		5.50±0.14	12	4.87±0.20	11	$P < 0.01$
(μg/g fresh weight)	kidney	12.67±0.80	12	5.28±0.70	11	$P < 0.001$
	heart	6.00±0.26	12	3.94±0.17	11	$P < 0.001$

*None detectable. The control value shown represents absorbance at 540 nm/0.1 ml serum/15 min incubation with p-phenylenediamine hydrochloride at 37° and pH 5.2.

control, riboflavin- and doubly-deficient rat liver preparations were statistically significant ($P < 0.001$). Mean specific activities (± standard errors) were 33.7±2.72, 13.3±1.58 and 16.7±2.51 nmoles of 4-hydroxyquinoline produced per mg of protein, respectively, during 20 minutes of incubation with kynuramine at 37° and pH 7.0. Inositol-deficiency *per se* was without effect on MAO activity, however. (Mean specific activity (± SE) was 32.6±2.95 nmoles of 4-hydroxyquinoline produced per mg of protein per 20 minute incubation.)

SUMMARY

1. A method has been developed for measuring monoamine oxidase (MAO) activity of the rat *in vivo*.

2. The specificity of the method has been confirmed by pretreating the experimental animals with inhibitors of MAO.

3. The method is a convenient and sensitive means of assessing the effects of different types of MAO inhibitor directly in the living animal.

4. Application of the method to a study of cofactor requirements of MAO confirmed that adequate amounts of dietary iron are essential for full activity of the enzyme.

5. The data suggest that iron may participate in the biosynthesis of the holoenzyme.

4. EFFECTS OF SOME INDUCED ANEMIAS ON MAO ACTIVITY IN THE RAT (IN VIVO AND IN VITRO)

Introduction

It has been demonstrated that the MAO activity in livers of iron-deficient rats decreases progressively when measured *in vitro* (161, and this Thesis) and *in vivo* (349, 350, this Thesis), and returns to normal when the rats are given iron (349, 350, this Thesis). Moreover, certain iron-chelating agents inhibit the rat liver enzyme. Parallel experiments conducted on copper-deficiency and with copper-chelators, respectively, showed no effects on MAO activity (161, 349, 350 and this Thesis).

The results of those nutritional and chelation studies suggested that iron is necessary for full activity of the enzyme, *in vitro* and *in vivo*.

The present investigations were undertaken to find out: (a) whether or not other means of interfering with iron metabolism (e.g., its storage and mobilization) in the rat might have some effect on MAO activity; and (b) to make some attempt to characterize the interactions which occur between those agents and MAO. It was decided, therefore, to test the effects of hemolytic and porphyriogenic agents as a means of accelerating development of the deficiency.

A rapid, long-lasting decrease in enzymic activity resulted when rats were injected with phenylhydrazine (PHZ), a classical hemolytic agent, and acetylphenylhydrazine (APHZ). This effect was more pronounced in liver than in brain.

Bernheim (419) and Green (420) had reported that PHZ and arylalkylhydrazines inhibit particulate MAO in vitro. Bernheim's study was mainly a comparison of the effects of PHZ on dehydrogenases, and did not include any kinetic data.

Many other derivatives of hydrazine have been synthesized and several have been tested in clinical studies as MAOIs (241, 421, 422) after iproniazid was found to have antidepressive effects (225, 226, 391). PHZ had been included in comparative investigations on the relative potencies of several substituted hydrazines as inhibitors of MAO and DAO in vitro (423). It has also been used as a tool in attempts to elucidate the mechanism of the reaction occurring at the catalytic site of the bovine kidney enzyme (424). However, neither PHZ nor APHZ have been studied for their anti-MAO properties in any detail, probably because of their high toxicity.

It seemed important to carry out these investigations to gain some knowledge of how these particular substances affect mitochondrial MAO activity as this aspect of the chemistry of the enzyme has long been neglected.

RESULTS

Effects of Injections with Allylisopropylacetamide (AIA)

The experimental animals were porphyruric and debilitated physically. However, the appearance of their tissues and blood was normal at the time of sacrifice, and their hemoglobin levels were normal. The treatment was without any effect on MAO activity of the livers.

Effects of Acetylphenylhydrazine (APHZ) in vivo

(i) The minimum effective dose (80 mg/kg body weight) of this drug was arrived at by the following criteria: that dosage level which was given once daily (subcutaneously) to test animals for 6 consecutive days and caused a significant decrease in hemoglobin concentration and permitted survival of the animals for at least one more day after the last injection.

(ii) There was a rapid, long-lasting decline in MAO activity of rats that were given APHZ either as a series of injections or in a single injection only. Fig. 10 shows that the loss in activity observed was of the same order ~~for~~ when more than one substrate was tested. Regeneration of the liver enzyme was very slow. Somewhat more than 3 weeks was required for complete restoration of its activity. This is in contrast to brain MAO activity which was inhibited by APHZ to a lesser extent and recovered completely by about 12 days (Fig. 11).

(iii) The effects of APHZ on the activity of liver MAO in normal and riboflavin-deficient rats were dose-dependent as well as time-dependent, as may be seen in Fig. 12. Not only was this so, but also, the depression of enzymic activity was much greater in the riboflavin-deficient than in the riboflavin-supplemented group of rats. This is similar to the observation made earlier by Distler and Sourkes (335) that MAO from livers of riboflavin-deficient rats was more susceptible to inhibition than the enzyme from control rat livers.

Figure 10

Inhibition of rat liver MAO *in vivo* by APHZ. A single (s.c.) injection of APHZ (80 mg/kg) was given to rats. The animals were killed at various intervals afterwards and homogenates of the livers were examined as described in Methods for MAO activity using as substrates: kynuramine (KA), isoamylamine (i-AA) and tyramine (TA). Experimental points represent the means (± SE) of at least 6 determinations expressed as a percentage of control activity.

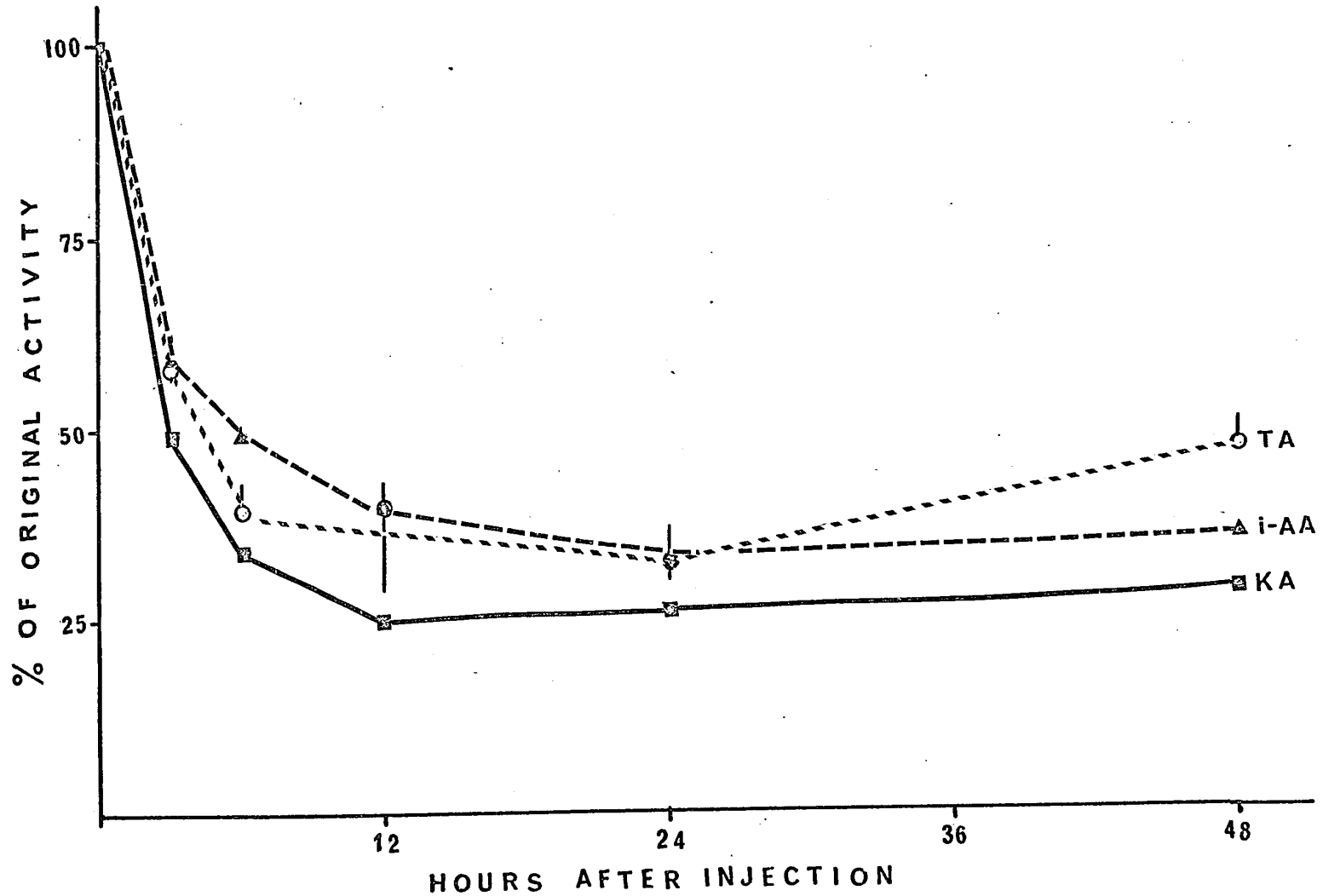

100
75
50
25
% OF ORIGINAL ACTIVITY
12
24
36
48
HOURS AFTER INJECTION
TA
i-AA
KA

Figure 11

Recovery of rat MAO activity from inhibition by APHZ. A single (s.c.) injection of APHZ (80 mg/kg) was given to rats. The animals were killed at various intervals afterwards and homogenates of the tissues were examined for MAO activity using kynuramine as substrate. Experimental points represent the means (± SE) of at least 6 determinations expressed as a percentage of control activity.

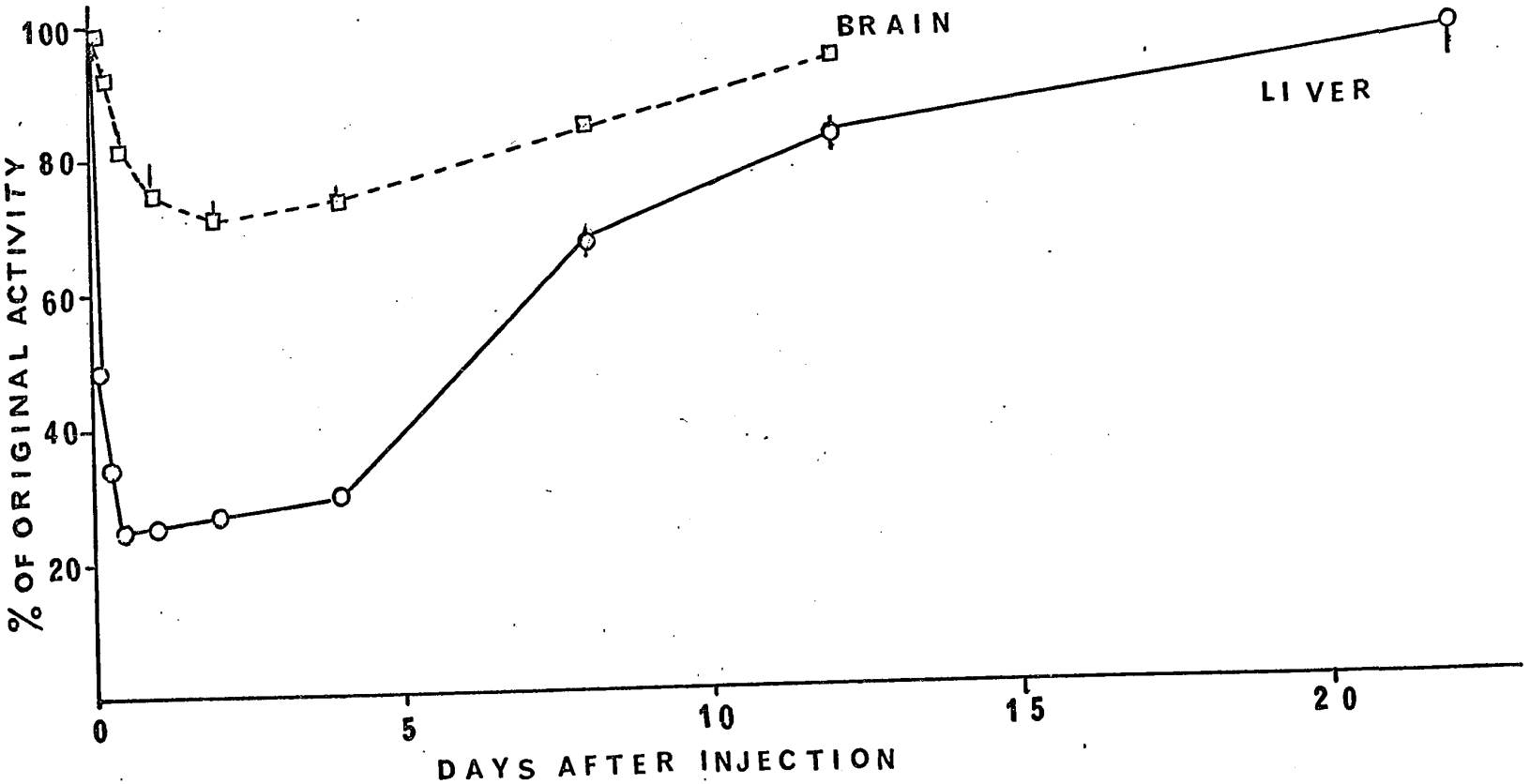
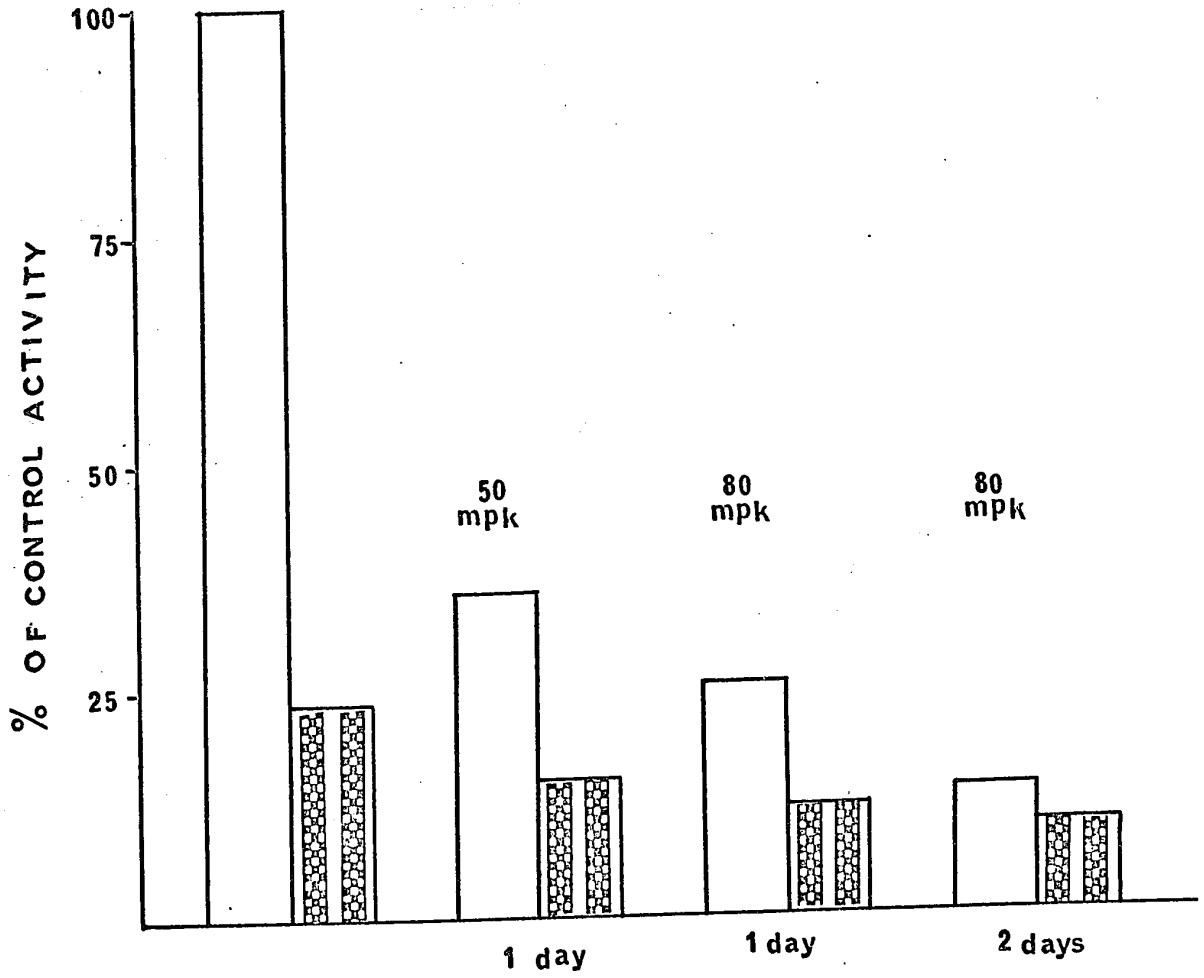

