Human Liver Mitochondrial Monoamine Oxidase. II. Determinants of Substrate and Inhibitor Specificities*

Charles M. McEwen, Jr., Gary Sasaki, and Douglas C. Jones

ABSTRACT: Kinetic data are presented describing interactions of the monoamine oxidase of human liver mitochondria with biogenic amines, pharmacological agents, and substrate analogs. The effect of pH values upon apparent Michaelis and inhibitor constants of amines suggests that typical substrates and analogous competitive inhibitors are bound at the enzyme active center by an electrophilic site that reacts with nonprotonated amines. Kinetic constants concerning enzyme complexes formed by substrates with multiple ionizable groups (*e.g.*, tyramine, octopamine, serotonin, and kynuramine) suggest that completely un-ionized species of these substrates interact with the free enzyme. The affinities of substrates and competitive inhibitors for the enzyme are adversely affected by polar substituents. Phenyl groups of aliphatic amines and alcohols may contribute to the affinities of these compounds for the enzyme. The inhibitory effects of mixtures of phenylethanol and ammonia suggest that the hydroxy and amino groups of these competitive inhibitors are bound at the same enzyme site. β-Naphthol, but not α-naphthol, is a potent competitive inhibitor of the enzyme. Certain 2-(β-naphthyl)ethylamines are also potent competitive inhibitors. These and other related findings may be interpreted as evidence that a hydrophobic area is immediately adjacent to the electrophilic binding site of the enzyme active center. These findings, however, do not indicate that nonpolar substrate residues are merely extracted from aqueous media by an unlimited hydrophobic surface of the enzyme. An unusual substrate activation at relatively high pH values and substrate inhibition at relatively low pH values were observed with kynuramine as substrate. On the other hand, we were unable to detect discrepancies in the kinetic data, to suggest the presence of multiple amine oxidase activities. The mitochondrial monoamine oxidase activity differed in several respects from that of the soluble monoamine oxidase of human plasma.

The preceding paper (McEwen *et al.*, 1968) in this series described the partial purification of a monoamine oxidase (EC 1.4.3.4) from human liver mitochondria, which catalyzes the oxidative deamination of amines acording to the general equation

$$RCH_2NHR' + O_2 + H_2O \longrightarrow RCHO + R'NH_2 + H_2O_2 \quad (1)$$

where R′ may represent either a hydrogen atom or a methyl residue. Kinetic data obtained with this enzyme preparation indicated that the nonprotonated species of model monoamines without other ionizable groups (*e.g.*, benzylamines and simple *n*-alkylamines) or substrate analogs (*e.g.*, ammonia) interact with the enzyme to form enzyme–substrate or enzyme–inhibitor complexes. The formation of these complexes was found to be determined by the following equation

$$K_m = K_i = \tilde{K}[1 + \text{antilog}\,(pK_a - pH)] \quad (2)$$

which relates apparent Michaelis or inhibitor constants measured at a given pH value to the pK_a values of the model amines and to kinetic dissociation constants ($\tilde{K}$ values) that are independent of pH. Electronic analogs of nonprotonated amines (*e.g.*, simple aliphatic alcohols) were found to be competitive inhibitors of the enzyme activity. Data were also presented which indicate that the apparent inhibitor constants of alcohols, unlike those of amines, do not vary with pH and that amino groups and hydroxy groups are bound by the same enzyme site. In addition, hydrophobic residues of amines and alcohols were found to enhance the affinities of these substrates and inhibitors for the enzyme. The combined data obtained with these model compounds were interpreted as evidence that the enzyme active center contains both electrophilic and hydrophobic binding sites.

The present report concerns the interactions of the mitochondrial monoamine oxidase of human liver with more complex substrates and inhibitors, including "typical" substrates of mitochondrial monoamine oxidases. The results presented in this paper extend the observations of the preceding paper and suggest that the unusual conclusions derived from model interactions are pertinent to human metabolism and pharmacology. In particular, the data cast doubt upon the widely held but unproven hypothesis that mitochondrial monoamine oxidases, in general, interact with protonated amines (Blaschko, 1952; Soep, 1961) and may explain conflicting quantitative data pertaining to the interactions of ionizable compounds with these enzymes, which were recently reviewed in detail (Biel *et al.*, 1964; Zirkle and Kaiser, 1964; Burger and Nara, 1965). They also allow speculation that changes in intramitochondrial pH values (Addanki *et al.*, 1968) may control the degradation of biologically active amines. In addition, the present paper reports new kinetic studies concerning instantaneous interactions of substrate analogs with the enzyme, instantaneous inhibitions by nitrogenous compounds with pharmacological activities, and differences between this en-

* From the Departments of Medicine, Sinai Hospital of Detroit and Wayne State University School of Medicine, Detroit, Michigan 48235. *Received April 15, 1969.* This investigation was supported by a U. S. Public Health Service grant (HD 01782) from the National Institute of Child Health and Human Development.

zyme and the soluble monoamine oxidase of human plasma (McEwen, 1965a,b).

Experimental Procedure

Materials. Partially purified mitochondrial monoamine oxidase (specific activity 44) was prepared from human liver by two Triton X-100 extractions of isolated mitochondria, ammonium sulfate fractionation of the second extract, extensive dialysis of this soluble activity, and a final high-speed centrifugation (27,000*g* for 2 hr) of the dialyzed preparation, as previously described (McEwen *et al.*, 1968). The source of the horseradish peroxidase and the dialysis of this enzyme before use have also been described.

Amantadine hydrochloride (Symmetrel, E. I. du Pont de Nemours and Co., Newark, Del.), guanethidine sulfate (Ismelin, CIBA Pharmaceutical Co., Summit, N. J.), hydrocortisone (Merck Sharp and Dohme Research Laboratories, Rahway, N. J.), 2-(β-naphthyl)-2-hydroxyethylamine, *N*-isopropyl-2-(β-naphthyl)-2-ketoethylamine, and *N*-isopropyl-2-(β-naphthyl)-2-hydroxyethylamine (I. C. I. compounds 41,427, 38,604, and Nethalide, Imperial Chemical Industries, Cheshire, England), and pronethanol and propranolol (Alderlin and Inderal, Ayerst Laboratories, New York, N. Y.) were kindly supplied as chemically pure compounds by research divisions of the indicated pharmaceutical companies. Other amines and substrate analogs were usually purchased from commercial laboratories (CalBiochem, Fisher Scientific, Mann, and Regis Chemical) that furnished evidence of the chemical purity of their products. Enzyme substrates and inhibitors purchased from other commercial sources were purified before use as previously described (McEwen *et al.*, 1968). All inorganic chemicals were analytical reagents. Doubly distilled water, containing less than 20 ppb of copper, was used throughout.

Methods. A Cary recording spectrophotometer (Model 15) fitted with a thermostatically controlled cuvet chamber, which maintained reaction mixtures at 25.0 + 0.2°, was used for the direct determination of enzymic reaction velocities as well as for colorimetric measurements of ammonia production and spectral determinations.

Initial rates of benzylamine, *N*-methylbenzylamine, and veratrylamine (3,4-dimethoxybenzylamine) oxidations were measured directly (McEwen *et al.*, 1968) by recording the increases in absorbance, respectively, at 250 mμ due to the production of benzaldehyde and at 308 mμ due to production of veratraldehyde. The molar absorptivities of benzaldehyde at 250 mμ and of veratraldehyde at 308 mμ were taken to be 12×10^3 M^{-1} cm^{-1} (Dearden and Forbes, 1958) and 9.4×10^3 M^{-1} cm^{-1} (McEwen *et al.*, 1966), respectively. Initial rates concerning kynuramine oxidations were measured by recording increases in absorbance at 316 mμ due to the production of 4-hydroxyquinoline (Weissback *et al.*, 1960). The molar absorptivity of 4-hydroxyquinoline at 316 mμ is constant between pH 7.5 and 9.8 and amounts to 12×10^3 M^{-1} cm^{-1} (Tucker and Irwin, 1951).

Initial rates of hydrogen peroxide production were measured by a modification (McEwen, 1965a) of the *o*-dianisidine–peroxidase assay for hydrogen peroxide concentrations in tissues (Aebi *et al.*, 1959). As previously described (McEwen *et al.*, 1968), the coupled assay had no effect upon maximal velocities of substrate oxidations, but *o*-dianisidine was found to be a competitive inhibitor. For this reason, K_m values derived from Lineweaver–Burk plots were corrected (McEwen *et al.*, 1968) and, as indicated under Results, these corrected values agreed well with estimates of substrate affinities by other methods.