100
80
60
40
20
% OF ORIGINAL ACTIVITY
BRAIN
LIVER
0
5
10
15
20
DAYS AFTER INJECTION

Figure 12

Effect of APHZ on MAO activity in livers of riboflavin-deficient rats. Groups of rats fed on riboflavin-deficient and -supplemented (control) semisynthetic diets for 28 days were injected with APHZ subcutaneously at dosage levels of 0, 50 and 80 mg/kg. The animals were killed at the indicated times after injection and homogenates of their tissues were assayed for MAO activity using kynuramine as substrate. Data obtained from 6-8 determinations per group of animals were expressed as a percentage of the values found for control rats injected (s.c.) with alcoholic saline and plotted as histograms. The paired bars shown closest to the ordinate represent pooled data (both days of the experiment) from saline-treated control rat livers (open bars) and saline-treated deficient rat livers (checkered bars).


% OF CONTROL ACTIVITY
100
75
50
25
50 mpk
80 mpk
80 mpk
1 day
1 day
2 days

There was no effect of riboflavin deficiency alone on MAO activity of rat brain. The effect of APHZ in this condition was to depress the activity of the enzyme in brains from the deficient group to the same extent as in the control group.

(iv) Most of the iron-deficient rats were too anemic after 5 weeks on the iron-deficient diet to survive treatment with 80 mg/kg of APHZ. The data in Table XIV show that a reduced dose of the drug (35 mg/kg) when administered to less anemic rats (iron-deficient for 3 weeks) caused the activity of MAO *in vivo*, as measured by the rate of catabolism of radioactive pentylamine-[1-^{14}C] to $^{14}CO_2$ in the whole animal, to decrease considerably more than is observed in simple iron-deficiency.

The increased sensitivity of MAO in the iron-deficient rat to inhibition is reminiscent of the work of Distler and Sourkes (335) on riboflavin deficiency. Those and other authors (131, 332-334) had postulated a role for riboflavin in the action of MAO long before the vitamin was proven to be a prosthetic group of the mitochondrial enzyme. These and other findings (349, 350 and this Thesis) strengthen previous conclusions (161) that iron is necessary for the action of mitochondrial MAO *in vivo* as well as *in vitro*.

Effects of Phenylhydrazine (PHZ) *in vivo*

PHZ was injected subcutaneously into rats at a dosage level of 80 mg/kg. Table XV shows that this treatment produced a decrease in MAO activity of liver and brain after 24 hours. Liver MAO

Table XIV

The effect of APHZ on the metabolism of pentylamine-[1-^{14}C] in the iron-deficient rat, in vivo. The animals were maintained on the test diets for 3 weeks. They were injected with the indicated dose of APHZ subcutaneously 16 hours before they were given the radioactive amine (5 μCi/kg) diluted with unlabeled n-pentylamine in a dose of 100 mg/kg body weight by intraperitoneal injection. Means ± SE are shown.

Diet	Injections	n	% of administered radioactivity recovered as $^{14}CO_2$ during the first hour after injection		
Control	Saline	4	32.9±0.98		
Control	35 mg/kg	4	12.2±0.52	$P < 0.001$	
Iron-deficient	Saline	2	23.5±0.54	$P < 0.01$	with respect to
Iron-deficient	35 mg/kg	4	6.6±0.44	$P < 0.001$	saline-treated controls

activity towards isoamylamine decreased by 48 hours following the injection.

The concentrations of serotonin were estimated in the brains of 24 rats. Six experimental animals were injected with APHZ and 6 others with PHZ. The remainder served as controls. Although each drug produced a decrease in brain MAO activity when given to rats at 80 mg/kg body weight, neither of them caused any increase in brain serotonin after 24 hours. PHZ caused the level of this amine in brain to increase by 22% (± 9.4%) relative to control values after 48 hours. However, the increase was not statistically significant. Chessin et al. (241) and Pletscher (234) had emphasized that 50-100% of inhibition of brain MAO may be required to elevate brain serotonin levels significantly. The amounts of APHZ and PHZ administered to the experimental rats were insufficient to cause such an inhibition of enzymic activity, probably because of the non-specificity of the effects of these two inhibitors toward MAO. (MAOIs with hydrazine structure inhibit several other enzymes, e.g., DAO, and aromatic amino acid decarboxylases, and probably other pyridoxal-dependent enzymes also (227, 234, 425)). Pletscher (234) had also reported that the MAOI-induced accumulation of monoamines in brain is of shorter duration than inhibition of the enzyme.

In vitro experiments:

(i) Data obtained for MAO activity measured in the presence of differing concentrations of APHZ and PHZ are shown as semi-logarithmic plots (% of inhibition vs. log (inhibitor concentration))

Table XV

The effect of a single injection of PHZ (80 mg/kg) administered subcutaneously on the MAO activity in livers and brains of normal rats. The rats were killed at the indicated times. Means ± SE are shown.

Injection	Days after Injection	MAO Specific Activity[a] Liver isoAmylamine	Liver Tyramine	Liver Kynuramine	Brain Kynuramine
0	1	3.98±0.24 (5)[b]	3.56±0.30 (7)	26.31±0.52 (6)	9.54±0.40 (6)
PHZ	1	3.48±0.37 (7)	2.11±0.31 (12)**	15.44±0.42 (6)***	6.09±0.26(6)***
0	2	5.66±0.59 (5)	3.40±0.14 (5)	27.63±0.69 (6)	9.43±0.17 (6)
PHZ	2	2.70±0.17 (8)***	2.25±0.17 (7)***	13.64±0.17 (6)***	5.81±0.17(6)***

[a] Enzymic activity is expressed as the rate of consumption of oxygen (µl/hr) per mg of protein at 37° in the presence of isoamylamine and tyramine (pH 7.4 and 7.0, respectively), and as the rate of production of 4-hydroxyquinoline (nmole/20 min) per mg protein at 37° and pH 7.0.

[b] Mean concentrations (±SE) of hemoglobin (g/100 ml blood) were as follows: 12.9 (±0.2) and 7.9 (±0.4) for 6 control and 11 PHZ-treated rats, respectively, on day 1 after the injection; 13.1 (±0.3) and 5.9 (±0.3) for 5 control and 7 experimental rats, respectively, on day 2.

** $P < 0.01$; *** $P < 0.001$ (Student t-test).

in Fig. 13. Concentrations of PHZ and APHZ theoretically capable of producing 50% inhibition of enzyme activity (I_{50} concentrations) were derived from the graphs by interpolation. From the I_{50} concentrations which appear in Table XVI, it is evident that PHZ inhibits MAO more actively than does APHZ.

(ii) Kynuramine was found to protect MAO against inhibition by APHZ but not PHZ. The data in Table XVII show that the usual extent of inhibition of MAO by APHZ was observed when the I_{50} concentration was increased some twenty-fold. In my hands, prior addition of kynuramine did not prevent the usual amount of inhibition from taking place when the PHZ was present at its I_{50} concentration. The reason for such a difference in behavior towards these two structurally similar inhibitors is not immediately apparent. Bernheim (419) and Davison (243) had reported that MAO inhibition by phenylhydrazine could be prevented or reversed if the reaction mixture contained product (aldehyde) or substrate, respectively. Green (420) had also found that the prior addition of substrate (tyramine) to incubation mixtures could suppress, but not reverse, the inhibitory action of several hydrazine derivatives toward particle-bound MAO. It is interesting that while these data for PHZ are contradictory in one sense, they are in excellent agreement with values published by Bernheim (419) for MAO inhibition by PHZ with isoamylamine as substrate.

(iii) The inclusion of sodium cyanide or semicarbazide in incubation media which contained PHZ or APHZ increased the extent

Figure 13

Inhibition of MAO by APHZ and PHZ *in vitro*. Rat liver mitochondrial preparations containing 0.80 and 0.94 mg protein in separate experiments with the respective inhibitors were incubated with buffered aqueous solutions of the inhibitors for 15 min at 37° and pH 7.0. Kynuramine (1 mM final concentration) was introduced into the incubation media and the reaction allowed to proceed for 20 min. The complete reaction mixtures contained 33.3 mM phosphate in a total volume of 3 ml. The experimental points represent the mean values (± SE) of duplicate determinations.

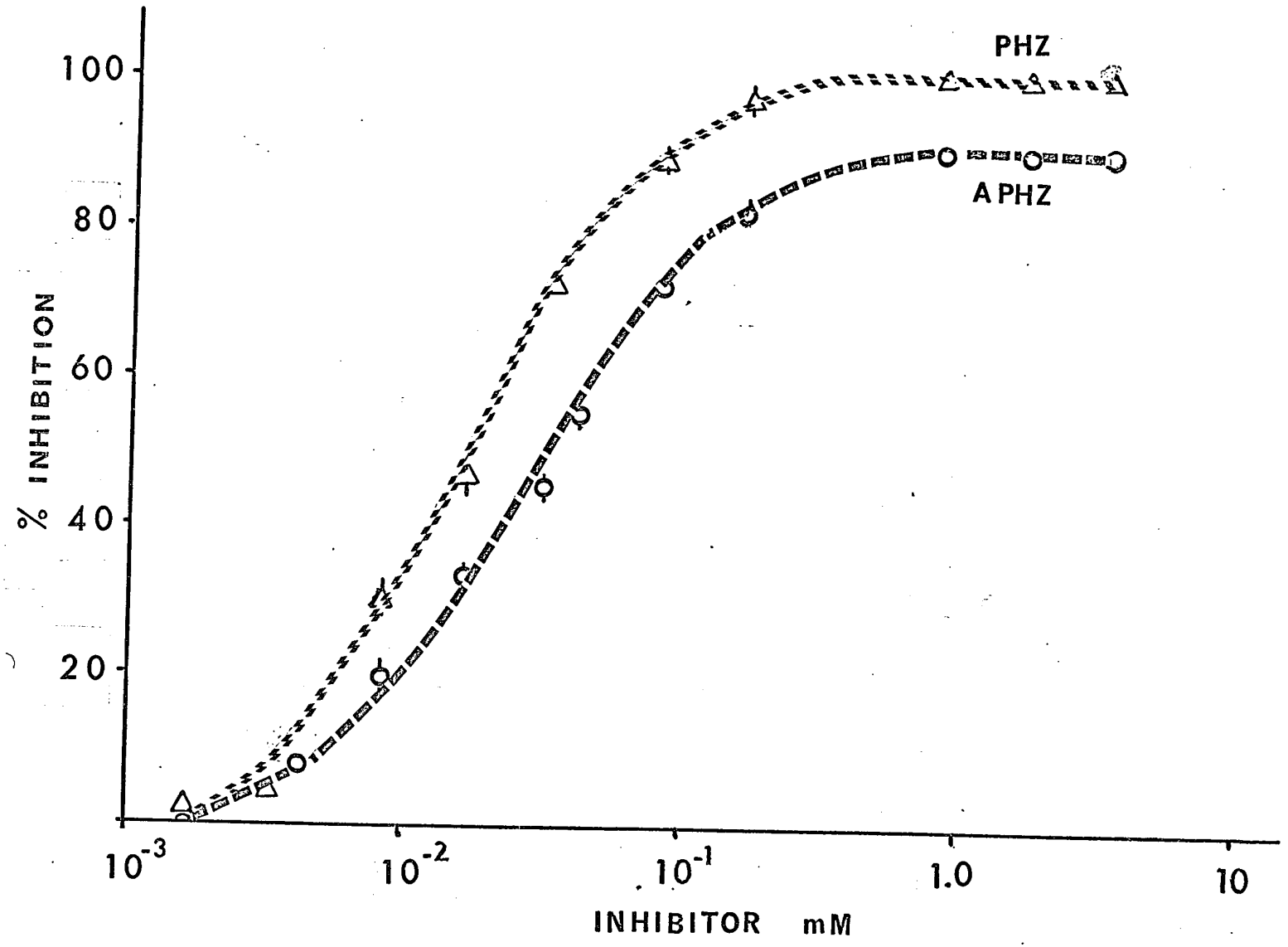

PHZ
APHZ
100
80
60
40
20
% INHIBITION
10^{-3}
10^{-2}
10^{-1}
1.0
10
INHIBITOR mM

Table XVI

Concentrations of compounds inhibiting MAO activity towards kynuramine by 50% *in vitro* at pH 7.0 and 37° and 15 min preincubation. The values shown were obtained by interpolation from graphs of % inhibition plotted against log (inhibitor concentration).

Inhibitor	I_{50} (M)
APHZ	3.6×10^{-5}
PHZ	1.6×10^{-5}

Table XVII

Effect of prior addition of substrate (kynuramine on the inhibition of rat liver mitochondrial MAO by APHZ and PHZ *in vitro*. Means ± SE are shown.

Inhibitor (mM)	Inhibition (%)
APHZ 4.2×10^{-2}	2.81 ± 0.0 (2)[a]
APHZ 8.3×10^{-1}	49.7 ± 0.48 (3)
PHZ 1.7×10^{-2}	57.6 ± 0.42 (3)
PHZ 3.3×10^{-1}	98.7 ± 0.40 (3)

[a] Figures in parentheses indicate the numbers of determinations that were performed.

of inhibition of MAO over that observed in their absence (Table XVIII). The presence of cyanide caused a more marked increase in inhibition by both inhibitors. The effect of semicarbazide was smaller, the net increase which it produced in the action of APHZ being half that observed when it was added to mixtures that contained PHZ. Similar phenomena have been remarked on previously (243, 420). A study was not made of the effects of cyanide and semicarbazide added in combination on the inhibition of MAO activity by APHZ and PHZ. Such a combination was itself inhibitory towards MAO (Table XIX), causing the activity of the rat liver enzyme towards kynuramine to decrease by about 24%. Semicarbazide alone inhibited the enzyme by approximately the same amount, while sodium cyanide enhanced the activity of the enzyme slightly (about 4% relative to control values). The anomalous behaviour of semicarbazide appears to be unique for kynuramine. This amine is a poor substrate for DAO (171), which does not occur in mitochondria of rat liver (69). Semicarbazide may be reacting with kynuramine or its deaminated oxidation product 4-hydroxyquinoline, thereby apparently reducing MAO activity. It may be useful to examine these possibilities at some future date.