Measurements (Brown *et al.*, 1957) of ammonia production were used for estimations of rates of substrate deaminations as previously described (McEwen, 1965a). Although velocity estimations were, at times, based upon ammonia production after 1 hr of incubation (Table III) and did not necessarily furnish initial rates of deamination, kinetic constants were derived under conditions (Figure 3) demonstrated to furnish linear rates of ammonia production over a 1-hr period.

Ionization Constants and pH Values. The pK_a values of the phenolic and amino groups of 4-hydroxyphenethylamine (tyramine) at 25° in aqueous media are 9.74 and 10.52, respectively (Kappe and Armstrong, 1965). The pK_a values of the phenolic and amino groups of 4-hydroxyphenylethanolamine (octopamine), 9.57 and 9.66, and 5-hydroxytryptamine (serotonin), 11.1 and 10.0, under the same conditions have also been reported (Vane, 1959; Kappe and Armstrong, 1965). The single pK_a value of amantadine at 25° is reported to be 9.0 (Harmon *et al.*, 1965). Unpublished data, kindly provided by members of the research divisions of the Imperical Chemical Industries and the Ayerst Laboratories, indicate the pK_a values at 25° in aqueous media for the single amino groups of 2-(β-naphthyl)-2-hydroxyethylamine, *N*-isopropyl-2-(β-naphthyl)-2-hydroxyethylamine, *N*-isopropyl-2-(β-naphthyl)-2-ketoethylamine, and *N*-isopropyl-3-(α-naphthyloxy)-2-hydroxypropylamine to be 8.47, 9.42, 8.72, and 9.45, respectively. The pK_a values of α-naphthol and of β-naphthol are reported to be 9.39 and 9.59 at 25° in aqueous media (Barlin and Perrin, 1966). Other dissociation constants at 25° in aqueous media, used in computations, were taken from previously cited (McEwen, 1965b) references, or from publications cited by Kortüm *et al.* (1961) and Perrin (1965).

Certain ionization constants in aqueous media were predicted when we were not aware of reported values. With the use of Taft's constant for benzoyl residues (Taft and Lewis, 1958) and the pK_a value of ethylamine (10.63), the pK_a value for the aliphatic amino group of kynuramine (2′,3-diaminopropiophenone) may be estimated (Clark and Perrin, 1964) to be 9.94 at 25°. The aromatic amino group of kynuramine may be assumed to have a pK_a value similar to that (2.3 at 25°) of the analogous amino group of 2-acetylaniline. With respect to the identical pK_a values (4.89) of other long-chain 1-carboxy-*n*-alkanes, the pK_a value of dodecanoic acid is assumed to be 4.89 at 25°.

By analogy with the effect of temperature upon the ionization of tyrosinamide, the pK_a value of the phenolic group of tyramine may be predicted to be 9.59 at 37°; the pK_a value of the amino group of tyramine at 37° may be estimated (Perrin, 1964) from the pK_a value obtained at 25° to be 10.13. In a similar manner, the pK_a values at 37° for the phenolic and amino groups of octopamine (9.32 and 9.31) and of serotonin (10.9 and 9.6) may be estimated.

The Model G Beckman pH meter was used to determine pH values of reaction mixtures. We assume the variation of these measurements to be ±0.02 pH unit. With respect to this variation, we were unable to detect pH changes during the enzyme reactions studied in the buffered mixtures described in this report.

Calculation of Kinetic Constants. Unless otherwise indicated,

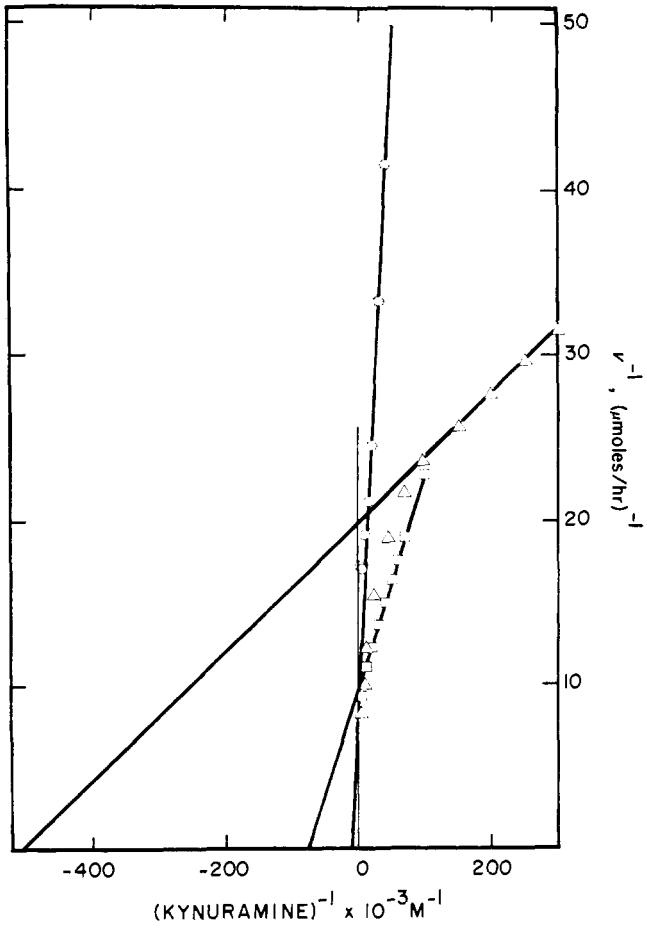

FIGURE 1: Lineweaver–Burk plots of initial rates of kynuramine oxidation at 25° and at different pH values. Reaction mixtures: 10 units of mitochondrial monoamine oxidase and the indicated final concentrations of kynuramine in 3.0 ml of 0.10 M sodium pyrophosphate buffer, pH 7.55 (○), pH 8.47 (□), or pH 9.42 (△).

apparent maximal velocities (V_{max} values), apparent Michaelis constants (K_m values), and apparent inhibitor constants (K_i values) were derived graphically from Lineweaver–Burk plots (Lineweaver and Burk, 1934). In the case of ionizable interactants, Michaelis and inhibitor constants were also derived with respect to the completely un-ionized species of interactants ($\tilde{K}_m$ and $\tilde{K}_i$ values) from apparent K_m or K_i values with the use of modifications of the Henderson–Hasselbalch equation. For example, in the case of competitive inhibitions, the following equations obtain, respectively, for monoamines without other ionizable groups:

$$\tilde{K}_i = K_i/[1 + \text{antilog}\,(pK_a - pH)] \quad (3)$$

for diamines without other ionizable groups:

$$\tilde{K}_i = K_i/[1 + \text{antilog}\,(pK_{a_1} - pH) + \text{antilog}\,(pK_{a_2} - pH)] \quad (4)$$

for monoamines with a single phenolic substituent:

$$\tilde{K}_i = K_i/[1 + \text{antilog}\,(pK_a - pH) + \text{antilog}\,(pH - pK_a')] \quad (5)$$

TABLE I: Oxidation of *C*-Phenyl-*n*-alkylamines at pH 8.72.[a]

Substrate	Substrate pK_a Value	V_{max}[b] (mμ-moles/hr)	K_m[b] (μM)	$\tilde{K}_m$[c] (μM)
Benzylamine	9.37	133	28	5.1
2-Phenylethylamine	9.79	68	1.2	0.097
3-Phenylpropylamine	10.16	92	5.5	0.19
β-Phenylethanolamine	8.90	21	13	5.1

[a] Reaction mixtures: 7.0 units of the mitochondrial monoamine oxidase, 300 μg of horseradish peroxidase, 60 μg (82 μM) of *o*-dianisidine (in 30 μl of methanol), and (at least five) concentrations of substrate in 3.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.72). [b] Derived from Lineweaver–Burk plots concerning initial rates of hydrogen peroxide production at 25°. [c] Obtained from eq 3.

and for phenols or carboxylic acids without other ionizable groups:

$$\tilde{K}_i = K_i/[1 + \text{antilog}\,(pH - pK_a')] \quad (6)$$

where pK_a, pK_{a_1}, and pK_{a_2} values represent ionization constants of amino groups and where pK_a' values represent ionization constants of phenolic or carboxylic residues. $\tilde{K}_m$ values were derived from K_m values in a similar fashion.