(iv) The pH-activity curves of the uninhibited and inhibited enzymic reactions were parallel. Reference to Fig. 14A and B will show that pH optima occurred between pH 8.6 to pH 9.0 for the inhibited as well as the uninhibited enzyme preparations. The ratios of inhibited to uninhibited enzymic activity at the corresponding pH values were calculated for both APHZ and PHZ. They all fell within (±) 2 standard deviations of the mean values of the respective

Table XVIII

Effects of cyanide and semicarbazide on the inhibitory action of APHZ and PHZ toward MAO activity of rat liver mitochondria *in vitro*. The enzyme preparations and all test substances were incubated together for 20 min at 37° and pH 7.4 before the reaction was initiated. Means ± SE are shown. Figures in parentheses indicate the numbers of replicate determinations that were performed.

Inhibitor	mM	% Inhibition	Net change (%)
APHZ	4.17×10^{-2}	40.1±1.2 (2)	-
plus NaCN	6.7×10^{-1}	63.6±0.5 (3)	+ 23.5 $P < 0.001$
" Semicarbazide	6.7	48.9±1.3 (3)	+ 7.7 $P < 0.05$
PHZ	1.67×10^{-2}	76.9±0.9 (3)	-
plus NaCN	6.7×10^{-1}	96.2±1.3 (3)	+ 19.3 $P < 0.001$
" Semicarbazide	6.7	92.4±0.4 (3)	+ 15.4 $P < 0.001$

Table XIX

Effects of cyanide and semicarbazide on the MAO activity of rat liver mitochondria as measured *in vitro* using kynuramine as substrate. The test substances and enzyme preparation were equilibrated for 5 min at 37° and pH 7.4 before the reaction was initiated. Means ± SE are shown. Figures in parentheses indicate the numbers of replicate determinations that were performed.

Addition	mM	% Inhibition
Sodium cyanide	6.7×10^{-1}	Nil
Semicarbazide (as HCl)	6.7	25.2±0.7 (4)
Sodium cyanide plus semicarbazide	as above	24.2±0.7 (2)

ratios. The results obtained in these experiments suggest two things: in the first place, binding of the inhibitors to the enzyme did not seriously alter any of the charge-dependent components of the enzyme molecule that are responsible for maintaining it in an active configuration. Secondly, binding of PHZ and APHZ to MAO, and consequent inhibition of its activity towards kynuramine did not depend on the pH value of the reaction media.

(v) (a) Effects of dilution: The data presented in Table XX are evidence that PHZ and APHZ bind MAO irreversibly since extensive dilution (thirty-fold with respect to inhibitor) did not significantly change the degree of inhibition originally observed on 20 min incubation of inhibitors and enzyme.

(b) Effects of incubation time: Linear plots of log (% residual activity) were obtained as a function of incubation time for both inhibitors (Figs. 15 and 16). The time-dependence of the inhibitions indicated that APHZ and PHZ bind irreversibly to MAO. The inhibition of MAO observed in both instances is referred to as "immediate" and not "progressive", because the lines, on being produced back to the Y-axis (zero time), do not pass through their origins and indicate very avid instantaneous binding of inhibitor to enzyme, possibly like a chemical reaction gone to completion. The change in slope of the APHZ line may be indicative of a mixed, partly reversible association of enzyme and inhibitor beyond 30 min of incubation.

(vi) The data obtained in these experiments were subjected to analysis by Lineweaver-Burk plots. Values derived for the

Figure 14

Effect of preincubation time on PHZ inhibition of MAO *in vitro*. Rat liver mitochondrial preparations (containing 0.59 mg protein) were incubated with buffered aqueous solutions of the inhibitor at the indicated concentrations for various intervals of time at 37° and pH 7.4. Kynuramine (0.51 mM final concentration) was added at the end of each preincubation period and allowed to react with the enzyme for 20 min. The complete reaction mixtures contained 33.3 mM phosphate in a total volume of 3 ml. The experimental points represent the mean values of duplicate determinations.

PHZ INHIBITION OF RAT LIVER MAO

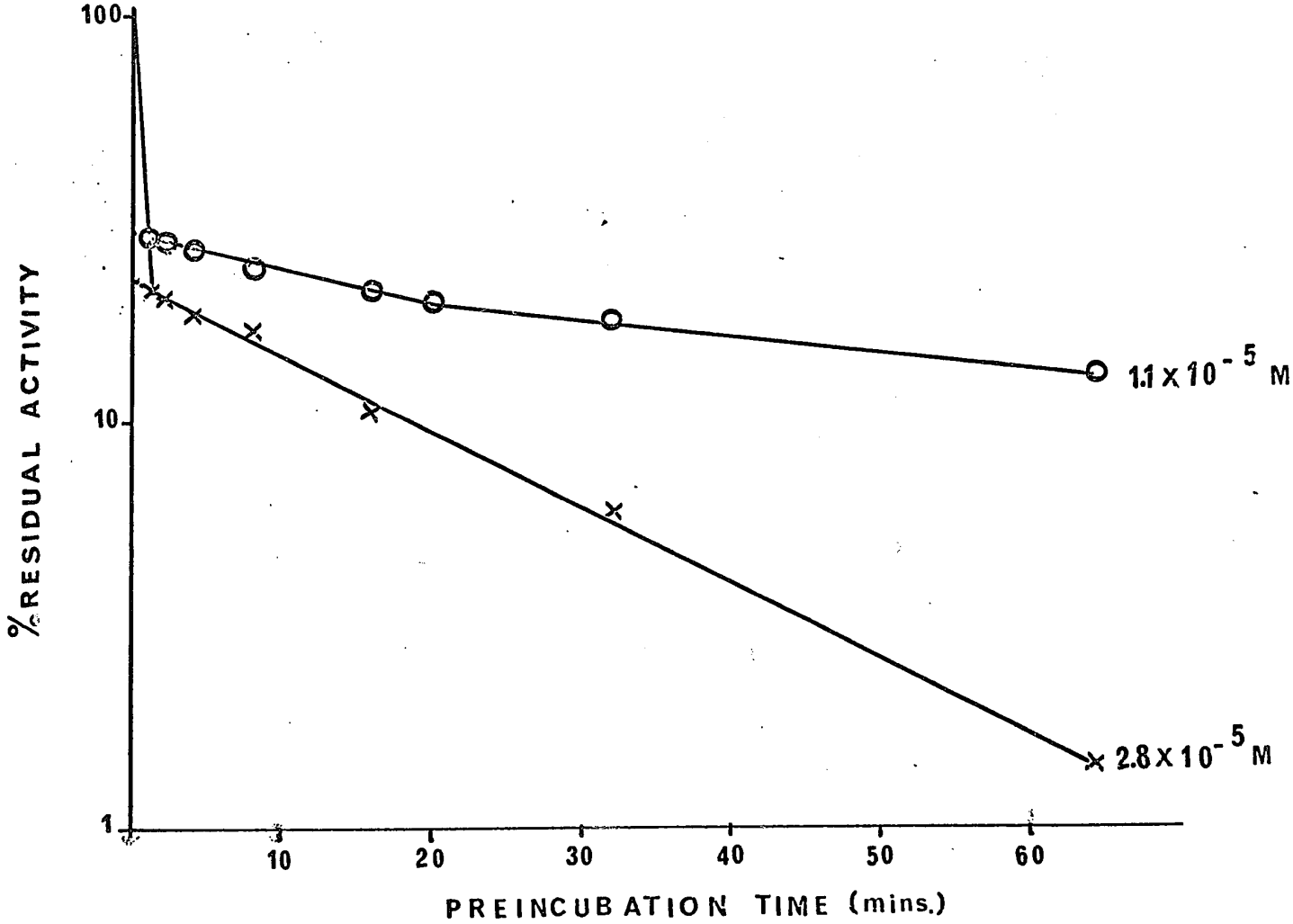


Table XX

Effects of dilution on MAO inhibition by APHZ and PHZ. The enzyme preparation, consisting of washed mitochondria from rat liver, and the inhibitors were incubated together for 20 min at 37° and pH 7.4. At the end of that interval, aliquots of the mixtures were withdrawn and transferred to vessels containing buffer, 1 mM kynuramine as substrate and either additional inhibitor to maintain the original concentration or no more inhibitor. The reaction was allowed to proceed for another 20 min. Fluorescence of the reaction product was compared with that of control and undiluted inhibited enzyme preparations. Means ± SE are shown. The figures in parentheses represent the numbers of determinations made.

Sample		Inhibitor (mM)	Inhibition (%)
Undiluted experimental	APHZ	4.31×10^{-2}	70.2±0.83 (2)
Diluted experimental	APHZ	1.4×10^{-3}	67.51±0.78 (3)
Diluted experimental with additional APHZ	APHZ	4.31×10^{-2}	72.06±1.39 (3)
Undiluted experimental	PHZ	2.77×10^{-2}	85.14±0.95 (4)
Diluted experimental	PHZ	9.23×10^{-4}	83.21±0.76 (4)
Diluted experimental with additional PHZ	PHZ	2.77×10^{-2}	84.33±0.68 (3)

Figure 15

Effect of preincubation time on APHZ inhibition of MAO *in vitro*. Rat liver mitochondrial preparations containing 0.58 mg protein were incubated with 4.31×10^{-2} mM APHZ for various intervals of time at 37^{o} and pH 7.4. Kynuramine (0.51 mM final concentration) was added at the end of each preincubation period and allowed to react with the enzyme for 20 min. The complete reaction mixtures contained 33.3 mM phosphate in a total volume of 3 ml. The experimental points represent the mean values of duplicate determinations.

APHZ INHIBITION OF RAT LIVER MAO

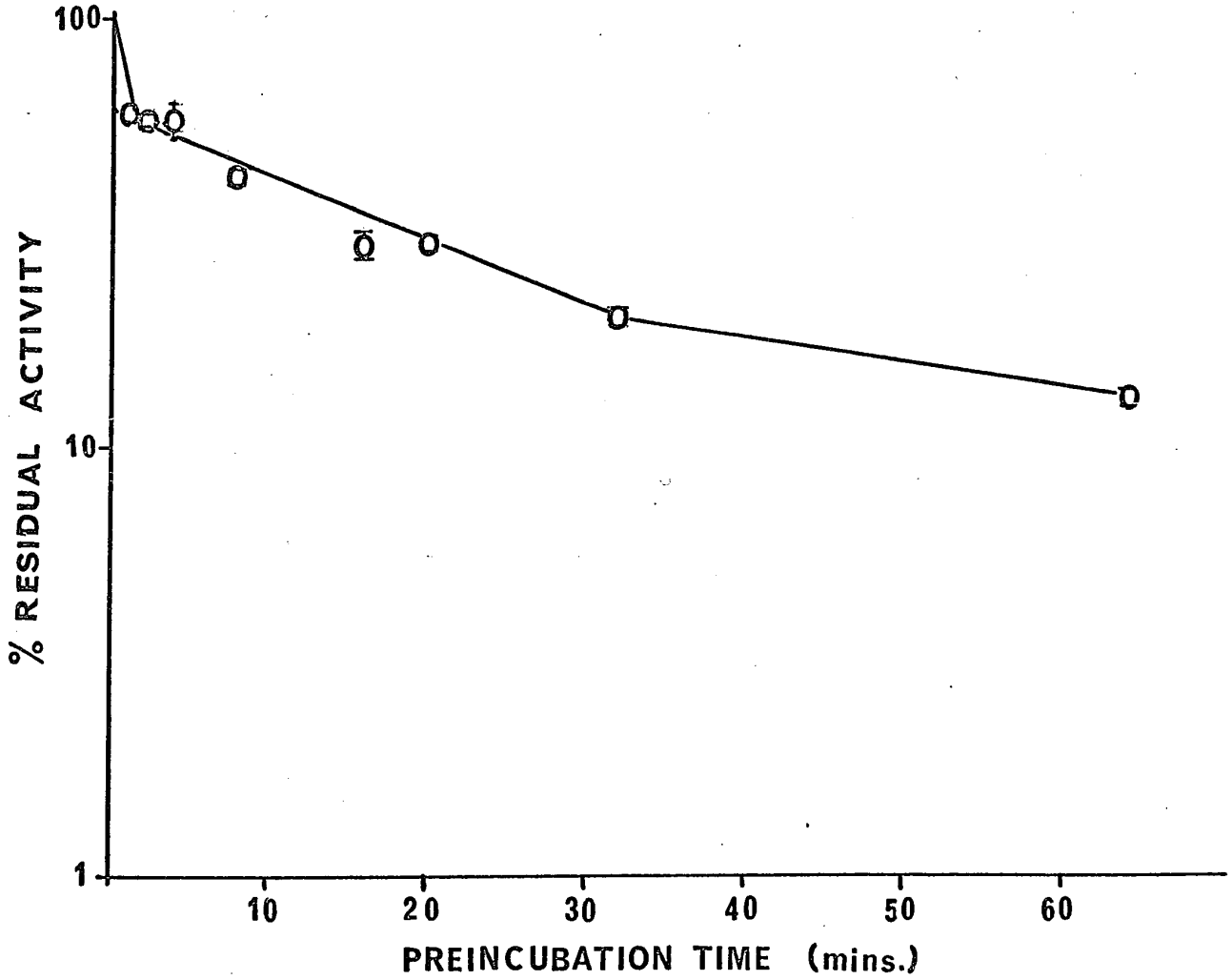


Figure 16

pH-Activity profiles of MAO in the absence and presence of (A) APHZ and (B) PHZ. Rat liver mitochondria were incubated in separate experiments with the indicated concentrations of inhibitors for 20 min at 37° and pH 7.4. Kynuramine (1 mM final concentration) was added and allowed to react with the enzyme for 20 min. The complete reaction mixtures contained 33.3 mM phosphate in a total volume of 3 ml. The experimental points represent the mean values (± SE) of duplicate determinations. The compositions of the buffers used are mentioned in the text (Experimental). The amounts of protein used in the experiments were: (A) 0.84 mg and (B) 1.14 mg.

pH-Activity profiles of MAO

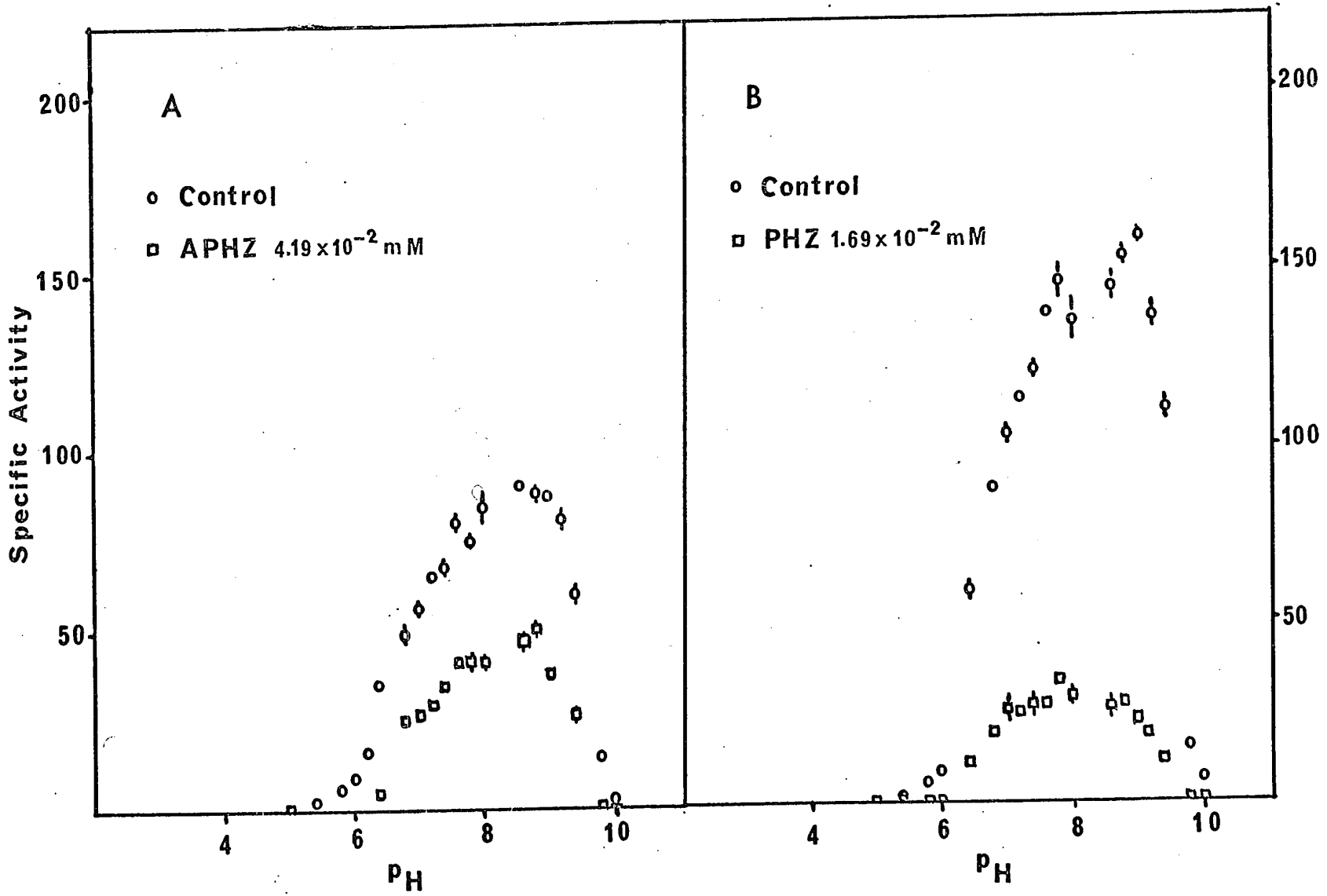


Table XXI

Effects of APHZ and PHZ on "kinetic constants" of MAO *in vitro*. The enzyme preparation, consisting of washed mitochondria from rat liver, and the inhibitors were incubated together for 20 min at 37° and pH 7.4 before the reaction was initiated. Determinations were performed in duplicate for each concentration of substrate used.

[Inhibitor] (mM)	Apparent K_m (mM)	V_m (nmole 4HOQ/20 min)
APHZ - None	4.10×10^{-2}	108.9
APHZ 5.39×10^{-3}	3.58×10^{-2}	83.1
APHZ 1.08×10^{-2}	3.76×10^{-2}	81.1
APHZ 4.31×10^{-2}	3.64×10^{-2}	33.8
PHZ - None	2.84×10^{-2}	534.8
PHZ 1.75×10^{-3}	2.78×10^{-2}	78.4
PHZ 8.75×10^{-3}	2.78×10^{-2}	24.7

apparent Michaelis constant under the experimental conditions used are in the range of 30-40 μM, in the presence and absence of the respective inhibitors (Table XXI). The values shown for the maximal reaction velocities were obtained in separate experiments and do not represent specific activities. Moreover, the difference in uninhibited values of V_{max} may be ascribed to the different operations followed in their derivation, i.e., fewer concentrations of substrate were used in the experiment in which the effect of APHZ was tested, owing to substrate inhibition being observed at higher concentrations of kynuramine. A wider range of kynuramine concentrations was used in the experiment with PHZ as inhibitor. This experiment seems to give a more reliable value for V_{max} since very high substrate concentrations capable of inhibiting MAO activity were not used. From the results of this set of experiments, both APHZ and PHZ are found to be irreversible, non-competitive inhibitors of MAO, a property which they share with several other hydrazine derivatives (234, 243, 426).

DISCUSSION

Zeller (9, 69) had based the distinction of MAO from DAO on the inability of the former to be inhibited by carbonyl reagents which inhibit the latter. Most of the common carbonyl reagents, including hydrazine itself do not inhibit MAO (373). However, several derivatives of hydrazine, including phenylhydrazine, inhibit MAO *in vivo* as well as *in vitro* (237). Thus the inhibitory effects of PHZ and APHZ on MAO reported here seem to be due chiefly to their

direct action on the enzyme itself and not merely to an induced anemia which can make an iron cofactor unavailable for the synthesis or activity of the enzyme. This seems to be true especially for the brain enzyme. There was no effect of riboflavin-deficiency on brain MAO activity, confirming previous findings by Youdim (402) and Leodolter and Genner (418). Although the vitamin is known to be a prosthetic group of several mammalian MAOs its concentration in rat brain does not decrease appreciably in comparison with heart and liver, in spite of long periods of dietary deprivation (427, 428).

The recovery of brain MAO from inhibition by APHZ occurred much sooner than it did for the liver enzyme (Fig. 11). These results are in contrast to those of Horita (429) who reported that the same dose of pargyline (20 mpk) administered to adult rats (i.p.) could cause total inhibition of both the liver and brain enzyme and recovery of activity in the liver was 2-3 times as rapid as that of brain. Injection of iron- and riboflavin-deficient rats with APHZ caused greater losses of enzymic activity than were due to either deficiency alone (Fig. 12 and Table XIV, respectively). These findings are significant. With regard to riboflavin, Sourkes (333) and Distler and Sourkes (335) had postulated cofactor functions for this vitamin in MAO activity on the basis of similar results. The rat liver enzyme is now known to contain riboflavin (16, 17), a property which it shares with mitochondrial MAOs from other mammalian organs (10-15, 18). A similar interpretation may

be made of the results from the present experiments on iron-deficiency, although the exact function of iron in MAO activity has not yet been established (161, 349, 350).

Hellerman and Erwin (353) found that incubation of a purified preparation of the beef kidney enzyme with limiting amounts of PHZ, pargyline and the d-isomer of tranylcypromine (equal concentrations of each inhibitor) resulted in additive inhibition. Initial addition of substrate (benzylamine) could protect the enzyme against inhibition but its later addition did not reverse the inhibition caused by PHZ and the other inhibitors. In this work, prior addition of kynuramine afforded the rat liver enzyme protection against inhibition by APHZ but not PHZ. These contrary results suggest that APHZ and PHZ act at different binding sites of the enzyme. It remains to be seen whether or not more highly purified preparations of rat liver MAO will be similarly affected by PHZ, with kynuramine as substrate.

APHZ was a more potent inhibitor of rat liver MAO *in vivo* than was PHZ, while the converse was true for the brain enzyme. With regard to their action *in vitro*, both drugs inhibit the rat liver enzyme non-competitively and irreversibly, PHZ being the more active of the two. The data presented here suggest that each inhibitor forms a non-dissociable complex with the enzyme. Complex formation and MAO inhibition *in vitro* were both independent of pH.

SUMMARY

1. Injection of rats with APHZ and PHZ rapidly produced a long-lasting inhibition of MAO. The effect was more pronounced

in liver than in brain at the levels of dosage employed (80 mg/kg body weight).

2. MAO activity in riboflavin- and iron-deficient rats was more susceptible to inhibition by APHZ than that of normal rats.

3. APHZ and PHZ also inhibited MAO *in vitro*. Both substances inhibited the enzyme immediately, in an irreversible, non-competitive manner which was independent of pH.

4. PHZ was a more active inhibitor of MAO than APHZ *in vitro*. Concentrations of the respective chemicals which caused 50% inhibition (*in vitro*) after 15 min of preincubation with the enzyme at 37° and pH 7.0 were ca. 1.6×10^{-5} and 3.6×10^{-5} M.

5. APHZ inhibited rat liver MAO *in vivo* more actively than did an equivalent dose of PHZ. The former compound caused 65-75% inhibition of activity by 24 hours after injection which persisted at that level for another day. This was true for three different substrates. On the other hand, PHZ inhibited by about 40% at 24 hours and 50% at 2 days. The situation with regard to the brain enzyme was different. Equal doses of PHZ caused 39% and APHZ 30% inhibition of activity after 2 days.

6. Kynuramine protected MAO against inhibition *in vitro* by APHZ but not PHZ.

7. Cyanide was more active than semicarbazide in enhancing the inhibitory action of both substances on MAO *in vitro*.

5. ADDITIONAL PROPERTIES OF SOLUBILIZED MAO PREPARED FROM RAT LIVER MITOCHONDRIA

Introduction

In previous work (161) and elsewhere in this Thesis the activity of MAO from normal and iron-deficient rat livers towards three different substrates, and the substrate-specific inhibitory effects of iron-chelating agents on MAO suggested that at least two types of rat liver MAO exist.

Purification of rat liver MAO was undertaken, partly to obtain some information on this aspect of its chemistry, and partly to verify and extend observations that were made on its cofactors previously (402). The data reported in this section supply additional evidence for the existence of multiple MAOs based on the electrophoretic separation of several bands of activity towards tryptamine and tyramine. In addition, partially purified preparations of the enzyme, shown to be homogeneous by gel filtration on Sephadex, were resolved by SDS electrophoresis into bands which represent polypeptide chains of MAO.

A proportionality existed between the specific activity of the partly purified MAO and the amount of iron which it contained, at each stage of its purification. That relationship suggested that iron may be a part of the enzyme.

The absorbance spectra of the oxidized and reduced enzyme as studied with some of the partially purified preparations were indicative of the presence of riboflavin in the enzyme. The fluorescence emission spectra of a peptide separated from the

enzyme by proteolytic digestion confirmed that the enzyme contains riboflavin.

The kinetics of inhibition of MAO by some thiol-reacting compounds were studied, and its content of sulfhydryl groups was determined. The number of .SH groups found in the enzyme increased on denaturation with 8 M urea. These experiments were conducted to augment previous data accumulated on the essential sulfhydryl groups of rat liver MAO (340, 402).

RESULTS

Enzyme Purification

(a) Table XXII shows an example of the results generally obtained in MAO purification. A 67-fold purification (from mitochondria as starting material) was regularly achieved, while the usual recovery of original activity up to the ion exchanger step varied from 15-21%. Similar levels of activity have been reported for other purified mitochondrial amine oxidases from different sources. Fig. 17 represents the usual elution pattern of the solubilized enzyme from DEAE cellulose ion exchange columns.

(b) The concentration of iron in the enzyme was determined at successive stages of its purification. Data from those experiments were plotted as a graph of specific activity (rate of oxygen consumption per mg protein) vs iron concentration (μg iron per mg protein). A typical example of the results obtained is shown in Fig. 18. The relationship that exists between the specific activity of the enzyme and its content of iron at different stages in its

purification is a linear one. (Both axes of the graph are drawn to a logarithmic scale for convenience and compactness of presentation). These data indicate that MAO from rat liver, purified at least up to 70-fold from mitochondria may contain iron. It has already been shown that rat liver MAO requires iron for full activity (161, 359 and this Thesis) and that more highly purified preparations contain relatively high amounts of iron (315, 337).

(c) The molecular weight of the partly purified MAO was estimated to be 280,000 and 290,000 by gel filtration on Sephadex G-150 and G-200 respectively. These estimates agree with the value of 290,000 reported previously by Youdim and Sourkes (315). A calibration curve from which an estimate was derived is shown in Fig. 19.

Detection of the Presence of Riboflavin in MAO

(a) Absorbance spectra of the partially purified enzyme are shown in Fig. 20. The spectrum of the enzyme in its oxidized state suggests the presence of riboflavin from the absorption band having its maximum at 415 nm. Addition of solid sodium tetrathionate (dithionite) reduced much of the absorbance, causing a slight displacement of the maximum towards a lower wavelength (405 nm). The difference spectrum represents absorption bands having maxima at wavelengths characteristic of riboflavin (370, 415 nm)*. These results, taken in conjunction with data from nutritional experiments (335, 336, 349, 350, and this Thesis), suggest that rat liver mitochondrial MAO contains riboflavin.

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*These spectral characteristics are common to MAO purified from several other sources (10 - 18) and also suggest that the enzyme contains iron (12, 161, 315, 337, 348), probably as non-heme iron (NHI).

Table XXII

Protocol for the purification of MAO from rat liver mitochondria

Step	Total Units*	Total Protein (mg)	Specific Activity (Units/mg)	Purification Fold	Yield (% of starting Units)
Mitochondria	6540	1815	3.6	1	100
Sonicate	5880	496	11.85	3.3	90
Ammonium sulfate 30-55% saturation	3180	139	22.87	6.4	48.7
DEAE Cellulose	1387	5.77	240.3	66.8	21.2

*Units of activity are defined as the rate of consumption of oxygen measured polarographically and expressed as μl/hr. Measurements were made in air-saturated media at 37.5° and pH 7.4. The reaction mixtures (3 ml final volume) contained 0.03 M phosphate, 20 μmoles semicarbazide, 2 μmoles cyanide and 40 μmoles isoamylamine as substrate.

Figure 17

Elution pattern of solubilized rat liver MAO from DEAE cellulose. The enzyme solution containing 36.4 mg of protein and 975 units of isoamylamine oxidizing activity was applied to a column (15 x 2.5 cm) of ion exchanger equilibrated with 0.05 M phosphate buffer, pH 7.4, and eluted with the same buffer containing stepwise increasing concentrations of sodium chloride. Fractions were collected at an average flow rate of approximately 30 ml per hour. Units of enzyme activity refer to the rate of consumption of oxygen expressed as µl per hr, measured polarographically in the presence of isoamylamine (40 µmoles), at 37° and pH 7.4.