Results

Amine Oxidations Measured by Hydrogen Peroxide Production. Phenylalkylamines are considered to be typical substrates of mitochondrial monoamine oxidases. Although catecholamines and related phenolic substrates are substrates of peroxidase (Saunders *et al.*, 1964) and therefore interfere with the measurement of hydrogen peroxide production by any coupled peroxidase assay, kinetics relating to oxidation of phenylalkylamines without ring substituents can be studied by such measurements. Of the four phenylalkylamines studied at pH 8.72 (Table I), phenethylamine (2-phenylethylamine) had the greatest affinity for the enzyme, but benzylamine had the largest V_{max} value. It may be noted that the hydroxy group of β-phenylethanolamine appears to be responsible for the low V_{max} value and the low affinity of β-phenylethanolamine for the enzyme relative to the parent compound, phenethylamine. Lineweaver–Burk plots concerning the oxidation of these amines were found to be linear up to substrate concentrations that were at least fivefold the K_m value.

Kynuramine Oxidation Measured by 4-Hydroxyquinoline Production. The monoamine oxidase prepared from human hepatic mitochondria converts kynuramine stoichiometrically into 4-hydroxyquinoline. For example, when 300 mμmoles of kynuramine was incubated at 25° with 25 units of enzyme in 3.0 ml of 0.10 M sodium pyrophosphate buffer (pH 8.96), the production of 4-hydroxyquinoline ceased after 2 hr and amounted to 301 mμmoles. The ultraviolet (245–400 mμ) difference spectrum concerning this product, obtained with a ref-

TABLE II: Variation of Kinetic Constants of Kynuramine Oxidations with pH Values.

pH Value	V_{max}[a] (mμmoles/hr)	K_m[a] (μM)	$\tilde{K}_m$ (μM)
7.55	131	112	0.46
8.47	102	13.7	0.45
9.42	50.5	1.98	0.46

[a] Obtained from the Lineweaver–Burk plots of Figure 1.

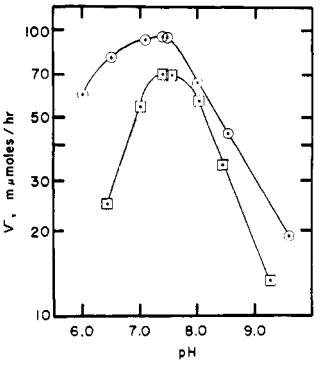

FIGURE 2: Effect of pH upon oxidative deaminations of tryptamines. Reaction mixtures containing 12 units of the monoamine oxidase and 500 mμmoles (1.0 mM) of tryptamine (○) or serotonin (□) in 0.5 ml of 0.10 M sodium pyrophosphate buffer were incubated at 37° and the indicated pH values for 1 hr.

erence reaction mixture containing 25 units of enzyme, but no kynuramine, was found to be identical with the spectrum of authentic 4-hydroxyquinoline in the same buffer.

Kinetic constants derived from the Lineweaver–Burk plots of Figure 1 (Table II) indicate that both maximal velocities (V_{max} values) and apparent Michaelis constants (K_m values) depend upon the pH values of the determinations. While V_{max} values progressively decreased with increasing pH values, the decrease in the apparent K_m values of kynuramine (2′,3-diaminopropiophenone) with increasing pH was more marked. The quantitive changes in both apparent kinetic constants with respect to pH changes are remarkably similar to those previously observed with benzylamine as substrate (McEwen *et al.*, 1968). Michaelis constants defined with respect to the completely nonprotonated species of kynuramine ($\tilde{K}_m$ values of Table II) were found to be constant over a relatively broad pH range. Also in keeping with kinetic data previously obtained with benzylamine as substrate Lineweaver–Burk plots (Figure 1) revealed substrate activation at the higher pH value and substrate inhibition at the lower pH value.

Amine Oxidations Measured by Ammonia Production. Point measurements of ammonia production (Figure 2) suggested that the enzymic oxidations of 1.0 mM concentrations of tryptamine and serotonin (5-hydroxytryptamine) at 37° are optimal at a pH value of approximately 7.4. At this pH value, but not necessarily at more extreme pH values, rates of ammonia production were found to be constant for more than 1 hr. For this reason, a pH value of 7.40 was selected for the measurement of rates of deamination of 1.0 mM concentrations of biogenic amines (Table III) and the determination of kinetic constants concerning the oxidations of typical substrates of mitochondrial monoamine oxidases (Table IV).

Biogenic amines that were unequivocally deaminated under the conditions of Table III (*i.e.*, deamination of greater than 10% of the amine concentrations initially present) included tyramine, dopamine, 3-methoxy-4-hydroxyphenethylamine, β-phenylethanolamine, tryptamine, serotonin, and 5-methoxytryptamine. The relative rates of oxidation of such substrates as tyramine, dopamine, tryptamine, and serotonin were found to be remarkably similar to those obtained, under comparable conditions, with highly purified preparations of the mitochondrial monoamine oxidases of rat liver (Guha and Krishna Murti, 1963), beef liver (Nara *et al.*, 1966), and beef kidney (Erwin and Hellerman, 1967). We interpret the finding that 1,4-diaminobutane is an exceedingly poor substrate as evidence that the mitochondrial preparation is not significantly contaminated with diamine oxidase activity (Zeller, 1951; McEwen, 1964). With respect to the oxidations of ethanolamine and octopamine, the substrate specificity of the mitochondrial monoamine oxidase differs markedly from that of the soluble monoamine oxidase purified from human plasma (McEwen, 1965a).

TABLE III: Rates of Amine Deaminations at pH 7.40.[a]

Substrate	NH_3 Production (mμmoles/hr)
4-Hydroxyphenethylamine (tyramine)	164
3,4-Dihydroxyphenethylamine (dopamine)	101
3-Methoxy-4-hydroxyphenethylamine	70
β-Phenylethanolamine	54
β-[4-Hydroxyphenyl]ethanolamine (octopamine)	30
Tryptamine	95
5-Hydroxytryptamine (serotonin)	71
5-Methoxytryptamine	83
Spermidine	15
1,4-Diaminobutane (putrescine)	3
Ethanolamine	2

[a] Monoamine oxidase (12 units) and 500 mμmoles of substrate (1 mM) were incubated in 0.50 ml of 0.20 M phosphate buffer[1] (pH 7.40) for 1 hr at 37°.

A comparison of the kinetic constants of Table IV derived from the linear Lineweaver–Burk plots of Figure 3 revealed that serotonin had the greatest maximal velocity but the least apparent affinity for the enzyme at pH 7.40. In comparison with the other phenolic amines tested, tyramine had, by far, the greatest affinity for the enzyme. The kinetic constants derived for octopamine (β-[4-hydroxyphenyl]ethanolamine) and its parent compound, tyramine (4-hydroxyphenethylamine), suggests that the β-hydroxy group of octopamine may be re-

[1] Phosphate buffers were made from dibasic sodium phosphate and monobasic potassium phosphate.

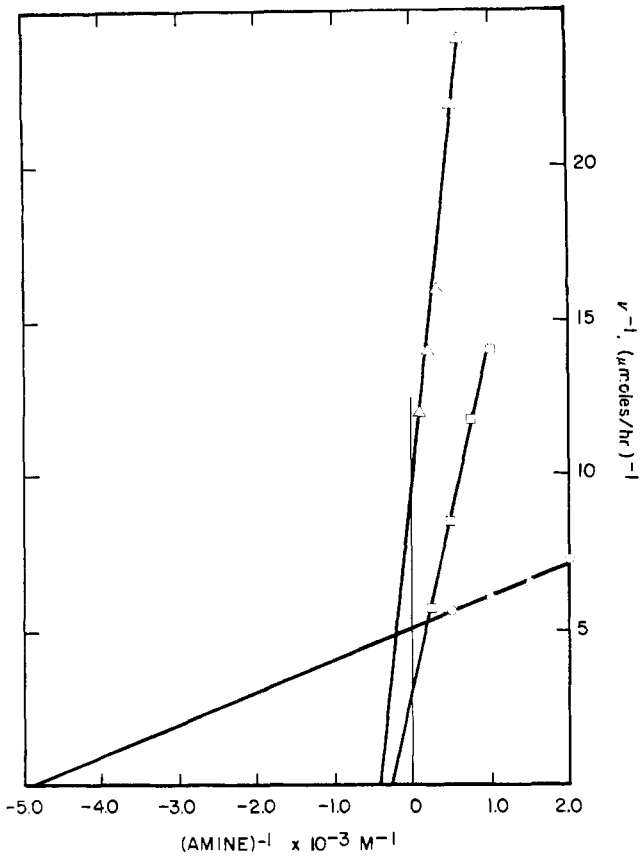

FIGURE 3: Lineweaver–Burk plots concerning the deaminations of phenolic amines. Reaction mixtures containing 12 units of monoamine oxidase and the indicated concentrations of octopamine (△), serotonin (□), or tyramine (○) were incubated in 0.50 ml of 0.20 M phosphate buffer[1] (pH 7.40), for 1 hr at 37°.