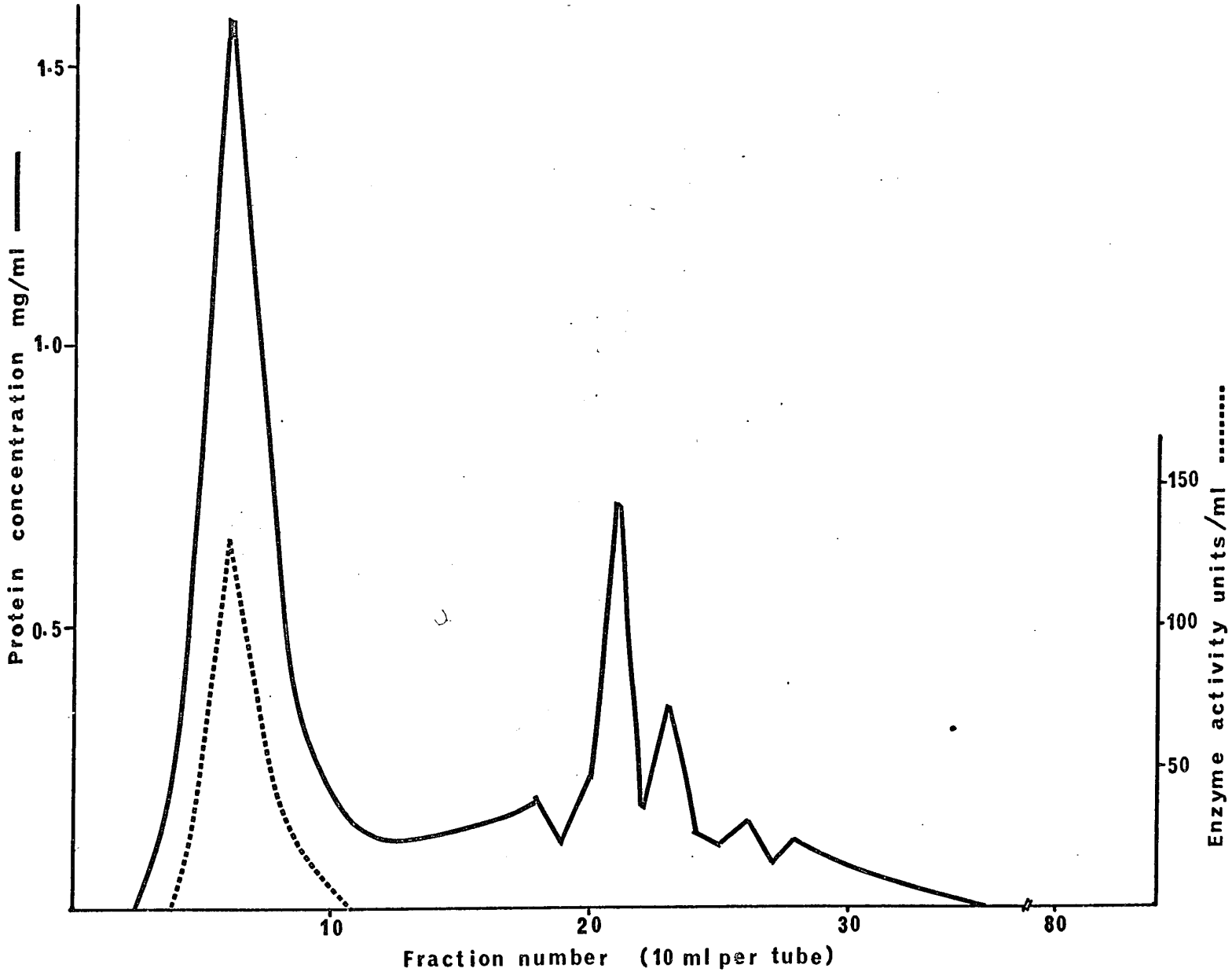
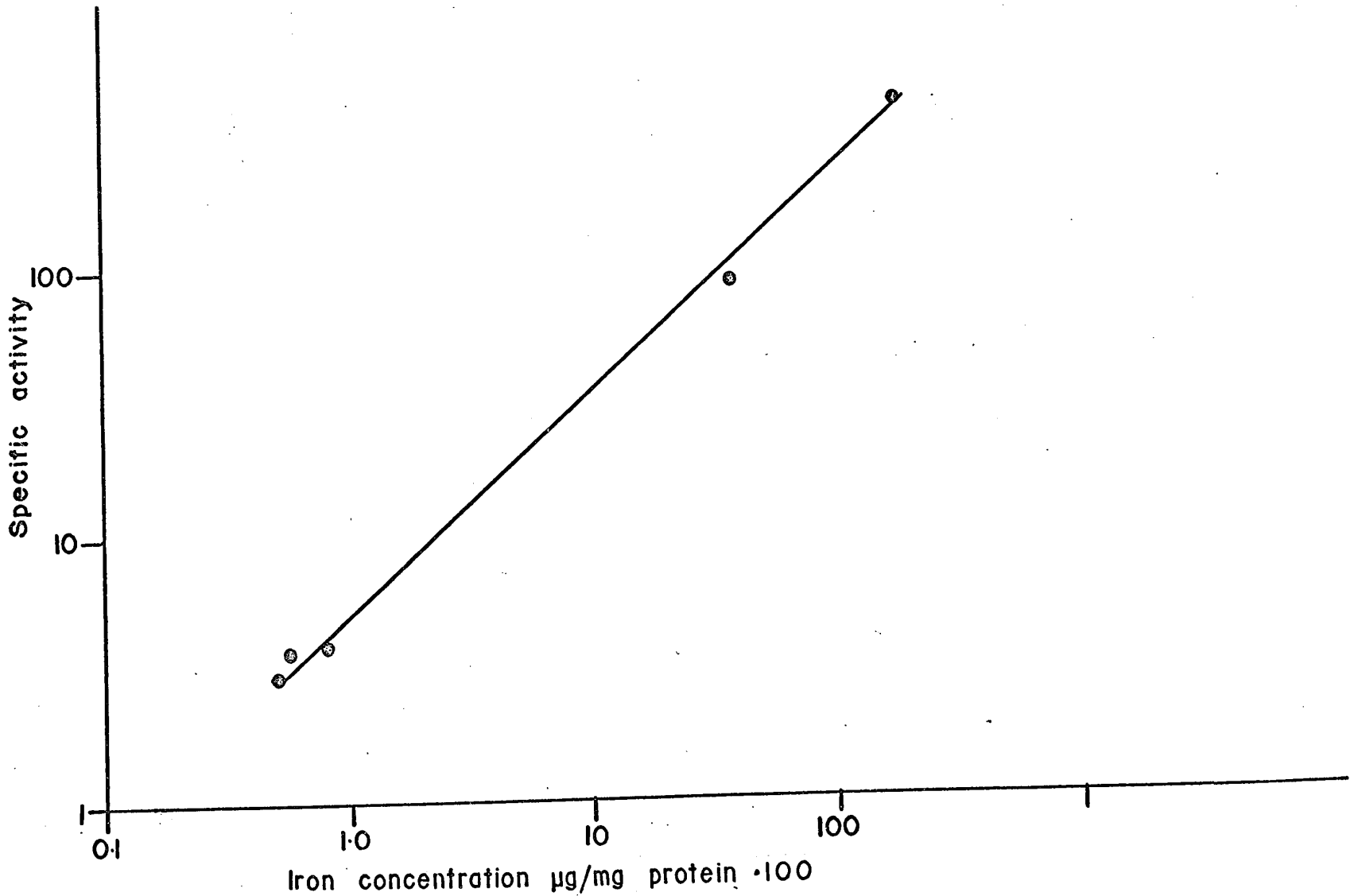

Protein concentration mg/ml
1·5
1·0
0·5
Enzyme activity units/ml
150
100
50
10
20
30
80
Fraction number (10 ml per tube)

Figure 18

Relationship of iron concentration and specific activity of rat liver MAO during its purification. Units of specific activity are defined as the rate of consumption of oxygen (μl/hr) per mg of protein, measured polarographically in the presence of isoamylamine (40 μmoles) at 37° and pH 7.4.


Specific activity
100
10
1
0·1
1·0
10
100
Iron concentration µg/mg protein ·100

(b) The native enzyme did not fluoresce strongly when it was excited at 450 nm, the wavelength of activation of riboflavin. Only a small peak was evident at 520 nm, the wavelength of maximum emission of fluorescence of riboflavin (Fig. 21). The product obtained upon digestion of the enzyme with Pronase fluoresced strongly, however. Its fluorescence emission spectrum resembled that of riboflavin. Like riboflavin itself, the peak of fluorescence emission of the hydrolysate was abolished upon reduction with dithionite. Figure 21 also shows that the results of fluorescence measurements made at pH 3.2 were almost identical to those obtained at pH 7.4, indicating that the flavin moiety under consideration might be bound FAD and not free flavin (430). Youdim (402) and other authors (11, 16, 17, 431) concluded that the isolated flavin was bound to a peptide from their observations of its ninhydrin-staining characteristics following chromatography.

If one assumes the Pronase-released flavin to be FAD, its content may then be calculated to be approximately 1.5 moles per mole of enzyme from the spectrophotometric data (ε = 1.13 x 10^4/M/cm; (12)), or 0.8 mole per mole of enzyme, from the fluorescence data. Both these values are probably underestimated, however, since the measurements were not made under strictly anaerobic conditions and the flavopeptide was not purified.

Acrylamide Gel Electrophoresis

(a) Preparations of MAO at different stages of its purification were subjected to gel electrophoresis either in discontinuous

Figure 19

Molecular weight determination of MAO by gel filtration chromatography. Sephadex G-200 was equilibrated with 0.05 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. The protein was eluted in the same buffer from a column (1.5 x 90 cm) of the gel in 2.5 ml fractions at an average flow rate of approximately 17 ml per hour. Standard proteins used to calibrate the column were: (a) cytochrome c; (b) ovalbumin; (c) bovine serum albumin; (d) γ-globulin; (e) catalase; (f) apoferritin.

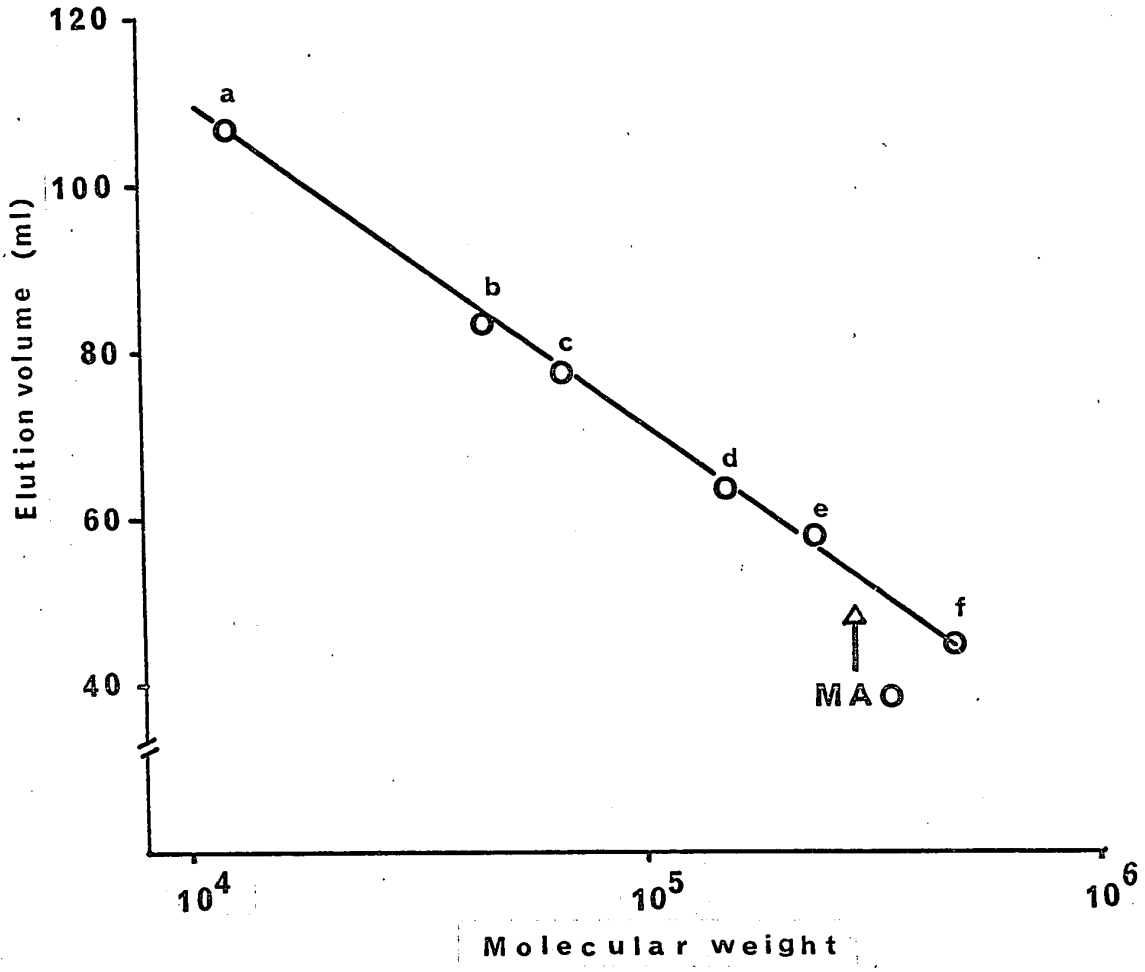
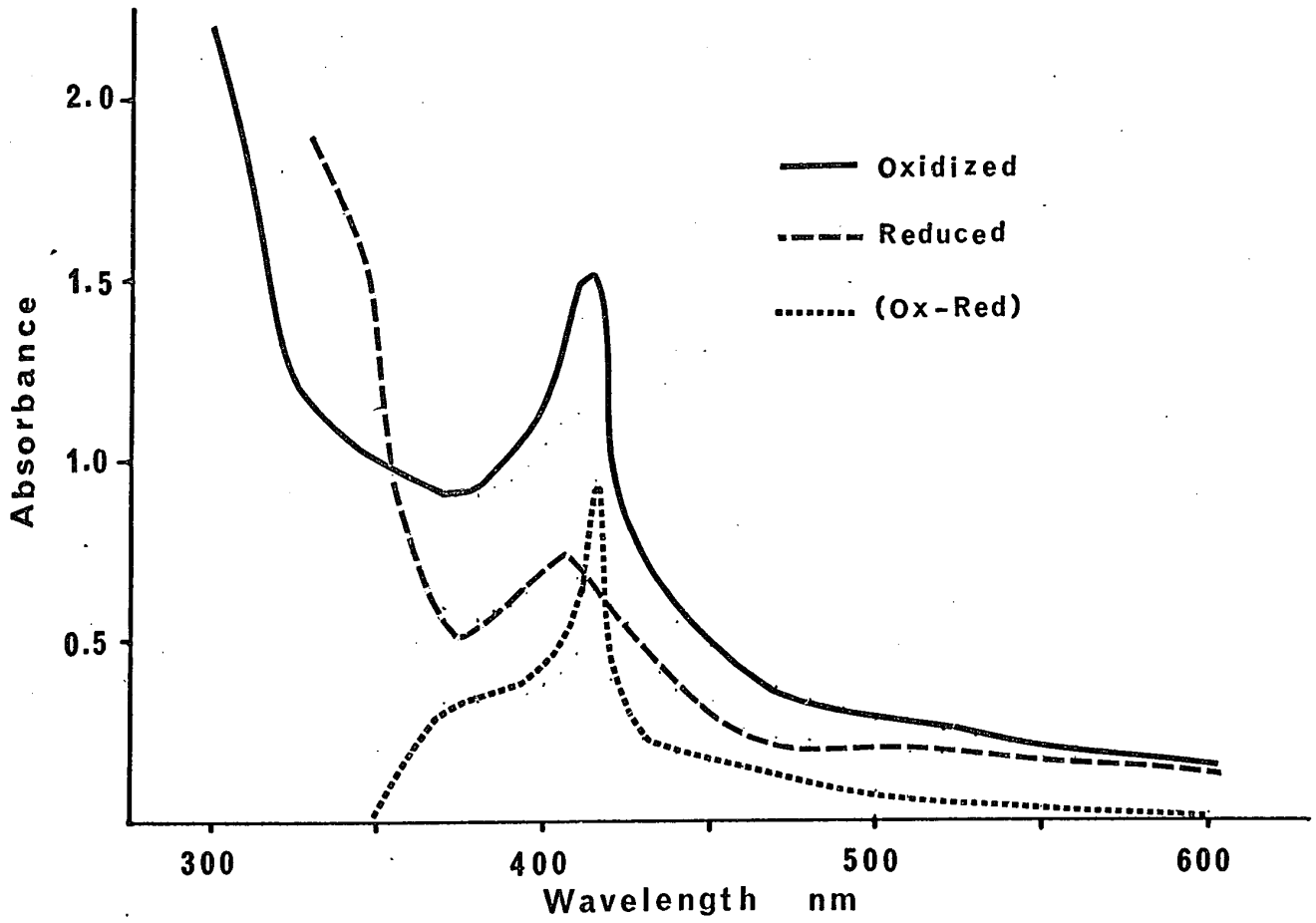

120
100
80
60
40
Elution volume (ml)
a
b
c
d
e
f
MAO
10^4
10^5
10^6
Molecular weight

Figure 20

Absorption spectra of partly purified rat liver MAO. Enzyme purified about 65-fold and containing 5 mg protein per ml in 0.05 M phosphate buffer, pH 7.4, was used in these experiments. The enzyme was reduced with sodium tetrathionate (dithionite).


Absorbance
2.0
1.5
1.0
0.5
300
400
500
600
Wavelength nm
Oxidized
Reduced
(Ox-Red)

systems at pH 8.9 or in continuous systems at pH values of 7.0 and 7.4. All stages except the sonicate displayed 3 or 4 purple-colored bands of formazan, indicating MAO activity when the gels were incubated with the tetrazolium-substrate mixtures. The sonicate usually showed a single, broad band of activity towards both those substrates. Diagrams representing the electrophoretic patterns usually observed are shown in Fig. 22. The positions of the bands of enzymic activity generally coincided with areas of stained protein on gel electropherograms from corresponding preparations that were run at the same time.

(b) SDS electrophoresis. Purified MAO, homogeneous when chromatographed on Sephadex, was denatured by incubation with SDS. Four to 5 bands of protein were visualized on stained electropherograms of the denatured enzyme (Fig. 22). The molecular weights of the dissociated polypeptide chains of MAO, represented by those bands, were estimated to range in size from 60,000 to 80,000 daltons. The molecular weights were calculated by interpolation from calibration curves of log (molecular weights) vs mobility ratios of standard proteins known to contain dissociable subunits which were examined by SDS electrophoresis simultaneously with the samples of MAO. An example of the calibration curves obtained by this procedure is shown in Fig. 23.

The Sulfhydryl Groups of MAO

The results of the experiments conducted on the inhibition (in vitro) of partly purified rat liver mitochondrial MAO by

Figure 21

Fluorescence emission spectra of native rat liver MAO and its Pronase-liberated component. Enzyme purified about 67-fold and containing 4.25 mg protein per ml in 0.05 M phosphate buffer, pH 7.4, was excited at 450 nm. The procedure used in the Pronase-mediated proteolysis of the enzyme is described in the text (Experimental). Sodium tetrathionate (dithionite) was used as the reducing agent.

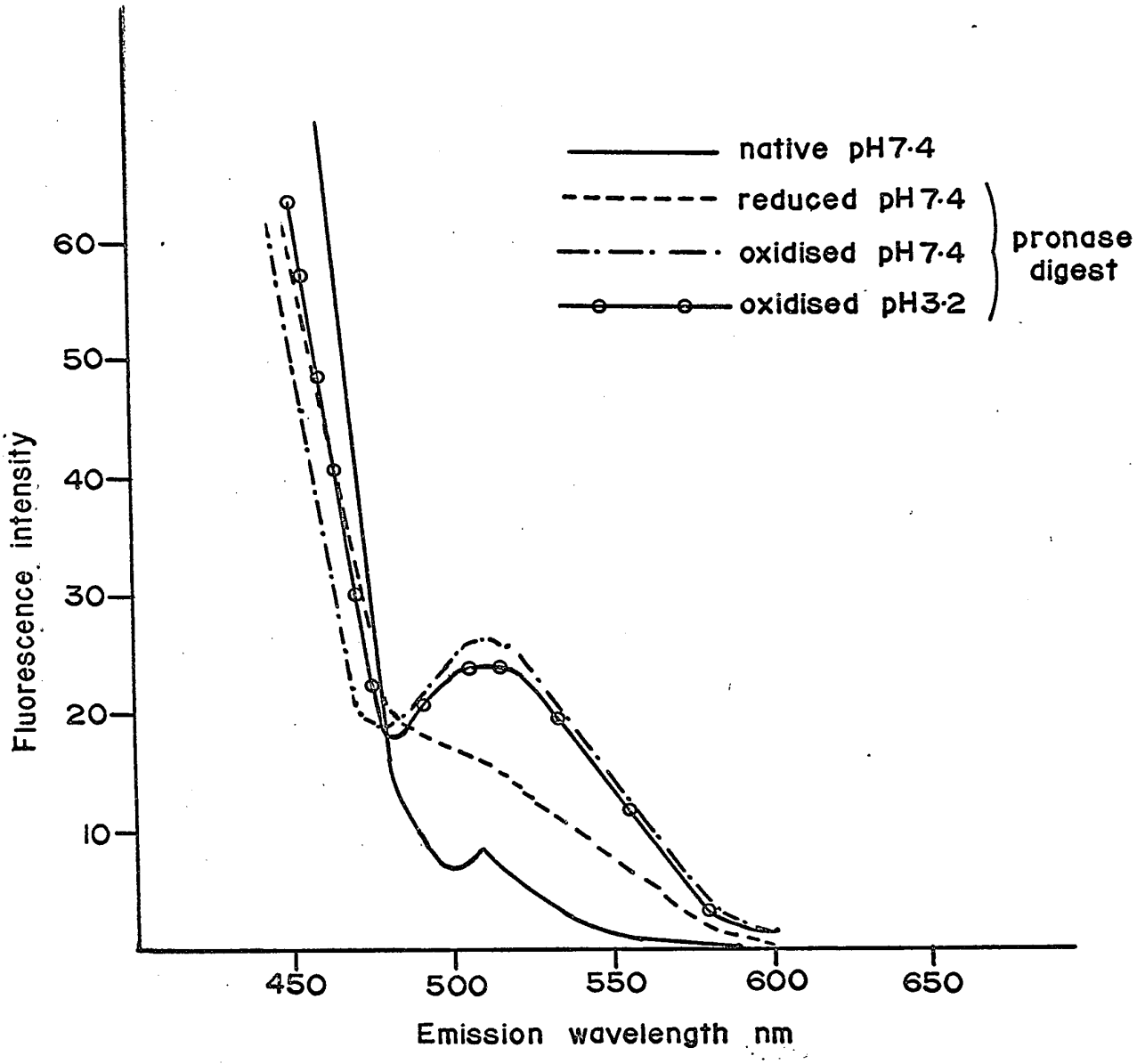
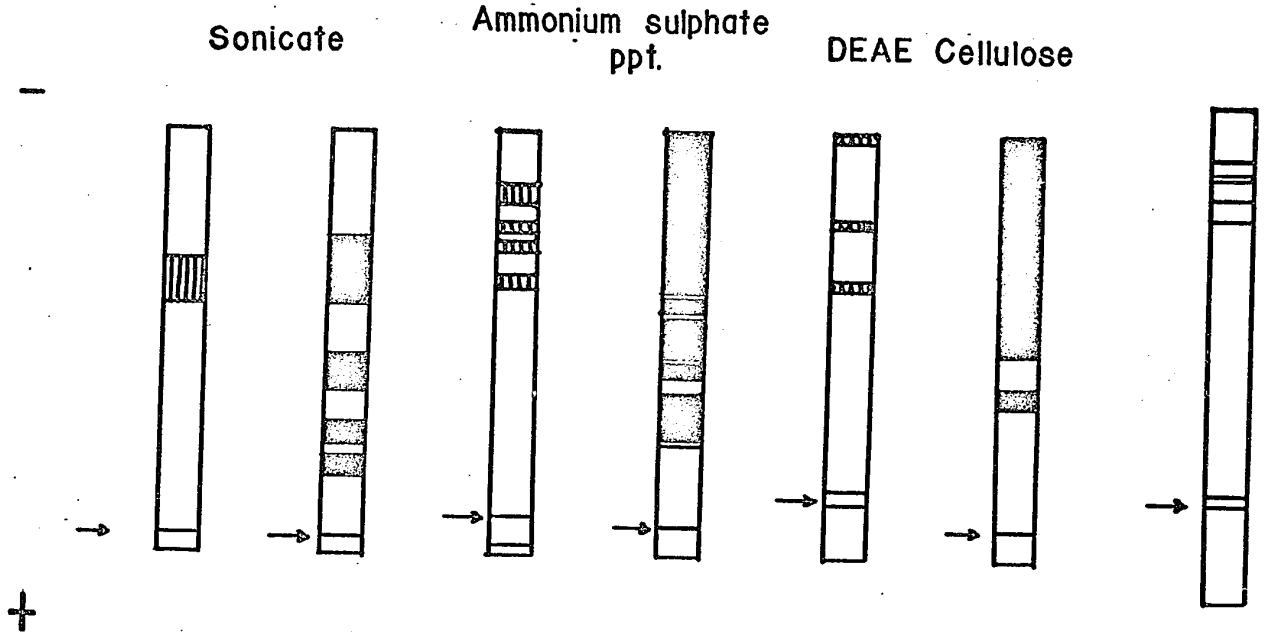

native pH7·4
reduced pH7·4
oxidised pH7·4
oxidised pH3·2
pronase digest
Fluorescence intensity
60
50
40
30
20
10
450
500
550
600
650
Emission wavelength nm

Figure 22

Acrylamide gel electrophoresis of rat liver MAO at different stages of its purification. The paired diagrams represent gel electropherograms of MAO preparations at the indicated purification stage. The hatched areas represent enzyme activity staining (left column) and the solid darker areas, protein staining (right column of each pair) visualized on actual columns of gel. Protein migrated towards the anode in a discontinuous system (7% gels buffered to pH 8.9 with the anodic electrolyte, 0.4 M Tris-HCl, pH 8.9) under a constant current of 2.5 mA per gel. The lone column at the extreme right represents an electropherogram of partly purified MAO that was denatured by SDS and stained for protein after electrophoresis was performed in a continuous system (10% gels were buffered to pH 7.0 with 0.2 M sodium phosphate containing 0.2% SDS. The electrolyte consisted of the same buffer diluted to half the concentrations of those ingredients). A running current of 8 mA per gel was used in these experiments. The arrows indicate the distances to which the tracking dye, bromophenol blue, migrated.

Acrylamide Gel Electrophoresis of Rat Liver MAO



various chemical agents are summarized in Table XXIII. Potassium thiocyanate, a chemical which reacts with iron did not inhibit kynuramine oxidation. Similarly, none of the reducing thiol compounds nor sodium arsenite were inhibitory at any of the concentrations tested. However, the sulfhydryl reagents DTNB and sodium nitroprusside were active inhibitors of rat liver MAO, the former being the more reactive of the two. Concentrations of DTNB and nitroprusside capable of inhibiting MAO activity by 50% (I_{50} values) were calculated by interpolation from graphs of percentage of inhibition plotted against log (inhibitor concentration) shown in Fig. 24. The I_{50} values obtained for 15 min preincubation periods with MAO at 37° and pH 7.0 are shown in Table XXIV and are for DTNB, 4.4×10^{-5} M; for nitroprusside, 5.8×10^{-4} M. Both these inhibitors seem to bind MAO reversibly, because the degree of inhibition observed in each case did not increase when the periods of preincubation with enzyme were prolonged (up to 25 min).

The number of .SH groups in MAO was determined by Ellman's method (400). The enzyme contained about 3.6 (ranged between 3 and 4) freely reactive .SH groups per molecule of a total of approximately 18.4 (determined in the presence of 8 M urea). The MAO used in these experiments was assigned a molecular weight of 290,000. The method was standardized by estimating the numbers of reactive .SH groups per molecule of cysteine, dithiothreitol and methionine at different concentrations of those substances. The data from those experiments are shown in Table XXV.

Figure 23

SDS gel electrophoresis: Calibration curve for the molecular weight determination of polypeptides derived from denatured MAO. Details of the experimental procedure that was followed appear in the text (Experimental) and the caption to Figure 22. The following proteins were used to standardize the system: (a) cytochrome c; (b) γ-globulin; (c) ovalbumin; (d) catalase; (e) bovine serum albumin. The bar at the lower right represents the range of molecular weights estimated for the oligomers of MAO that were detected.

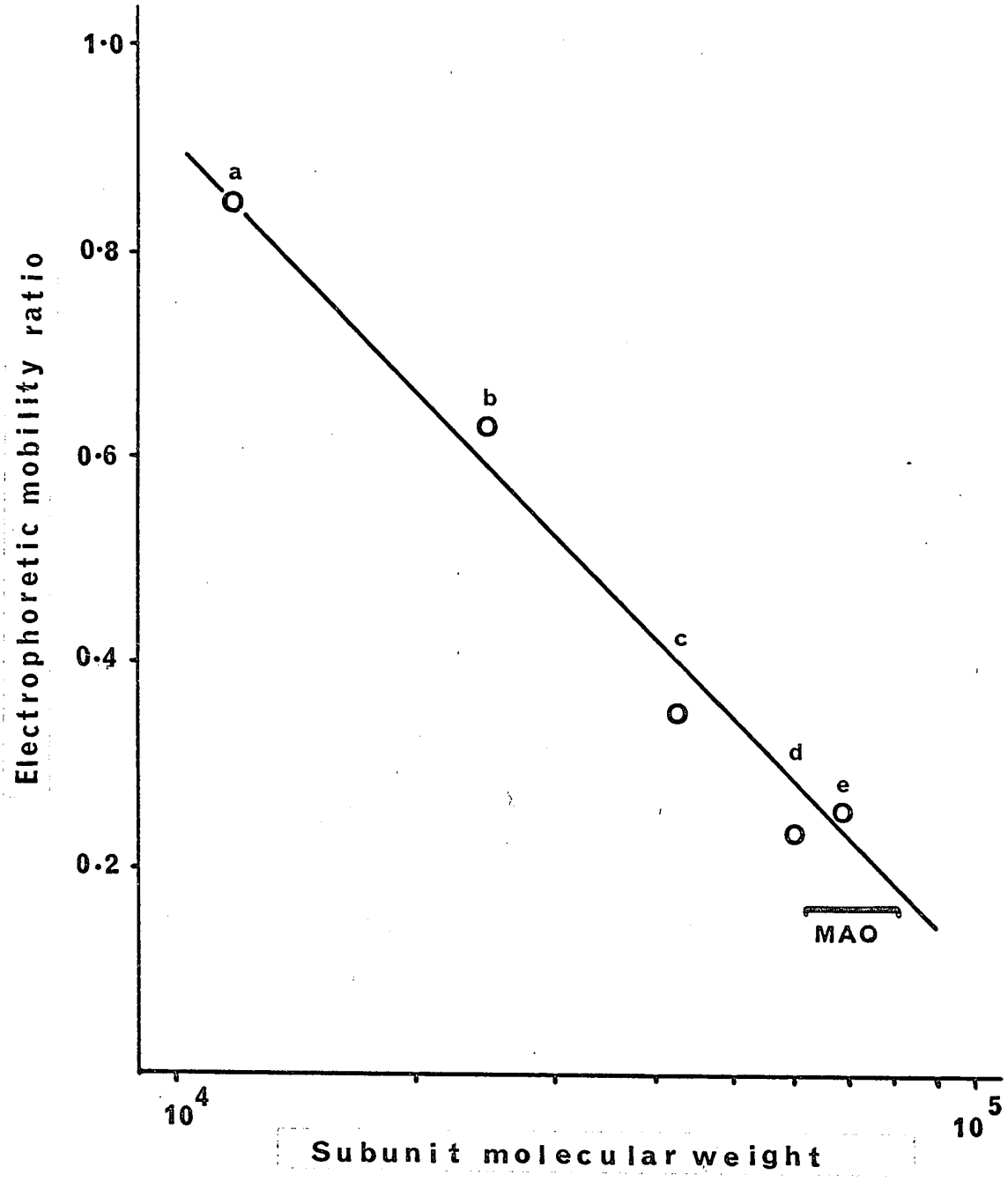

1·0
0·8
0·6
0·4
0·2
Electrophoretic mobility ratio
a
b
c
d
e
MAO
10^4
10^5
Subunit molecular weight

Table XXIII

Effect of sulfhydryl reagents and thiol compounds on MAO purified from rat liver mitochondria

Inhibitor	Concentration (M)	Relative Specific Activity* % (Mean ± SE)
Sodium Arsenite	3.33×10^{-7}	105.7 ± 0.00
	3.33×10^{-6}	105.7 ± 0.00
	3.33×10^{-5}	107.2 ± 0.00
	1.67×10^{-4}	103.6 ± 0.95
	1.67×10^{-3}	103.9 ± 0.40
Cysteine	1.67×10^{-4}	99.6 ± 2.35
	1.67×10^{-3}	91.3 ± 0.00
DTNB	3.33×10^{-7}	97.7 ± 0.00
	3.33×10^{-6}	90.0 ± 0.00
	3.33×10^{-5}	58.6 ± 0.00
	1.67×10^{-4}	10.0 ± 0.04
	3.33×10^{-4}	3.0 ± 0.10
Dithiothreitol	3.00×10^{-6}	101.1 ± 0.40
	3.00×10^{-5}	97.8 ± 2.70
	3.00×10^{-4}	95.8 ± 0.70
2-Mercaptoethanol	3.33×10^{-7}	101.5 ± 0.00
	3.33×10^{-6}	99.6 ± 0.00
	3.33×10^{-5}	98.9 ± 0.00
	1.67×10^{-4}	102.2 ± 0.75
	1.67×10^{-3}	91.3 ± 0.00

Table XXIII continued

Sodium Nitroprusside	1.67×10^{-5}	96.8 ± 3.45
	1.67×10^{-4}	86.1 ± 0.30
	1.67×10^{-3}	17.6 ± 1.00
Potassium Thiocyanate	1.67×10^{-4}	96.6 ± 1.25
	3.33×10^{-4}	96.8 ± 1.35
	1.67×10^{-3}	95.3 ± 2.00

*Specific activity expressed as a percentage of uninhibited control values were calculated from duplicate or triplicate determinations. Mean specific activity (± SE) for control determinations (n = 2) was 720 (± 0.0) nmoles of 4-hydroxyquinoline produced per mg protein during a 20 min incubation at 37° and pH 7.0. The reaction mixtures containing 33 mM phosphate buffer, enzyme (0.84 mg), inhibitor and water were incubated for 15 min before kynuramine (1 mM final concentration) was added to start the reaction. The enzyme preparation used was purified 64-fold from rat liver mitochondria (183 units of isoamylamine oxidizing activity).