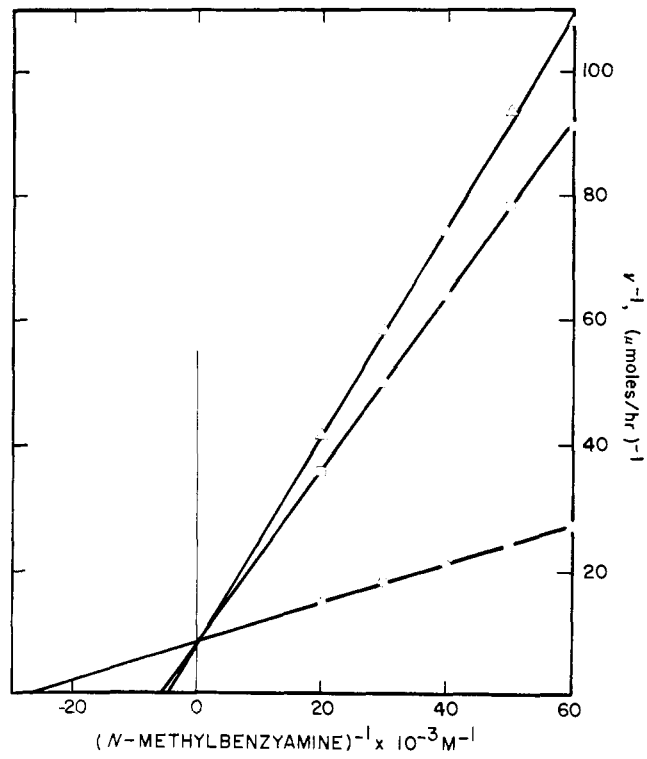

FIGURE 4: Competitive inhibitions by β-phenylethanolamine and β-naphthol. Initial rates of benzaldehyde production at 25° were obtained from 3.0-ml reaction mixtures: 12 units of monoamine oxidase, indicated *N*-methylbenzylamine concentrations, 0.10 M sodium pyrophosphate buffer (pH 8.61), and 67 μM β-phenylethanolamine (△), 50 μM β-naphthol (□), or no inhibitor (○).

sponsible for its relatively low maximal velocity and enzyme affinity. In keeping with the kinetic data (Table I) for β-phenylethanolamine and its parent compound, phenethylamine, the β-hydroxy group of octopamine had a more marked effect upon substrate binding than upon maximal velocity.

Inhibitions of the Oxidation of Benzylamines by Phenylalkylamines. A number of *C*-phenylalkylamines with and without substituents were found (Table V) to inhibit the oxidations of benzylamines in a strictly competitive fashion. A Lineweaver–Burk plot concerning the inhibition of *N*-methylbenzylamine oxidation by β-phenylethanolamine at pH 8.61 is presented in Figure 4 as an example. As expected, these competitive inhibitors included substrates of the mitochondrial monoamine oxidase, as well as phenylalkylamines that have not been demonstrated to be oxidized by the enzyme preparation.

Apparent inhibitor constants (K_i values) derived for the phenylalkylamines listed in Table V were found to depend upon the pH values of the determinations. Inhibitor constants ($\tilde{K}_i$ values) derived from K_i values with the use of eq 3 and 5 were found to be independent of pH values. Despite the disparate affinities of benzylamine ($\tilde{K}_m$ = 5.0 μM), *N*-methylbenzylamine ($\tilde{K}_m$ = 4.1 μM), and veratrylamine ($\tilde{K}_m$ = 204 μM) for the enzyme (McEwen *et al.*, 1968) the K_i values derived for phenylalkylamines did not depend upon the particular substrate used in the kinetic determinations. Furthermore, $\tilde{K}_i$ values for a particular substrate agreed well with the $\tilde{K}_m$ values derived for phenylalkylamines with (Table IV)[2] or without (Table I) phenolic groups. In conjunction with kinetic data concerning model interactions (McEwen *et al.*, 1968) we must interpret these data as evidence that nonprotonated amino groups of typical substrates of mitochondrial monoamine oxidase, as well as nonprotonated amino groups of analogous amines, interact with the free enzyme to form Michaelis com-

TABLE IV: Kinetic Constants at pH 7.40 Measured by Ammonia Production.

Substrate	V_{max}[a] (mμ-moles/hr)	K_m[a] (mM)	$\tilde{K}_m$ (μM)
Tyramine	197	0.204	0.38
Octopamine	106	2.53	31
Serotonin	342	3.84	24

[a] Obtained from the Lineweaver–Burk plots of Figure 3.

[2] The remarkable agreement between the $\tilde{K}_m$ values at 37° and the $\tilde{K}_i$ values at 25° derived for phenolic amines was unexpected. In retrospect, this unexpected agreement may be related to the opposite effects of temperature upon hydrophobic bonding by the polar and nonpolar groups of the un-ionized species of phenolic amines.

TABLE V: Inhibitor Constants for Phenylalkylamines.

Inhibitor	Inhibitor Concn (mM)	Inhibitor pK Value(s) pK_a	pK_a'	Benzylamine Substrate	pH Value	Inhibitor Constants K_i[a] (mM)	$\tilde{K}_i$ (μM)
Benzylamine	0.10	9.37		Veratrylamine	8.20	0.076	4.8
N-Methylbenzylamine	0.040	9.54		Veratrylamine	8.65	0.036	4.1
N,N-Dimethylbenzylamine	1.0	8.91		Veratrylamine	7.51	1.47	56
N,N-Dimethylbenzylamine	0.20	8.91		Veratrylamine	8.65	0.15	55
2-Phenylethylamine	0.020	9.79		Veratrylamine	7.51	0.019	0.097
2-Phenylethylamine	0.0080	9.79		Veratrylamine	8.11	0.0044	0.090
2-Phenylethylamine	0.0013	9.79		Veratrylamine	8.72	0.0012	0.096
2-Phenylethylamine	0.0050	9.79		*N*-Methylbenzylamine	8.15	0.0043	0.096
3-Phenylpropylamine	0.10	10.16		*N*-Methylbenzylamine	8.15	0.019	0.19
3-Phenylpropylamine	0.0050	10.16		Veratrylamine	8.72	0.0056	0.20
4-Phenylbutylamine	0.0050	10.36		*N*-Methylbenzylamine	8.15	0.011	0.067
4-Phenylbutylamine	0.0050	10.36		Veratrylamine	8.72	0.0032	0.071
α-Methylbenzylamine	0.20	9.08		Veratrylamine	8.72	0.17	49
4-Methylbenzylamine	0.010	9.36		Veratrylamine	8.72	0.0095	1.8
β-Phenylethanolamine	0.0080	8.90		Veratrylamine	8.72	0.013	5.1
β-Phenylethanolamine	0.067	8.90		*N*-Methylbenzylamine	8.61	0.015	5.0
ω-Aminoacetophenone	0.0033	*b*		*N*-Methylbenzylamine	8.15	0.0028	
Tyramine	0.50	10.52	9.74	Benzylamine	7.50	0.40	0.38
Tyramine	0.10	10.52	9.74	*N*-Methylbenzylamine	8.15	0.090	0.38
Tyramine	0.50	10.52	9.74	Veratrylamine	7.51	0.40	0.39
Tyramine	0.10	10.52	9.74	Veratrylamine	8.11	0.098	0.38
Tyramine	0.050	10.52	9.74	Veratrylamine	8.72	0.025	0.39
Octopamine	2.0	9.66	9.57	Veratrylamine	7.50	4.4	30
Octopamine	0.33	9.66	9.57	Veratrylamine	8.72	0.30	31
Octopamine	2.0	9.66	9.57	Benzylamine	7.50	4.4	30
Octopamine	0.83	9.66	9.57	*N*-Methylbenzylamine	8.15	1.0	30
4-Methoxyphenethylamine	0.0020	9.89		*N*-Methylbenzylamine	8.15	0.0065	0.12
4-Methoxyphenethylamine	0.0040	9.89		Veratrylamine	8.70	0.0022	0.13
3-Methoxy-4-hydroxyphenethylamine	0.33	*b*	*b*	Veratrylamine	8.72	0.23	
3,4-Dimethoxyphenethylamine	0.039	*b*		Veratrylamine	8.72	0.043	
Mescaline	1.0	*b*		Veratrylamine	8.72	1.9	

[a] Derived from Lineweaver–Burk plots concerning initial rates of benzaldehyde or veratraldehyde production at 25° from 3.0-ml reaction mixtures: 0.10 M sodium pyrophosphate buffer at the indicated pH value, 7.0 or 12.0 units of monoamine oxidase, and at least five concentrations of the benzylamine substrate above and below the relevant, apparent K_m value with and without the given concentrations of phenylalkylamine inhibitor. All Lineweaver–Burk plots revealed the inhibitions to be strictly competitive with respect to substrate. [b] To our knowledge, reliable estimates of these pK_a values are not available.

plexes. We interpret the data concerning phenolic amines as evidence that the species of these substrates that interact with the free enzyme may be characterized by a nonprotonated amino substitutent and a protonated phenolic group. In other words, the combined data (Tables I, IV, and V) suggest simple interactions of completely un-ionized compounds with the free enzyme to form Michaelis and inhibitor complexes (Dixon and Webb, 1958; Webb, 1963).