Figure 24

Inhibition of rat liver MAO by sulfhydryl-reactive compounds. The values used to prepare these plots were derived from the data recorded in Table XXIII. A summary of the experimental procedure that was followed appears in the footnote to Table XXIII.

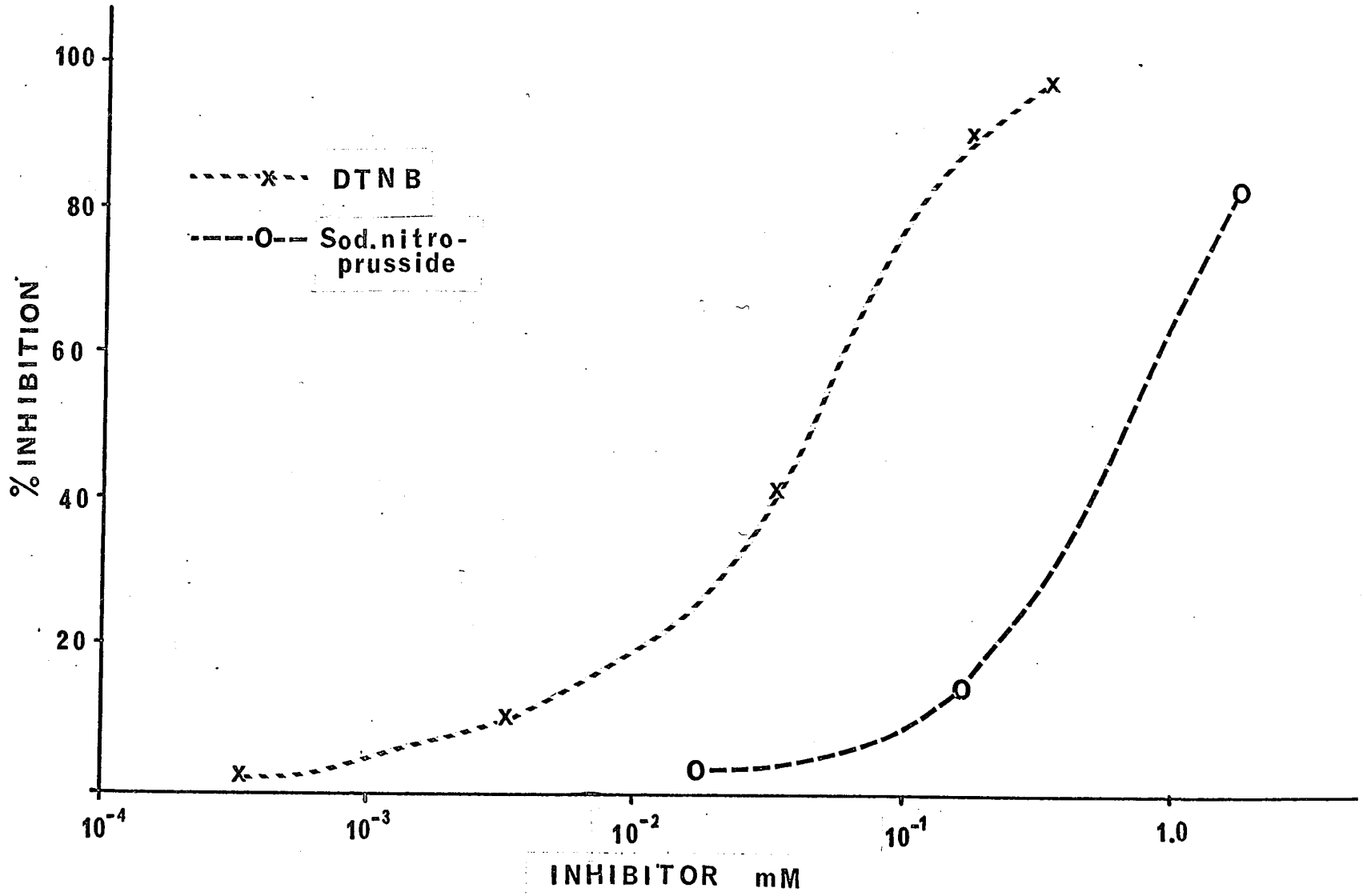

100
80
60
40
20
% INHIBITION
DTNB
Sod.nitro-prusside
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1.0
INHIBITOR mM

Table XXIV

Concentrations of sulfhydryl compounds inhibiting MAO activity by 50% *in vitro* at pH 7.0 and 37° and 15 min preincubation. Values are obtained by interpolation from graphs of % inhibition plotted against log (inhibitor concentration).

Inhibitor	I_{50} (M)
DTNB	4.35×10^{-5}
Sodium nitroprusside	5.80×10^{-4}

Table XXV

Determination of the number of sulfhydryl groups in MAO using DTNB

Thiol compound	Concentration (M)	No. of .SH groups found per mole	
		Duplicates	(Means ± SE)
Cysteine	0.025×10^{-3}	1.00, 1.02	
	0.50×10^{-3}	1.02, 1.02	
	1.00×10^{-3}	1.07, 0.995	1.02 ± 0.00
Dithiothreitol	0.020×10^{-3}	2.16, 2.22	
	0.050×10^{-3}	2.05, 2.01	2.11 ± 0.04
Methionine	0.020×10^{-3}	Nil	
	0.050×10^{-3}	Nil	
MAO: freely reacting	0.552×10^{-6}	3.10, 3.48	
	1.00×10^{-6}	3.66, 4.11	3.59 ± 0.21
MAO: total sulfhydryls	1.00×10^{-6}	17.3, 19.4	18.35 ± 1.05

Reactions were carried out at room temperature, and were initiated by the addition of aliquot amounts of DTNB (final concentration ranging from $1.2\text{-}1.3 \times 10^{-4}$M) dissolved in 0.1M pH 8.0 phosphate buffer that contained 0.01M EDTA to solutions of the indicated test substances which were prepared in the same buffer mixture. The final volume of the reaction mixtures was 2.0 ml. Blank solutions were prepared in which equivalent volumes of the buffer mixture replaced DTNB. Extinction values were measured at 412 nm after the addition of DTNB until there was no further increase. The numbers of .SH groups in the thiol compounds were calculated from the molar absorptivity of the reaction product ε (412 nm) = 1.36×10^{4}/M/cm (400). Determinations of the total .SH groups in MAO were conducted in 8M urea.

DISCUSSION

The Riboflavin Prosthetic Group of MAO

The results of the spectral studies on the isolated rat liver MAO and of nutritional experiments confirm that rat liver MAO contains riboflavin. In my hands, native MAO purified from rat liver mitochondria did not fluoresce strongly when excited at 450 nm. This was similar to the observation of Harada and Nagatsu (13) for the beef brain enzyme but differed from published reports on mitochondrial MAO from other sources (12, 15). Tipton (15) and Youdim and Sourkes (17) had mentioned that less highly purified preparations of pig brain MAO and rat liver MAO respectively did not fluoresce in the native state. Recent work by Harada et al. (14) tends to support this observation. These workers have obtained a purified preparation of beef brain MAO which is fluorescent in the native state. In the present work, marked fluorescence of the flavin cofactor was evident only after it was released by Pronase digestion. The flavin prosthetic group of some other mammalian mitochondrial amine oxidases is covalently linked to the apoenzymes with varying degrees of tenacity (10-15, 432). In contrast to that, the flavin moiety of bacterial amine oxidases is readily dissociable (33, 433).

Youdim and Sourkes (17) had identified the Pronase-released peptide-bound flavin of rat liver MAO as FAD from its chromatographic behavior and its fluorescence properties. They estimated its content at 2 moles of FAD per mole of native MAO. The fluorescence spectrum of the corresponding material scanned at pH 3.2 (Fig. 21) suggests strongly that the peptide-bound fluorescent species may be FAD.

Weber (430) had shown that at pH values near 3 the intrinsic fluorescence of riboflavin itself declines, while that of FAD in equimolar concentrations increases. Advantage has been taken of this property of FAD to measure its concentration in tissue preparations (434, 435). Harada and Nagatsu (13) also identified the acid-releasable flavin cofactor of beef brain MAO by its chromatographic properties as FAD. Igaue et al. (10) and Erwin and Hellerman (12) used spectrophotometric data to identify the prosthetic groups of beef liver MAO and beef kidney MAO respectively, as FAD, and to estimate its content as 1 mole per 10^5 g of enzyme in each case. Tipton (15) characterized the heat-releasable prosthetic group of pig brain mitochondrial MAO as FAD by chromatography and also by its ability to reactivate the FAD-specific D-amino acid apooxidase in vitro. He also reported that only authetic FAD could reactivate apoMAO in vitro and that the holoenzyme contained 1 mole FAD per molecule.

Walker et al. (431) have shown recently that FAD is linked covalently to the peptide chain of beef liver mitochondrial MAO by means of a cysteinyl thioether. Ghisla and Hemmerich (432) have prepared a synthetic flavopeptide whose physicochemical properties are identical to the enzyme-derived one (431). The mode of attachment of the flavin to the peptide arising from Pronase hydrolysis of the rat liver enzyme is not yet known.

Electrophoresis of MAO and Multiplicity of the Enzyme

It seems likely that MAO consists of isomeric subunits of approximately equal size which migrate in an electric field at

different rates owing to slight differences in their conformation and charge. The reason for a single band of enzymic activity originating from the sonicate is not immediately apparent. However, Youdim (private communication) has consistently observed this phenomenon, although Kim and D'Iorio (376) and Shih and Eiduson (53, 54) have reported the equally consistent occurrence of multiple bands of MAO activity in electropherograms of sonically treated preparations.

The question has been posed as to the likelihood of multiple bands of MAO originating as artefacts of preparation. Kim and D'Iorio (376) found that the occurrence and patterns of the possible isoenzyme bands were reproducible regardless of the method of preparation of the enzyme. It was also observed in the course of this work that untreated mitochondrial preparations often behaved identically to solubilized, purified preparations of MAO when they were subjected to gel electrophoresis on polyacrylamide. Youdim et al. (375) have found that re-electrophoresis of isolated individual bands always results in discrete single bands of enzymic activity having the same mobility as was originally observed. All these findings negate the likelihood of artefactual origin for the electrophoretic multiplicity of MAO.

Denatured samples of partly purified MAO subjected to SDS electrophoresis also yielded five polypeptide chains which varied in molecular weight between 60,000 and 80,000 daltons, and which were consistent with an approximate molecular weight of 300,000

daltons for the undissociated enzyme (assuming that these observed forms are subunits of MAO). Findings similar to this have now been published by Youdim and Collins (380, 381). These authors also found that the MAO subunits could be reassociated with restoration of activity if the exposure to denaturing conditions was of relatively short duration (less than 10 minutes)(381).

Mitochondrial MAO originating from several different tissues, and in different species, has been shown to consist of multiple forms (371). MAO from rat liver microsomes also contains multiple forms (376). The different forms of MAO possess characteristic patterns of substrate specificity (54, 379) as well as unique pH activity and thermal stability profiles, and distinct inhibitor sensitivities (375, 378). Gomes et al. (373), using column chromatography, had isolated multiple components of beef liver MAO which had similar substrate and inhibitor specificities. Hartman et al. (436) showed subsequently that those components were antigenically identical, i.e., they did not represent isoenzymes but could be, instead, fragments of the same protein in different physical states. These findings were supported by the results of Akopyan and coworkers (359) who found, using polyacrylamide gel electrophoresis, that MAO from beef liver mitochondria exists as a single enzymically active species while the rat liver enzyme exists as multiple forms.

Although MAO purified from mitochondria of pig brain (437) and beef brain (438) do not possess multiple forms, isoenzymes are present in MAO in the brains of the rat (54, 375), human being (378),

domestic fowl (53), and Xenopus Laevis (55). Harada, Mizutani and Nagatsu (14) recently reported the separation by continuous flow electrophoresis of two components from purified beef brain MAO. Both forms of the enzyme had similar physicochemical characteristics and inhibitor sensitivity patterns but they exhibited differences in their substrate preferences. Harada et al. believed that both components belong to the same mitochondrial enzyme protein and could be artefacts arising from structural alterations produced during purification. The MAO isoenzymes found in different regions of the human brain exhibit marked differences in their substrate preference and sensitivity towards various inhibitors (378). According to Collins and his colleagues (378) this finding may explain, in part, why some MAO inhibitors are not only more beneficial than others in treating depression of the central nervous system, but also may be more likely to induce undesirable side effects (e.g., hypertensive crises stemming from interactions with tyramine or dopamine of dietary origin). Ontogenic effects have been observed in the MAO isoenzyme activity patterns in animal tissues (53-55). That is, the isoenzyme components of embryonic and neonatal tissues differed qualitatively and quantitatively from those forms which occur in the corresponding adult tissues.

The Essential Sulfhydryl Groups of MAO

Reports from several laboratories based on inhibition studies with sulfhydryl group reagents have implicated .SH groups in the action of MAO purified from different sources (12, 89, 314, 319, 353-357).

However, relatively little quantitative work has been done on the .SH groups of rat liver MAO (356). Youdim (402) had compared the inhibitory potencies of several heavy metal salts and organic sulfhydryl agents on highly purified MAO prepared from rat liver.

In the present work, the failure of arsenite to inhibit MAO indicates a lack of vicinal sulfhydryl groups in the enzyme. Cysteine, dithiothreitol and 2-mercaptoethanol were also not inhibitory at the concentrations tested. Lagnado and Sourkes (340) and Youdim (402) had reported that significant inhibition of MAO activity was mediated by relatively high concentrations of cysteine and other thiol compounds. The slight acceleration of enzymic activity that occurred on incubation with low concentrations of arsenite and 2-mercaptoethanol (in these studies) suggested that these compounds might protect the .SH groups of MAO during the oxidation of kynuramine. Lagnado and Sourkes (340), Singer and Barron (352) and Friedenwald and Herrmann (351) had previously shown that glutathione could protect the enzyme from inhibition by thiol-reacting chemicals. Friedenwald and Herrmann (351) had also found cysteine to be of value in this respect.

The values of 3 to 4 "freely reacting" sulfhydryl groups and 17 to 19 total sulfhydryl groups (in the presence of 8 M urea) found per molecule of rat liver MAO differs considerably from the estimates of 8 (total) .SH groups per 100,000 g protein reported for the beef kidney enzyme (12, 353, 354) and 7 (total) .SH groups per 100,000 g of beef liver MAO (357). Klyashtorin and Gridneva

(356) had reported that soluble, 25-fold purified MAO from rat liver mitochondria contained 7 "freely reacting" .SH groups per 10^5 g protein and a total of 8.5 in the presence of concentrated urea. Since their preparation still contained much ballast protein and the method that they used could lead to overestimation, the discrepancies between their values and those found in the present experiments may be due to those circumstances.