The $\tilde{K}_i$ constants of Table V, as well as the $\tilde{K}_m$ constants of Tables I and IV, suggest that relatively polar substituents affect phenylalkylamine affinities for the mitochondrial monoamine oxidase adversely. For example, the affinity of the un-ionized species of tyramine for the enzyme is less than that of the nonprotonated species of its less polar methyl ether, 4-methoxyphenethylamine; both affinities are markedly less than that of the parent compound, phenethylamine. Even more marked are the different affinities derived for the un-ionized species of phenethylamine, its β-hydroxylated derivative (β-phenylethanolamine), and β-(4-hydroxyphenyl)ethanolamine (octopamine). On the other hand, hydrophobic residues of phenylalkylamines may either facilitate or hinder the formation of enzyme–substrate and enzyme–inhibitor complexes. For example, while the nonprotonated species of 4-methylbenzylamine and phenethylamine have distinctly greater enzyme affinities than that of benzylamine, the affinity of the nonprotonated species of 3-phenylpropylamine is mark-

TABLE VI: Inhibitor Constants for Other Substrates and Substrate Analogs.

Inhibitor	Inhibitor Concn (mM)	Inhibitor pK Values		Benzylamine Substrate	pH Value	Inhibitor Constants	
		pK_a	pK_a'			K_i[a] (mM)	$\tilde{K}_i$ (μM)
Tryptamine	0.020	10.2		Veratrylamine	7.51	0.033	0.065
Tryptamine	0.0080	10.2		Veratrylamine	8.11	0.0092	0.067
Tryptamine	0.0040	10.2		Veratrylamine	8.72	0.0022	0.065
Tryptamine	0.010	10.2		*N*-Methylbenzylamine	8.15	0.0067	0.066
N-Methyltryptamine	0.0020	*b*		Veratrylamine	8.72	0.0012	
5-Hydroxytryptamine	0.33	10.0	11.1	Veratrylamine	8.11	1.9	23
5-Hydroxytryptamine	0.50	10.0	11.1	Veratrylamine	8.72	0.54	26
5-Methoxytryptamine	0.67	10.3		Veratrylamine	8.72	0.36	8.7
Indole	0.33	−2.4		Veratrylamine	8.72	0.14	140
β-Aminopropionitrile	50	7.7		Veratrylamine	7.51	63	31,000
β-Aminopropionitrile	50	7.7		Veratrylamine	8.72	29	26,000
Amantadine	6.7	9.0		Veratrylamine	8.72	5.8	2.0
2,2′-Bipyridyl	1.0	4.3		Veratrylamine	8.11	2.0	2,000
2,2′-Bipyridyl	2.0	4.3		Veratrylamine	8.11	2.0	2,000
2-Phenylethanol	0.36			*N*-Methylbenzylamine	8.15	0.30	
2-Phenylethanol	0.36			Veratrylamine	8.72	0.31	
Octanoic acid	10		4.89	Veratrylamine	8.72	8.3	1.2
Dodecanoic acid	0.10		4.89	Veratrylamine	8.11	0.15	0.090
Dodecanoic acid	0.67		4.89	Veratrylamine	8.72	0.61	0.090

[a] Derived from Lineweaver–Burk plots concerning initial rates of oxidation of benzylamine substrates at 25° as described in the legend of Table V. All such plots revealed the inhibitions to be strictly competitive with respect to the benzylamine substrates. [b] To our knowledge, reliable estimates of relevant pK_a values are not available.

edly less than the affinities of nonprotonated species of both phenethylamine (2-phenylethylamine) and 4-phenylbutylamine.

Inhibitions of Benzylamine Oxidations by Other Substrates and Substrate Analogs. Other substrates and dissimilar substrate analogs (Table VI) were demonstrated to be competitive inhibitors of the enzyme activity. Examples of such strictly competitive inhibitions are presented in Figure 5.

Apparent inhibitor constants (K_i values) derived for the substrates, tryptamine and serotonin (5-hydroxytryptamine), were found to depend upon the pH values of the determinations (Table VI). Inhibitor constants computed with respect to the completely un-ionized species of these substrates ($\tilde{K}_i$ values) were independent of pH. The $\tilde{K}_i$ values obtained for serotonin agreed with the $\tilde{K}_m$ value[2] derived for this substrate (Table IV). In keeping with the different $\tilde{K}_i$ values obtained (Table V) for 2-phenylethylamine, 4-methoxyphenylethylamine, and 4-hydroxyphenylethylamine (tyramine), $\tilde{K}_i$ values for tryptamine, 5-methoxytryptamine, and 5-hydroxytryptamine (serotonin) suggest that polar residues of substituted tryptamines affect affinities adversely. K_i values (0.13 mM) derived for the indole inhibition of veratrylamine oxidation in 0.15 M sodium borate buffer (pH 9.23) with the use of both a Lineweaver–Burk plot (indole concentration 0.20 mM) and a Dixon plot (Dixon and Webb, 1958) (indole concentration varied between 0.13 and 0.80 mM) agreed well with the K_i value obtained in pyrophosphate buffer (pH 8.72) (Table VI).

Despite its relatively low pK_a value, β-aminopropionitrile (2-cyanoethylamine) was found to be a relatively poor competitive inhibitor of veratrylamine oxidation by the mitochondrial monoamine oxidase.

Amantadine (1-adamantamine), an aliphatic monoamine with ten methylene residues bridged in a spherical manner to provide a hydrophobic shell of carbon atoms (Harmon *et al.*, 1965), was also found to be a comparatively weak, competitive inhibitor of the mitochondrial monoamine oxidase. Its $\tilde{K}_i$ value indicates that its affinity for the enzyme is less than that derived for *n*-butylamine (McEwen *et al.*, 1968), an aliphatic amine with only four methylene residues but variable conformations. The relatively poor affinity of amantadine may be attributed to its rigid configuration or to other factors which may not allow its hydrophobic residues to interact optimally with apolar sites of the enzyme active center.

The enzyme activity is also competitively inhibited by 2,2′-bipyridyl which may be considered to be a substrate analog by virtue of either hydrophobic or nucleophilic characteristics. *o*-Phenanthroline, however, a bulky and more rigid analog of 2,2′-bipyridyl, was not found to be an effective, instantaneous inhibitor. For example, 1.5 mM *o*-phenanthroline has no inhibitory effect upon veratrylamine oxidation at pH 8.11. It should be noted that preincubation of the enzyme for 40 min at 25° in the presence of 0.50–1.5 mM concentrations of *o*-phenanthroline and 0.10 M sodium pyrophosphate buffer (pH 8.11) before addition of the veratrylamine substrate did not lead to detectable inactivation of the enzyme. Under the same conditions, preincubation of the enzyme in the presence of 2.0 mM 2,2′-bipyridyl did not enhance the inhibition observed instantaneously. With respect to another chelating agent, 1.0