The data in Table XXV show that all the .SH groups of rat liver MAO became available for mercaptide formation only when the enzyme was denatured. These results are in contrast to the results reported by Erwin and colleagues (12, 353, 354) and Gomes et al. (357) who did not find any additional .SH groups when they assayed for them in concentrated urea. It may be significant that the concentration of DTNB used to estimate the 3 to 4 "freely reacting" sulfhydryls of MAO could inhibit the oxidation of kynuramine by more than 80% under the conditions of enzyme activity determination (Fig. 24). How these particular .SH groups participate in the action of rat liver MAO is not known at the present time, however. Hellerman and Erwin (353) and Gomes et al. (357) presented evidence indicating that the .SH groups of the beef kidney and liver enzymes, respectively, are probably required to maintain them in stable conformations instead of being concerned directly with catalysis. This appears to be true also for other sulfhydryl enzymes such as urease (439), rabbit muscle aldolase (440), phosphoglucose isomerase (441) and phosphorylase (442). Gorkin and his colleagues have shown

that the oxidative "transformation" of some mammalian mitochondrial amine oxidases (146, 152, 359) and the soluble MAO (tyramine oxidase) derived from Sarcina lutea (89), to DAO-like enzymes is the result of the oxidation of some essential .SH groups, probably to disulfide bonds. Akopyan et al. (359) also proposed that the .SH groups of the beef liver enzyme might assist in the maintenance of its three-dimensional structure and do not participate directly in its catalytic activity. The sulfhydryl groups of some other biologically active proteins are known to be involved directly in their activity. For instance, Godeaux (443), Bailey and Perry (444) and Buchtal et al. (445) reported that the .SH groups of myosin are responsible for its contractility and its association with actin. Bailey and Perry (444) concluded from the results of inhibition studies that the same .SH groups of myosin were intimately connected with its ATP-ase activity as well as with its contractility. Buchtal et al. (445) however, presented evidence showing that those two activities of myosin were independent of each other. Evidence that .SH groups of glyceraldehyde-3-phosphate dehydrogenase prepared from yeast and from rabbit muscle bind the substrate to the enzyme during catalysis was presented first by Krimsky and Racker (446) whose findings were later substantiated by Koeppe, Boyer and Stulberg (447).

While it is likely that most of the essential .SH groups of rat liver MAO are responsible for maintaining the structure of the enzyme molecule, it is also possible that some of them could play a secondary role in catalysis. For instance, they might participate

in electron transfer between the catalytic site of the enzyme and its flavin coenzyme, or alternatively bind the metal cofactor to the protein. Further studies need to be done with the highly purified enzyme to test these possibilities.

SUMMARY

1. A linear relationship was found between the activity of mitochondrial MAO from rat liver and its iron content at different stages of purification. This finding suggests that the enzyme may contain iron.

2. The absorbance spectra of partially purified MAO indicate the presence of riboflavin as a cofactor. The fluorescence spectra of a substance liberated from the native enzyme by proteolytic digestion confirmed peptide-bound riboflavin which could be present as FAD. The approximate concentration of presumed FAD in the native protein was 1.5 moles per mole of MAO, and in the isolated flavopeptide it was about 0.8 mole per mole of original enzyme (290,000 daltons molecular weight was assumed).

3. Direct evidence in support of the concept of multiplicity of MAO has been found. Multiple forms of the enzyme which seemed to be similar in size but different in their conformation, or their surface charge, were detected by electrophoresis at different stages of its purification.

4. SDS electrophoresis of denatured MAO separated five distinct polypeptide chains which differed slightly in molecular

weight. The polypeptide chains, ranging in size from 60,000 to 80,000 daltons, could represent subunits of undissociated MAO.

5. The thiol-reacting agents, sodium nitroprusside and DTNB inhibited rat liver MAO activity indicating the presence of freely reacting, essential .SH groups in the enzyme. The mode of inhibition was apparently reversible. The non-inhibitory action of arsenite infers that dithiol residues are absent.

6. Thiol-containing substances did not inhibit the oxidation of kynuramine at the concentrations used. Results of experiments in which they were tested suggest that they may, in low concentrations, protect the essential .SH groups of MAO.

7. Eighteen .SH groups per molecule of rat liver MAO were found using DTNB. At least 3 to 4 of that number are classified as "freely reacting". The discrepancy between these values and values reported in the literature may be due to differences between species and methodological differences.

8. Possible roles for the essential .SH groups of MAO in its action have been discussed.

III. GENERAL DISCUSSION

The significance of metals to biological processes has long been recognized. The biological function of metals has been looked for in their association with proteins, especially those which display enzymic activity (448).

Nutritional studies in which organisms have been deprived of a particular metal and later repleted with it have often provided the first indication that the metal is an essential component of an enzyme either for its structure or for its activity. For example, alcohol dehydrogenase activity of Neurospora crassa decreased in cultures grown on zinc-deficient media and was restored when zinc was added to the media. These findings led to the discovery that zinc is part of that enzyme in organisms as diverse as yeast, the horse and man (449). Similar nutritional studies showed that molybdenum is an integral part of xanthine oxidase (XO) of mammalian intestine (449). Analyses of purified XO from milk, mammalian liver, and avian liver and kidney have confirmed the presence of molybdenum in this enzyme (450).

Observations made from similar nutritional experiments are reported in this Thesis and elsewhere (161, 349, 350) and they indicate that iron is essential for the activity of mitochondrial MAO in rat liver (see Tables II, IV, X and Figs. 4, 5 and 7A).

The linear relationship found to exist between specific activity of the partially purified enzyme and its iron content is shown in Fig. 18 and suggests that iron may be a prosthetic group

of the enzyme (see also 402). Youdim and Sourkes (315, 337) had earlier found relatively high amounts of iron in their purest preparations of the rat liver enzyme. Oreland (358) has found 2 atoms of iron per mole of pig liver MAO more recently.

The slight decrease in MAO activity found in tissue preparations of iron-deficient rats, compared to the lowering of blood hemoglobin and liver storage iron concentrations suggest that if iron is a cofactor of MAO, then it must be held very tightly to the apoenzyme. The fact that relatively high concentrations of chelators inhibited the enzyme are consistent with this suggestion. Additional evidence to support this was obtained in experiments with the well-known iron reagent potassium thiocyanate. Table XXIII shows that incubating the enzyme with concentrations of this chemical greater than 1 mM did not inhibit MAO activity appreciably. Oreland (358) also found that dialysis of the enzyme against chelators was unsuccessful in removing iron from the enzyme. Attempts made to restore enzymic activity in tissue slices, homogenates and mitochondrial suspensions prepared from livers of iron-deficient rats, by incubating those preparations with solutions of different salts of iron have been unsuccessful (Young, S., private communication). These findings are also consistent with the above suggestion.

In vivo tests of MAO activity in the whole animal demonstrated that iron is involved only at the oxidative deamination stage of the catabolism of a monoamine. It was also shown by these means that MAO in the iron-deficient rat was more susceptible to inhibition

by APHZ than in the normal rat (Table XIV). This finding is similar to one which led Distler and Sourkes (335) to propose that riboflavin is a prosthetic group of MAO long before the presence of this vitamin had been detected in MAO.

The failure of nutritional copper-deficiency to influence MAO activity in the rat militates against iron's role in the action of the enzyme being solely the maintenance of the ultrastructural integrity of liver mitochondria. Other possible roles of iron in the action of this enzyme are: maintenance of its active configuration, or alternatively, iron is a necessary cofactor for the synthesis of MAO.

The iron found in MAO may contribute to the absorbance band appearing in the Soret region of the enzyme's spectrum (12, 161, 337). Oreland found that amounts of heme which caused Soret absorption in the spectrum of pig liver MAO were considerably less than the amounts of flavin and iron found in that enzyme (358). Several purified metalloflavoenzymes obtained from vertebrates and microorganisms contain NHI in their prosthetic groups (450).

Effects of nutritional riboflavin-deficiency and repletion with the vitamin on MAO activity were also studied in the rat by the whole animal technique. Riboflavin was found to be an absolute requirement for the efficient catabolism of a monoamine in the rat (Fig. 8A). Figure 12 shows that the riboflavin-deficient rat liver MAO was more sensitive to inhibition by APHZ than was the enzyme from normal rat liver. The results of these experiments confirm

the earlier findings reported by Hawkins (332) and Sourkes and his coworkers (333-337) regarding the importance of this nutrient in the action of MAO.

Experiments conducted *in vitro* with the Pronase digest of the partly purified enzyme confirmed that riboflavin is bound covalently to MAO. The content of flavin (presumptive FAD) in the enzyme was estimated to be 1.5 moles per mole of MAO from spectrophotometric data, assuming that free and bound flavin have the same molar absorptivity (12, 86). Less flavin (0.8 mole per mole MAO) was found in the Pronase-released peptide. These values for MAO flavin content are lower than the value of 1 mole per 10^5 g protein reported for other more highly purified MAOs (12, 15-17, 358, 432a).

The results of the experiments conducted *in vivo* and *in vitro* with APHZ and PHZ add to the present knowledge of the chemistry of MAO, although these substances do not inhibit MAO only. As far as I am aware, no other detailed studies of their effects on MAO have been published. Preincubating the enzyme with kynuramine protected it only against APHZ inhibition (Table XVII). The differential effects observed for substrate protection of MAO against these agents and in their time courses of inhibition of the enzyme (Figs. 15 and 16) suggest either that APHZ and PHZ may bind at different sites on the enzyme molecule or that more than one kind of MAO may exist. The biphasic nature of the graph shown in Fig. 16 could also be an indication that MAO degrades APHZ to some other reactive substance which inhibits it. The suggestion that biodegradation

of hydrazine derivatives and their inhibitory effects on MAO are interdependent has been made earlier by several authors (245, 246, 270, 451-455). Hucko-Haas and Reed (456) have reported that beef plasma DAO also degrades hydrazine derivatives.

The pH-independence of MAO inhibition by APHZ and PHZ had been observed for other hydrazine compounds earlier, by Bloom (452).

Differential effects towards three different substrates were observed in nutritional experiments on iron-deficiency and in experiments with iron chelators. Those differences suggested that MAO may possess multiple forms. Multiplicity of the enzyme was confirmed by means of polyacrylamide gel electrophoresis in continuous and in discontinuous systems. MAO that appeared homogeneous by gel filtration chromatography was found to consist of about 4 to 5 polypeptide chains when subjected to gel electrophoresis in the presence of SDS and 2-mercaptoethanol. The oligomers of MAO varied in molecular weight from approximately 60,000 to 80,000, and may represent subunits of the enzyme.

Partly purified rat liver MAO was estimated to contain an average of about 18 (total) .SH groups per mole (290,000 daltons), of which 3 to 4 of them are regarded as freely reacting. The difference between these values and other estimates reported for numbers of .SH groups in MAO (353-357) may be ascribed to differences between species and to different analytical methods that were used.

The exact function of the freely reacting .SH groups of rat liver MAO is not known. Perhaps they take part in the transfer of electrons between the active site and the prosthetic groups of the enzyme. Alternatively, they may be needed for binding the iron cofactor to the apoenzyme. Atoms of NHI are bound to metallo-flavoprotein enzymes by .SH groups (450). The other .SH groups of MAO may serve to maintain the enzyme molecule in its active configuration, as has been suggested by Hellerman and Erwin (353), Gomes et al. (357) and Akopyan and coworkers (359) for MAO obtained from other sources.

IV. CONCLUSIONS*

Methods developed for measuring monoamine oxidase (MAO) activity of rat tissues *in vitro* and in the whole animal *in vivo* were used to study the cofactor requirements of the enzyme.

Chronic nutritional deficiencies of iron and riboflavin caused MAO activity to decrease significantly. Realimentation with the respective nutrients soon restored enzyme activity to normal levels. Prolonged feeding of copper- and inositol-deficient diets had no effect on MAO.

Iron- and riboflavin-deficiencies rendered MAO more susceptible to the inhibitory action of acetylphenylhydrazine.

It can be concluded from the results of the nutritional deficiency experiments, the inhibitory action of iron-chelating agents and chemical analysis of partly purified MAO that iron participates in the action of the enzyme on monoamines. This metal is not required for the metabolism of the immediate oxidation products of the amines, however, as judged from the results of the whole animal studies. Inhibition tests indicate that if iron is a prosthetic group of MAO then it may be bound tightly to the enzyme.

Similar conclusions regarding the role of riboflavin in MAO can be drawn from the results of experiments involving the deficiency of this vitamin. The presence of a flavin prosthetic group covalently

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*Summaries to the separate experimental sections of this Thesis appear on pages 68, 92, 117 and 146.

bound to the enzyme has been confirmed in these studies.

The hemolytic agents acetylphenylhydrazine (APHZ) and phenylhydrazine (PHZ) inhibited MAO activity of rats irreversibly *in vivo* and *in vitro*. They inhibited the enzyme non-competitively *in vitro*. The *in vivo* inhibitory action of APHZ towards the enzyme in rat liver was greater than that of an equivalent dose of PHZ. The latter compound was slightly more active than the former against the rat brain enzyme *in vivo*, and against rat liver MAO *in vitro*.

Multiple forms of MAO were detected by polyacrylamide gel electrophoresis. The enzyme may consist of 4 to 5 subunits which differ slightly in their charge properties and their molecular size.

Partly purified rat liver MAO contained an average of approximately 18 sulfhydryl (.SH) groups per mole of enzyme. Three to 4 of that total are considered to be freely reacting. Possible roles of .SH groups in the action of MAO have been discussed.

V. CLAIMS TO ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1. Methods were developed to study the action of monoamine oxidase (MAO) *in vitro* with tissue preparations and *in vivo* in the living animal.
2. The *in vivo* method referred to above was used to assess the relative potencies of several inhibitors of MAO in the living animal.
3. The above-mentioned tests were used in studies of nutritional deficiencies. It was found that the action of the enzyme on monoamines *in vivo* depends upon the presence of sufficient quantities of dietary iron and riboflavin.
4. MAO activity *in vivo* is not dependent upon the presence of either copper or inositol.
5. Iron-chelating agents inhibited MAO activity in mitochondrial preparations of rat liver. Potent copper-chelating chemicals were not inhibitory.
6. The requirement for iron resides only at the oxidative deamination stage of monoamine catabolism *in vivo*.
7. The concentration of iron in MAO increased linearly with respect to its specific activity during purification.
8. The results of inhibition experiments with chelators and potassium thiocyanate are in favor of the possibility that the metal is bound tightly to the apoMAO molecule.
9. A prosthetic group released from MAO by proteolytic digestion was found to contain flavin.

10. Acetylphenylhydrazine (APHZ) and phenylhydrazine (PHZ) both inhibited MAO in the rat rapidly and irreversibly, *in vivo* and *in vitro*.
11. APHZ and PHZ inhibited MAO noncompetitively *in vitro*.
12. The actions of APHZ and PHZ on MAO, *in vitro*, were independent of pH.
13. Concentrations of APHZ and PHZ capable of inhibiting MAO *in vitro* by 50%, following 15 minutes of preincubation with the enzyme were 3.6×10^{-5} M and 1.6×10^{-5} M respectively, at pH 7.0 and 37°.
14. The inhibitory effects of APHZ were enhanced in iron- and riboflavin-deficient rats.
15. Three to four bands of MAO activity were detected by acrylamide gel electrophoresis at different stages of enzyme purification.
16. Electrophoresis of MAO under denaturing conditions produced 4 to 5 bands of protein representing dissociated polypeptide chains of MAO. The molecular weights of the oligomers (60,000 to 80,000) were consistent with an approximate molecular weight of 300,000 for the undissociated enzyme.
17. Concentrations of sulfhydryl (.SH) reagents sodium nitroprusside and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) that inhibited MAO by 50% after a preincubation period of 15 minutes were 5.8×10^{-4} M and 4.4×10^{-5} M, respectively at 37° and pH 7.0.
18. Thiol-containing reducing agents did not affect enzymic activity significantly.

19. The present work provides evidence that MAO lacks vicinal .SH groups.
20. An average of 18.4 (total) .SH groups per molecule of MAO was found.
21. The MAO molecule was estimated to contain an average of 3.6 freely reacting .SH groups.

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