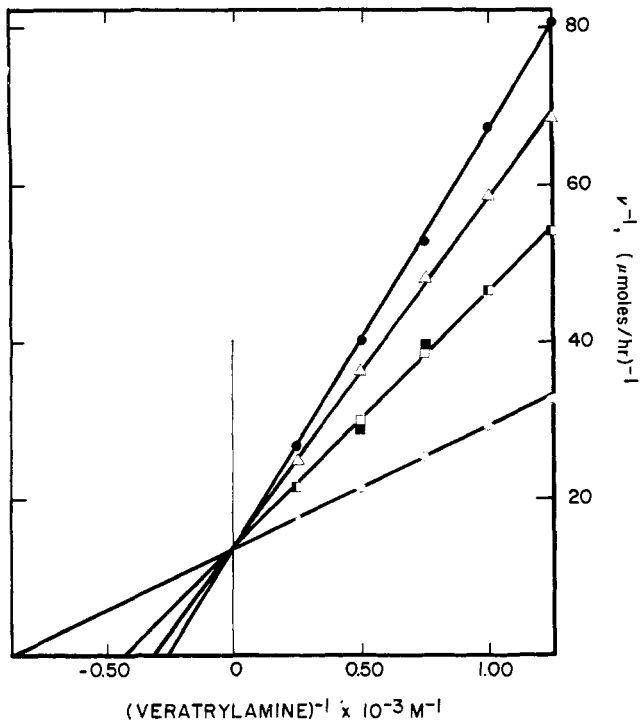

FIGURE 5: Competitive inhibitions by tryptamine, indole, amantadine, and lauric acid. Initial rates of veratraldehyde production at 25° were measured from 3.0-ml reaction mixtures: 7.0 units of the monoamine oxidase, indicated veratrylamine concentrations, 0.10 M sodium pyrophosphate buffer (pH 8.72), and 4.0 μM tryptamine (●), 0.33 mM indole (△), 6.7 mM amantadine (□), 0.67 mM lauric acid (■), or no inhibitor (○).

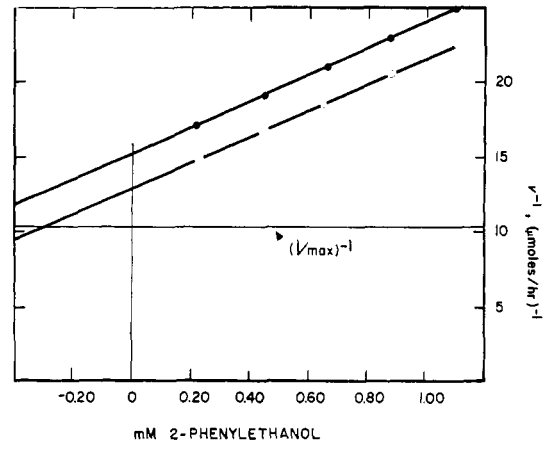

FIGURE 6: Mixed inhibition by 2-phenylethanol and ammonia. Initial rates of veratraldehyde production at 25° were measured spectrophotometrically with the use of 3.0-ml reaction mixtures: 9.5 units of monoamine oxidase, 2.0 mM veratrylamine, indicated 2-phenylethanol concentrations, and 0.15 M sodium borate buffer (pH 9.23) with (●) or without (○) 0.19 M buffered ammonia. The inverse of the maximal velocity of veratrylamine oxidation, $(V_{max})^{-1}$, at pH 9.23 was derived from a Lineweaver–Burk plot.

mM concentrations of ethylenediaminetetraacetate had no inhibitory effect either instantaneously or after preincubation with the enzyme.

Inhibitor constants derived for 2-phenylethanol, an electronic analog of the nonprotonated species of 2-phenylethylamine, were not found to depend upon the pH values of the determinations. The Dixon plot (Dixon and Webb, 1958) included in Figure 6 provides a K_i value (0.30 mM) for 2-phenylethanol at pH 9.23 that agrees well with the K_i values (Table VI) derived from Lineweaver–Burk plots at lower pH values. The Yonetani–Theorell plot (Yonetani and Theorell, 1964) also included in Figure 6 provides parallel lines which indicate that the kinetic constant (α) for the interaction between 2-phenylethanol and ammonia is infinite. In other words, these kinetic data are consistent with the hypothesis (McEwen *et al.*, 1968) that an electrophilic site at the enzyme active center may bind either hydroxy or nonprotonated amino groups of substrate analogs. On the other hand, alcohols with hydrophobic residues but without the other characteristics of substrate analogs of mitochondrial monoamine oxidase may not inhibit the enzyme activity. For example, 0.05–0.17 mM concentrations of the highly hydrophobic alcohol, hydrocortisone, had no detectable effect upon the oxidation of veratrylamine.

Because 1-hydroxy-*n*-alkanes and 1-amino-*n*-alkanes have been found to interact with the enzyme active center (McEwen *et al.*, 1968), 1-carboxy-*n*-alkanes were tested as enzyme inhibitors. Octanoic (*n*-caprylic) acid and dodecanoic (lauric) acid (Table VI) were found to inhibit the enzyme activity in a competitive fashion (*e.g.*, Figure 5). The apparent affinity of dodecanoic acid decreased exponentially with increasing pH values of the determinations (*i.e.*, $\Delta \log K_i/\text{pH} = 1.0$). In other words, inhibitor constants ($\tilde{K}_i$ values) computed with respect to the un-ionized species of dodecanoic acid did not appear to depend upon pH values. A comparison of the inhibitor constants of dodecanoic acid with those of its shorter chain homolog, octanoic acid, indicated that the additional methylene residues of dodecanoic acid enhance its affinity for the enzyme and suggested that *n*-alkane residues of carboxylic acids may interact with the hydrophobic binding sites of the enzyme active center.

Interactions with Naphthyl Derivatives. Naphthols have recently been reported to inhibit competitively activities of the mitochondrial monoamine oxidase purified from beef kidney (Erwin and Hellerman, 1967). At all pH values tested (Table VII), β-naphthol was found to be a potent, competitive inhibitor of the human liver monoamine oxidase (*e.g.*, Figure 4). Inhibitor constants defined with respect to the un-ionized species of this inhibitor ($\tilde{K}_i$ values) were found to be independent of pH changes (Table VII). In comparison with its effects upon the mitochondrial monoamine oxidase of beef kidney, β-naphthol appears to be, by far, a more potent inhibitor of the human mitochondrial enzyme. On the other hand, α-naphthol, which inhibits the beef mitochondrial enzyme in concentrations almost equal to β-naphthol, is not an effective inhibitor of the mitochondrial monoamine oxidase of human liver. For example, 40 μM concentrations of α-naphthol had no effect at 25° upon the oxidation of *N*-methylbenzylamine at pH 7.78, 8.15, or 8.61. This lack of effect may not be attributed to the slightly dissimilar pK_a values of α-naphthol (9.39) and β-naphthol (9.59). On the other hand, the configuration of α-naphthol may not permit optimal bonding to either the limited nonpolar areas or the electrophilic site of the enzyme active center.

Because of the β-naphthol inhibition, we have also included in this study other naphthyl derivatives and, in particular, pharmacological agents that inhibit responses to adrenergic

TABLE VII: Inhibitor Constants for Naphthalenes.

Inhibitor	Inhibitor concn (μM)	Inhibitor pK Values		pH Value	Inhibitor Constants	
		pK_a	pK_a'		K_i[a] (μM)	$\tilde{K}_i$ (μM)
β-Naphthol	20		9.59	7.78	13	13
β-Naphthol	4.2		9.59	8.15	13	13
β-Naphthol	50		9.59	8.61	14	13
2-(β-Naphthyl)-2-hydroxyethylamine	2.7	8.97		8.15	4.1	0.54
N-Isopropyl-2-(β-naphthyl)-2-ketoethylamine	6.7	8.72		8.15	13	2.8

[a] Obtained from Lineweaver–Burk plots concerning the oxidation of *N*-methylbenzylamine (*e.g.*, Figure 4).

sympathetic nerve activity, as well as analogous agents under present investigation. Neither of the β-adrenergic blocking agents, pronethalol (racemic *N*-isopropyl-2-(β-naphthyl)-2-hydroxyethylamine, pK_a = 9.42) and propranolol (racemic *N*-isopropyl-3-(α-naphthyloxy)-2-hydroxypropylamine, pK_a = 9.45), were found to be effective, instantaneous inhibitors of the mitochondrial monoamine oxidase. For example, 83 μM concentrations of either agent had no effect upon the oxidation of *N*-methylbenzylamine at pH 8.15. On the other hand, racemic 2-(β-naphthyl)-2-hydroxyethylamine, a parent compound of pronethalol, was found to be a potent, competitive inhibitor of the enzyme activity under the same conditions (Table VII). In comparison, the keto analog of pronethalol, racemic *N*-isopropyl-2-(β-naphthyl)-2-ketoethylamine, was found to be a somewhat less potent, competitive inhibitor.

Guanethidine Inhibition. Guanethidine, [2-(octahydro-1-azocinyl)ethyl]guanidine, was found to be an instantaneous inhibitor of the enzyme. Although relatively high concentrations of this antiadrenergic agent were required to demonstrate the *in vitro* inhibition, the relevant data of Figure 7 are of interest in two respects. Because both *N*-alkylazacycloalkanes and *N*-alkylguanidines have high pK_a values and because these residues of guanethidine may be assumed to be predominately ionized at pH values below 9.0, it seems unlikely that a guanethidine species analogous to the un-ionized substrates of the enzyme may interact with its active center. In the second place, both plots of Figure 7 indicate guanethidine to be a noncompetitive inhibitor. This finding could not be anticipated because a number of observations (Kuntzman and Jacobson, 1963; Dvornik *et al.*, 1963; Schoepe and Swett, 1967) indicate that other guanidines are competitive inhibitors of mitochondrial monoamine oxidases and, to our knowledge, relevant kinetic data concerning guanethidine inhibitions have not been reported. The K_i values for guanethidine at pH 8.11 derived from the Lineweaver–Burk plot (6.0 mM) and the Dixon plot (6.1 mM) of Figure 7 agreed well. Similar guanethidine concentrations have been reported (Dvornik *et al.*, 1963) to inhibit kynuramine oxidation at pH 7.4 by hepatic extracts of several laboratory animals. In the present study, the inhibition did not depend upon time. For example, preincubation of 7.0 units of the human mitrochondrial monoamine oxidase with 1.3 mM guanethidine in 0.10 M sodium pyrophosphate buffer (pH 8.11) for 30 min at 25° did not enhance the inhibition.

Discussion

We have previously assumed that the partially purified enzyme preparation that is the subject of this report represents an activity that oxidizes amines of biologic interest. The data of Tables, I, III, and IV indicate that phenethylamines and tryptamines are oxidized by the enzyme preparation. Data included in Tables V and VI indicate that the same enzymic activity is responsible for the oxidations of both model benzylamines and more typical substrates of mitochondrial monoamine oxidases. These latter substrates include octopamine, which accumulates in the sympathetic nerves after the inhibition of mitochondrial monoamine oxidase *in vivo* and may be the major "false neurochemical transmitter" responsible for the partial adrenergic blockade produced by inhibitors of mitochondrial monoamine oxidase (Kopin, 1966). They also include tryptamine, tyramine, phenethylamine, and other amines that are excreted by man in increased quantities during the administration of the same pharmacological agents (Sjoerdsma, 1966).

The kinetic data presented in this paper support the hypothesis that un-ionized amines interact with the monoamine oxidase of human liver to form enzyme–substrate complexes. In other words, eq 2 describes the basic interaction of the free enzyme with simple monoamines. In the case of amines with other ionizable groups, however, this basic equation requires modifications, which do not alter the primary hypothesis that the enzyme active center has an electrophilic site for the binding of nonprotonated amino groups. Such modifications, *e.g.*, eq 3–6, suggest that species of substrate and competitive inhibitors that react with the enzyme have no ionized groups. For example, the data concerning kynuramine oxidation (Table II) suggest that both amino groups of this substrate must be nonprotonated in order to permit the formation of an effective Michaelis complex. On the other hand, the kinetic data concerning monoamines with phenolic groups (Tables IV–VI) suggest that the species of these substrates that interact with the enzyme have nonprotonated amino and protonated phenolic groups. These kinetic data allow speculations concerning the effect of changes in intramitochondrial pH values (Addanki *et al.*, 1968) upon the activities of mitochondrial monoamine oxidases particularly because these enzymes appear to be localized primarily in the inner membrane system of mitochondria (Green *et al.*, 1968). If nonprotonated amines inter-

act with the human liver mitochondrial monoamine oxidase, it is likely that small changes in intramitochondrial pH values may control the oxidative deaminations of substrates with relatively low affinities for the enzyme (*e.g.*, serotonin).

We have previously suggested that a hydrophobic area within the enzyme active center and immediately adjacent to its electrophilic site permits binding of linear hydrocarbon chains and that such binding may represent partial enclosure of methylene residues by this enzyme hydrophobic area. Data of the present report describe interactions of *n*-alkylbenzenes with the enzyme and support the hypothesis that a limited hydrophobic area is present within the enzyme active center. The contributions of phenyl groups may be compared with those of methylene residues with the use of the following equation

$$\Delta\Delta F = 2.3RT \log (1/\tilde{K}_m(1) - 1/\tilde{K}_m(2)) = 2.3\ RT \log (1/K_i(1) - 1/K_i(2)) \quad (7)$$

where $\Delta\Delta F$ refers to differences in the changes in the standard free energy contributed by phenyl and methylene residues in the formation of Michaelis and inhibitor complexes at pH 8.72 and 25°, R indicates the universal gas constant, T indicates the temperature in degrees Kelvin, $\tilde{K}_m(1)$ and $\tilde{K}_m(2)$ are Michaelis constants computed with respect to the nonprotonated species of phenylalkylamines (Table I) and the analogous methylalkylamines (McEwen *et al.*, 1968), respectively, and $K_i(1)$ and $K_i(2)$ are the inhibitor constants derived for phenylethanol (Table VI) and 1-propanol (McEwen *et al.*, 1968), respectively. The comparisons of $\tilde{K}_m$ values for benzylamine (5.1 μM) and ethylamine (390 μM), $\tilde{K}_m$ values for 2-phenylethylamine (0.097 μM) and *n*-propylamine (19 μM), and K_i values for 2-phenylethanol (0.31 mM) and 1-propanol (72 mM) indicate that *n*-alkylbenzenes are bound more tightly than comparable *n*-alkanes by similar $\Delta\Delta F$ values (2.6, 3.1, and 3.2 kcal/mole, respectively). These $\Delta\Delta F$ values are reasonable, since the difference between the dispersion energies of a benzene ring and a methyl group interacting *on one* side with a hydrophobic protein surface has been estimated to be approximately 2.1 kcal/mole (Webb, 1963) and these $\Delta\Delta F$ values may represent more complete enclosure. A comparison of $\tilde{K}_m$ and $\tilde{K}_i$ values derived for β-phenylethanolamine and octopamine with the appropriate kinetic constants determined for the relevant parent compounds, 2-phenylethylamine and tyramine, respectively (Tables I, IV, and V), suggests that substrate affinities for the enzyme may be affected adversely by polar β substituents (*i.e.*, β-hydroxy groups) of substrates. These and other data included in the present report support the hypothesis that a hydrophobic area is immediately adjacent to the electrophilic binding site of the enzyme active center. On the other hand, data of the present report also suggest that hydrophobic binding sites within the enzyme active center are limited, presumably by steric factors, and that nonpolar substrate residues are not simply "extracted" from aqueous media by an unlimited hydrophobic surface of the enzyme. For example, a comparison of the $\tilde{K}_i$ values derived for α-methylbenzylamine and *N*,*N*-dimethylbenzylamine with those derived for benzylamine and *N*-methylbenzylamine (Table V) indicates that nonpolar substitutents close to the reactive amino group of substrates and inhibitors may adversely affect their interactions with the enzyme electrophilic site. The $\tilde{K}_i$ values derived for 4-phenylbutylamine (Table V), moreover, are distinctly greater than those previously reported for the analogous methylalkylamine, *n*-pentylamine, while the affinity of 3-phenylpropylamine for the enzyme is less than that of 2-phenylethylamine (Tables I and V).

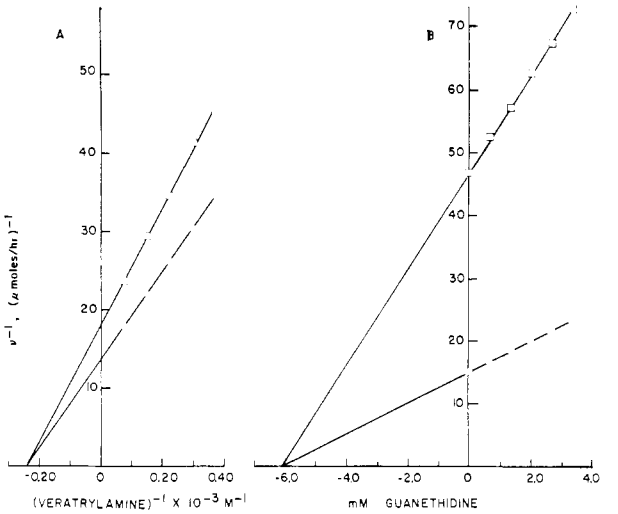

FIGURE 7: Noncompetitive guanethidine inhibitions. (A) Lineweaver–Burk plot of initial rates of veratraldehyde production at 25° measured from 3.0-ml reaction mixtures: 7.0 units of monoamine oxidase, indicated veratrylamine concentrations, and 0.10 M sodium pyrophosphate buffer (pH 8.11) with (□) or without (○) 2.0 mM guanethidine. (B) Dixon plot of initial rates of benzaldehyde production at 25° measured from 3.0-ml reaction mixtures: 7.0 units of monoamine oxidase, 50 μM (□) or 1.0 mM (○) *N*-methylbenzylamine, indicated guanethidine concentrations, and 0.10 M sodium pyrophosphate buffer (pH 8.11).

The inhibitions of mitochondrial monoamine oxidase activities by metal and particularly copper chelating agents have frequently been presented as evidence that these activities are catalyzed by metalloproteins. Unfortunately, most copper chelating agents may be considered, by virtue of amino or analogous nucleophilic groups, to be substrate analogs. In this respect, it is not surprising that the kinetics reported for the inhibitions of the mitochondrial monoamine oxidase of bovine liver by *o*-phenanthroline (Barbarto and Abood, 1963) and cuprizone (Nara *et al.*, 1966) suggest that these inhibitions are predominately competitive with respect to substrate. Although other data concerning the beef liver enzyme clearly indicate that this mitochondrial monoamine oxidase is a copper protein (Nara *et al.*, 1966), data relating to the mitochondrial monoamine oxidase of bovine kidney (Erwin and Hellerman, 1967) do not support the hypothesis that all mitochondrial monoamine oxidases are copper proteins. Certainly the data of the present report concerning the interaction of the mitochondrial monoamine oxidase of human liver with 2,2′-bipyridyl should not be interpreted as evidence that this enzyme is a copper protein. On the other hand, the human enzyme may be distinguished from the mitochondrial monoamine oxidase of bovine liver by the distinctly different effects of *o*-phenanthroline. We interpret the finding that 2,2′-bipyridyl is a more potent competitive inhibitor of the human enzyme than its close analog, *o*-phenanthroline, as evidence that the active centers of the mitochondrial monoamine oxidases of

human and bovine liver differ with respect to either conformation or configuration.

Assertions that certain mitochondrial preparations may contain "multiple" monoamine oxidase have frequently depended upon changes in rates of oxidation of fixed substrate concentrations produced by pH changes in the presence of fixed concentrations of nucleophilic inhibitors. For example, the instantaneous inhibitions of the bovine enzyme caused by fixed concentrations of the weak base, *o*-phenanthroline, and measured by the oxidation of a fixed concentration of the markedly more basic substrate, kynuramine, decrease with increasing pH values (Barbarto and Abood, 1963). We have suggested (McEwen, 1965b) that these, as well as comparable data, may be a consequence of the interactions of nonprotonated amines and related nucleophilic agents with the active centers of mitochondrial monoamine oxidases. On the other hand, factitious transformations (Gorkin and Tatyanenko, 1967) of mitochondrial monoamine oxidases may also be responsible for other findings concerning the multiplicity of these activities. Despite the complex substrates and competitive inhibitors studied in this report, we were not able to detect discrepancies in the kinetic data that might suggest the presence of multiple amine oxidase activities in the mitochondrial preparation. These data, of course, do not exclude the possibility that intact mitochondria of human liver may contain other amine oxidase activities. On the other hand, the data do not suggest that the enzyme preparation studied is contaminated with either the soluble monoamine oxidase of human plasma (McEwen, 1965a,b) or any diamine oxidase activity that has been reported (Gorkin and Tatyanenko, 1967) to result from a factitious transformation of mitochondrial monoamine oxidase.

References

Addanki, S., Cahill, F. D., and Sotos, J. F. (1968), *J. Biol. Chem. 243*, 2337.

Aebi, H., Gressly, R., Oestreicher, R., and Zuppinger, A. (1959), *Helv. Chim. Acta 42*, 2531.

Barbarto, L. M., and Abood, L. G. (1963), *Biochim. Biophys. Acta 67*, 531.

Barlin, G. B., and Perrin, D. D. (1966), *Quart. Rev. (London) 20*, 75.

Biel, J. H., Horita, A., and Drucker, A. E. (1964), *in* Psychopharmacological Agents, Vol. I, Gordon, M., Ed., New York, N. Y., Academic, p 359.

Blaschko, H. (1952), *Pharmacol. Rev. 4*, 415.

Brown, R. H., Duda, G. D., Korkes, S., and Handler, P. (1957), *Arch. Biochem. Biophys. 66*, 301.

Burger, A., and Nara, S. (1965), *J. Med. Chem. 8*, 859.

Clark, J., and Perrin, D. D. (1964), *Quart. Rev. (London) 18*, 295.

Dearden, J. C., and Forbes, W. F. (1958), *Can. J. Chem. 36*, 1362.

Dixon, M., and Webb, E. C. (1958), Enzymes, New York, N. Y., Academic.

Dvornik, D., Kraml, M., Dubuc, J., Tom, H., and Zsoter, T. (1963), *Biochem. Pharmacol. 12*, 229.

Erwin, V. G., and Hellerman L. (1967), *J. Biol. Chem. 242*, 4230.

Gorkin, V. Z., and Tatyanenko, L. V. (1967), *Biochem. Biophys. Res. Commun. 27*, 613.

Green, D. E., Allmann, D. W., Harris, R. A., and Tan, W. C. (1968), *Biochem. Biophys. Res. Commun. 31*, 368.

Guha, S. R., and Krishna Murti, C. R. (1963), *Biochim. Biophys. Acta 67*, 531.

Harmon, T. B., Hewes, W. E., Lynes, T. E., and Hermann, E. C. (1965), *J. Pharmacol. Exptl. Therap. 150*, 484.

Kappe, T., and Armstrong, M. D. (1965), *J. Med. Chem. 8*, 368.

Kopin, I. J. (1966), *Pharmacol Rev. 18*, 513.

Kortüm, G., Vogel, W., and Andrussow, K. (1961), Dissociation Constants of Organic Acids in Aqueous Solution, London, Butterworth.

Kuntzman, R., and Jacobson, M. M. (1963), *J. Pharmacol. 141*, 166.

Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56*, 658.

McEwen, C. M., Jr. (1964), *J. Lab. Clin. Med. 64*, 540.

McEwen, C. M., Jr. (1965a), *J. Biol. Chem. 240*, 2003.

McEwen, C. M., Jr. (1965b), *J. Biol. Chem. 240*, 2011.

McEwen, C. M., Jr., Cullen, K. T., and Sober, A. J. (1966), *J. Biol. Chem. 241*, 4544.

McEwen, C. M., Jr., Sasaki, G., and Lenz, W. R., Jr. (1968), *J. Biol. Chem. 243*, 5217.

Nara, S., Gomes, B., and Yasunobu, K. T. (1966), *J. Biol. Chem. 241*, 2774.

Perrin, D. D. (1964), *Australian J. Chem. 17*, 484.

Perrin, D. D. (1965), Dissociation Constants of Organic Bases in Aqueous Solution, London, Butterworth.

Saunders, B. C., Holmes-Siedle, A. G., and Stark, B. P. (1964), Peroxidase, Washington, D. C., Butterworth.

Schoepe, H. G., and Swett, L. R. (1967), *in* Medicinal Chemistry, Vol. 7, DeStevens, G., Ed., New York, N. Y., Academic, p 393.

Sjoerdsma, A. (1966), *Pharmacol. Rev. 18*, 673.

Soep, H. (1961), *Pure Appl. Chem. 3*, 481.

Taft, R. W., and Lewis, I. C. (1958), *J. Am. Chem. Soc. 80*, 2436.

Tucker, G. F., and Irwin, J. L. (1951), *J. Am. Chem. Soc. 73*, 1923.

Vane, J. R. (1959), *Brit. J. Pharmacol. 14*, 87.

Webb, J. L. (1963), Enzyme and Metabolic Inhibitors, Vol. 1, New York, N. Y., Academic.

Weissback, H., Smith, T. E., Daly, J. W., Witkop, B., and Udenfriend, S. (1960), *J. Biol. Chem. 235*, 1160.

Yonetani, T., and Theorell, H. (1964), *Arch. Biochem. Biophys. 106*, 243.

Zeller, E. A. (1951), *in* The Enzymes, Vol. II, Sumner, J. B., and Myrbäck, K., Ed., New York, N. Y., Academic, p 536.

Zirkle, C. L., and Kaiser, C. (1964), *in* Psychopharmacological Agents, Vol. I, Gordon, M., Ed., New York, N. Y., Academic, p 445.